A novel progesterone oral formulation for the treatment of endometrial hyperplasia with reduced adverse signalling compared to synthetic progestins.

Bentham, Zoe

A Novel Progesterone Oral Formulation for the treatment of Endometrial Hyperplasia with Reduced Adverse Signalling compared to Synthetic Progestins

Zoe Bentham

Submitted to Swansea University in fulfilment of the requirements for the degree of Doctor of Philosophy

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College of Medicine

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Background

Natural progesterone is currently unavailable for the treatment of endometrial hyperplasia. The first line of treatment for this condition is with the synthetic progestins Medroxyprogesterone Acetate (MPA) and Levonorgestrel (LNG). However, these hormones frequently trigger side effects which occur as a result of adverse gene and protein regulation. The current unavailability of natural progesterone is down to its poor bioavailability which is due to the low aqueous solubility and extensive first-pass metabolism of this compound.

Aims:

The first aim of this thesis is to compare natural progesterone with MPA and LNG in their regulation of a subset of genes and proteins in the endometrium whilst also assessing the selected proteins in biopsies from patients with endometrial hyperplasia before and after progestin therapy. The second aim is to study progesterone metabolism within the gastrointestinal tract and liver in order to direct the production of progesterone amorphous solid dispersions.

Methodology:

During this thesis both microbiological and pharmacological laboratory techniques were used. For microbiology investigations immunohistochemistry, polymerase chain reaction, InCell Analysis and enzyme-linked immunosorbent assay were the key research methods. For pharmacological research the main methods used were stability assays analysed by tandem mass spectrometry and high performance liquid chromatography, solvent emulsion evaporation, powder x-ray diffraction and scanning electron microscopy.

Conclusions:

Amorphous solid dispersions are an attractive option for the future production of a natural progesterone oral formulation with enhanced solubility. Such a formulation can be targeted for delivery to the distal small intestine where progesterone metabolism is reduced compared to other intestinal regions. This formulation would be of particular benefit to patients with endometrial hyperplasia as the most commonly used progestins to treat this disease were shown to differentially regulate genes and proteins in the human endometrium compared to the natural product.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT 1

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ACKNOWLEDGMENTS

I would like to dedicate this thesis to my father, Ian Coombes, and my grandmother, May Rowe, who started this journey with me and whom I wish were still here to see it come to an end. You both provided me with endless praise and encouragement and the memory of you both as hard-working, firm standing role models has helped see me through the last few years.

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Abbreviations

ABBREVIATION	FULL NAME
AF	Activation Factor
AKR	Aldo Keto Reductase
AR	Androgen Receptor
AREG	Amphireguline
ASD	Amorphous Solid Dispersion
BP	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic AMP/Cyclic Adenosine Monophosphate
cDNA	copy Deoxyrionucleic Acid
cT	Threshold Cycle
CYP	Cytochrome P450
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA Binding Domain
DMEM/F-12	Dulbecco's Modified Eagle Medium: Nutrient Mix F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differntial Scanning Calorimetry
E_1	Estrone
E_2	17-β Estradiol
E2V	Estradiol Valerate

E_3	Estriol
E_4	Estetrol
ECCAC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EE	Ethinyl Estradiol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen Receptor
ERE	Estrogen Response Element
Erα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
FaSSIF	Fasted State Simulated Intestinal Fluid
FBS	foetal bovine serum
FeSSGF	Fed State Simulated Gastric Fluid
FeSSIF	Fed State Simulated Intestinal Fluid
FOXO1	Forkhead Box O Protein 1
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GR	Glutocorticoid Receptor
GREB1	Gene Regulated in Breast Cancer 1

h	hours
H	Hyperplasia
HA	Hyperplasia with Atypia
HBSS	Hanks' Balanced Salt Solution
hCG	Human Chorionic Gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HESC	Human Endometrial Stromal Cells
HPLC	High Performance Liquid Chromatography
HPMC	Hypromellose
HPMCAS	Hypromellose Acetate Succinate
HRP	Horseradish peroxidase
HRT	Hormone Replacement Therapy
hTERT	Human Telomerase Reverse Transcriptase
ID	Inhibitory Domain
IGF1	Insulin-like Growth Factor 1
IGG	Imunoglobulin
IHC	Immunohistochemistry
k	Rate Constant
LBD	Ligand Binding Domain
LC MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LF	Lactoferrin
LH	Leutinising Hormone

LNG	Levonorgestrel
MAPK	Mitogen Activated Protein Kinase
MET	Mesenchymal to Epithelial Transition
MPA	Medroxyprogesterone 17-Acetate
MR	Mineralocorticoid Receptor
NADPH	nicotinamide adenine dinucleotide phosphate
P_4	Progesterone
P_{app}	apparent permeability coefficient
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCOS	Polycystic Ovarian Syndrome
PMSF	phenylmethanesulfonylfluoride
PR	Progesterone Receptor
PRE	Progesterone Response Element
PRL	Prolactin
PVDF	Polyvinylidene fluoride
PXRD	Powder X-Ray Diffraction
qRT-PCR	Quantitative Real-time polymerase chain reaction
RFU	Relative Fluorescent Units
RFU	Relative Fluorescent Units
RNA	Ribonucleic acid
RPM	Rotations per minute

RPM	Revolutions per Minute
RT	Reverse Transcription
SCF	Simulated Colonic Fluid
SD	Standard Deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
SIF	Simulated Intestinal Fluid
SRC	Steroid Receptor Coactivator
TBS	Tris-Buffered Saline
TEER	Transepithelial Electrical Resistance
TFA	Trifluoroacetic acid
Tg	Glass Transition
TGFα	Transforming Growth Factor Alpha
UV-HPLC	Ultra Violet - High Performance Liquid Chromatography
WHO	World Health Organisation

Chapter 1

Introduction

1. Introduction

1.1 The Female Reproductive System

The female reproductive system comprises two main parts; the ovaries and the uterus which are connected by the fallopian tubes. The uterus then joins the vagina at the cervix which opens to the external genitalia. At birth the reproductive organs are all present, though it is not until adolescence that they become fully mature and functional (Morton et al., 2011). The structure of the female reproductive system can be seen in Figure 1.1.

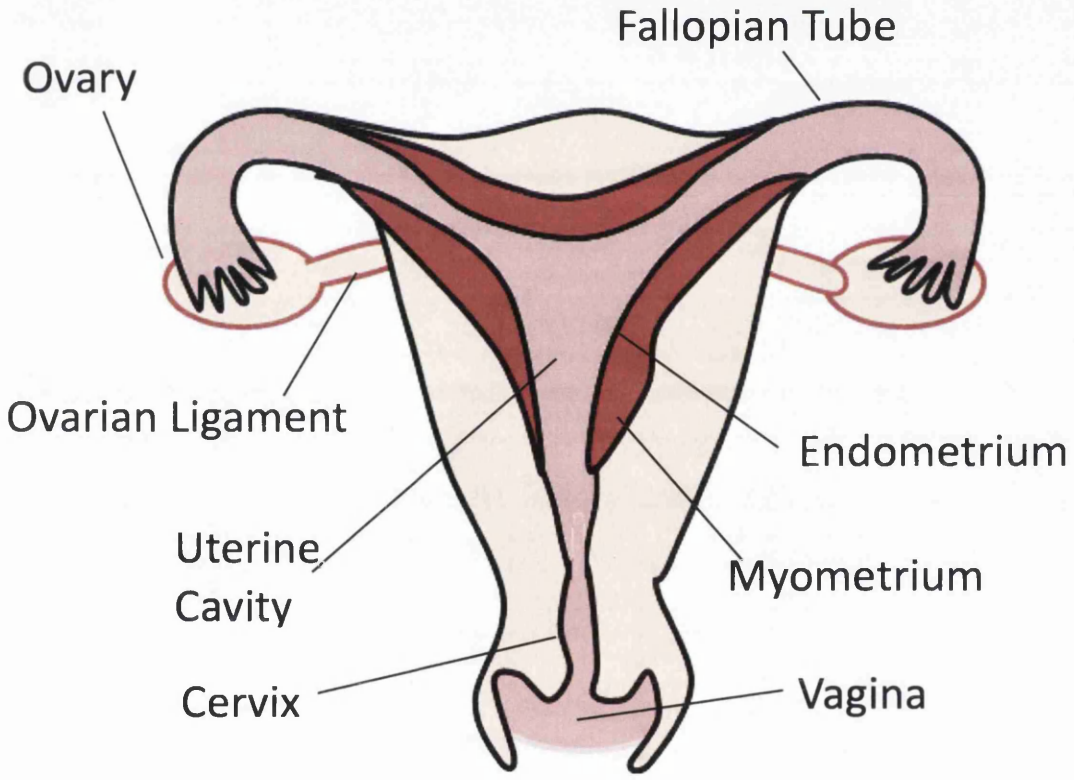


Figure 1.1: The structure of the female reproductive system. The system consists primarily of the two ovaries held in place by the ovarian ligaments which are connected to the uterus via the fallopian tubes. The uterus is made up of the uterine cavity and uterine wall consisting of layers including the myometrium and endometrium. The uterus connects to the cervix which opens into the vagina.

1.1.1 The Ovaries and Fallopian Tube

The ovaries lie in the pelvis and are kept in position through attachment to the pelvic peritoneum via two ligaments commonly known as the infundibulopelvic ligaments; they are also attached to the uterus through the utero-ovarian ligaments. The ovaries contain many follicles and each consist of an ovum (oocyte) surrounded by folliclular cells which are formed during the embryonic stage, upon puberty the follicle and oocyte begin to develop in a process called oogenesis. Once developed, the follicle ruptures, releasing the oocyte from the ovary in the process of ovulation (Sánchez and Smitz, 2012). The oocyte leaves the ovary and enters the fallopian tube where fertilisation occurs if sperm is present. The fallopian tubes are muscular tubes that extend from the superior corners of the uterus to the edge of the ovaries. The inside of each fallopian tube is covered in cilia that work with the smooth muscle of the tube to carry the ovum to the uterus (Saladin and Miller, 1998).

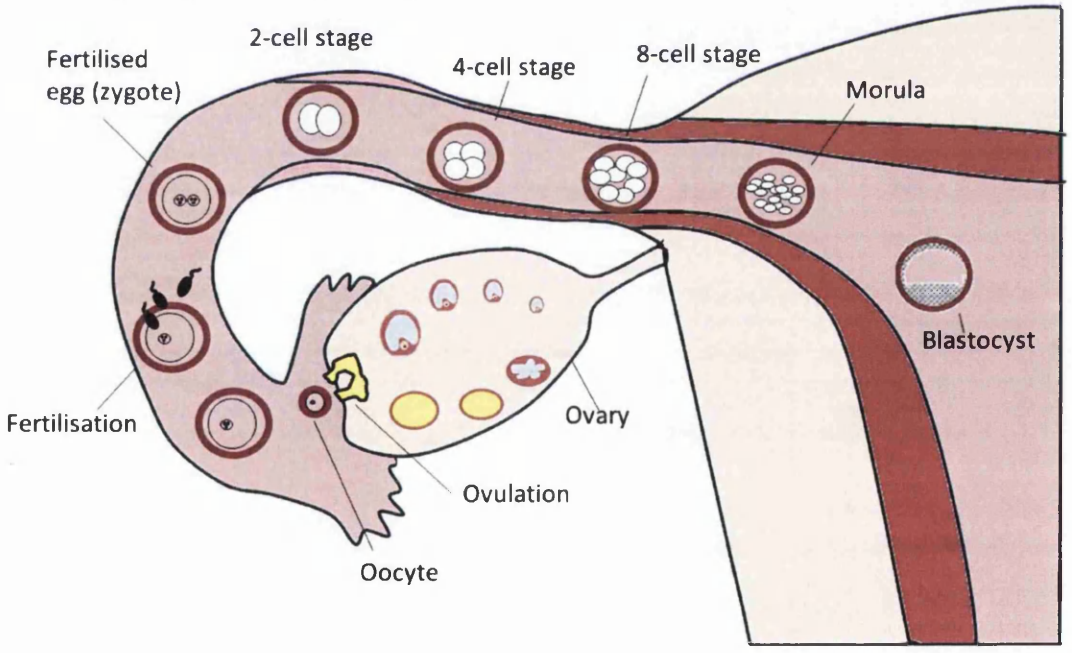


Figure 1.2: Development of the pre-implantation blastocyst in humans. The oocyte begins to mature within the follicle and is released into the fallopian tube during ovulation. After fertilisation the fertilised egg, now called the zygote, undergoes a series of cell divisions until becoming the morula and then the blastocyst.

If fertilisation occurs the oocyte begins to mature and undergoes cleavage as it travels down the fallopian tube becoming the morula. At 4 days post-fertilisation the morula enters the uterus where it undergoes further divisions to become the blastocyst (Moore et al., 2011) (Figure 1.2).

1.1.2 The Uterus

The uterus is a hollow, muscular organ which facilitates and maintains pregnancy. It is also the site of blastocyst implantation and home to the developing foetus for the whole of the gestation period. The uterus consists of three layers of tissue; the perimetrium, the myometrium and the endometrium. The perimetium is the outermost layer; it is a thin membrane that covers the uterus. The myometrium is the middle layer of tissue and is thick and muscular. The inner lining of the uterus, the endometrium, is thick and enriched with blood vessels and plays a role in protecting the cells of the myometrium; however the main role of the endometrium is in facilitating blastocyst implantation should fertilisation occur (Ramsey, 1994).

1.1.3 The Endometrium

The endometrium comprises of glandular and luminal epithelium which rests on blood vessel rich connective tissue called the stroma. The endometrium is described in terms of its layers; the stratum functionale or functional layer and stratum basalis or basal layer. The stratum basalis lays adjacent to the myometrium and contains stromal cells as well as deep glands and arteries which are retained throughout the menstrual cycle. The retaining of this layer is essential as it contains progenitor cells which serve as a regenerative source for the stratum functionale which is shed with each cycle (Nair and Taylor, 2010). Regeneration of the stratum functionale is characterised by extensive angiogenesis and an increase in estrogen and growth factor signalling (Maybin and Critchley, 2012). The stratum functionale makes up the superficial two-thirds of the endometrium and contains epithelial cells and glands; it also contains both densely and loosely packed stroma (Nair and Taylor, 2010). The stratum functionale and stratum basilis are depicted in Figure 1.3.

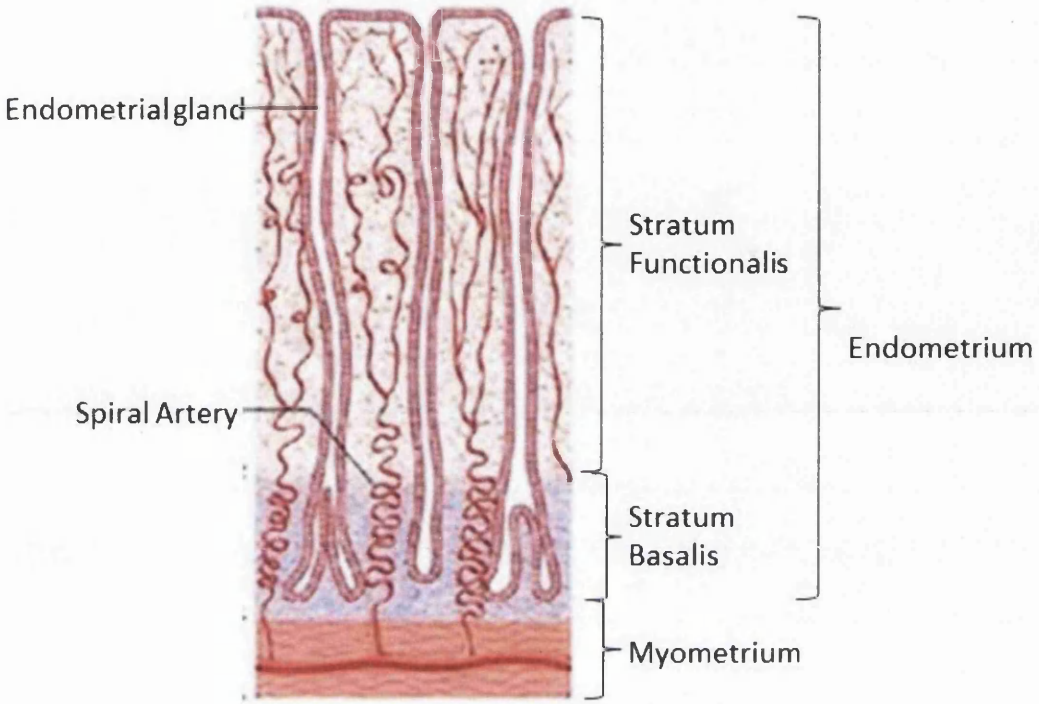


Figure 1.3: Layers of the endometrium. The endometrium is made up of the Stratum Functionalis and Stratum Basalis. The stratum basalis lays adjacent to the myometrium and contains stromal cells as well as deep glands and arteries which are retained throughout the menstrual cycle whereas the stratum functionalis makes up the superficial two-thirds of the endometrium and contains epithelial cells and glands and both densely and loosely packed stroma.

Adapted from http://quizlet.com/21043569/reproductive-system-flash-cards/

If fertilisation occurs then menstruation is halted due to progesterone secretion by the blastocyst. Progesterone maintains the thick, blood vessel rich endometrium providing a perfect environment for blastocyst implantation. Implantation occurs between days 20 and 24 post menstruation, the timing of implantation is crucial as it needs to be aligned perfectly with a change in shape of the underlying stromal cells in a process is known as decidualisation (Ozturk and Demir, 2010). If implantation is successful the blastocyst implants deep into the stroma and begins its development into the foetus. In the absence of fertilisation the oocytes degenerates and is sloughed along with the epithelial lining during menstruation and another oocyte begins to

mature within its follicle to begin another cycle. This process of cyclic shedding and regeneration is called the menstrual cycle.

1.2 The Menstrual Cycle

For the successful completion of the menstrual cycle each stage must be seamlessly controlled at a molecular level. Thus, the growth and differentiation of the human endometrium is regulated by the hypothalamic-pituitary axis and is under the coordinate control of the sex steroid hormones. There are four main hormones involved in controlling the menstrual cycle these are estrogen and progesterone (P_4) produced by the ovaries and luteinising hormone (LH) and follicle stimulating hormone (FSH) released by the pituitary (Sherman and Korenman, 1975).

Estrogen and progesterone are both key players in the regulation of the menstrual cycle with estrogen dominating the proliferative phase and progesterone dominating the secretary phase (Sherman and Korenman, 1975). As a result, the basal level of circulating plasma estrogen and progesterone changes throughout the menstrual cycle and can vary significantly between individuals. During the proliferative phase of the cycle progesterone levels have been measured with a mean concentration of 0.32 (+/- 0.25) ng/ml, this increases steadily during the luteal phase with a plateau to between 10-20ng/ml between days 16 and 20 of the cycle (Johansson, 1969, Sherman and Korenman, 1975). The basal level serum estradiol also changes drastically throughout the menstrual cycle. In healthy volunteers, prior to the surge in LH, estrogen levels rise sharply constituting the proliferative phase of the cycle and fall abruptly between one and two days after the LH surge (Hotchkiss et al., 1971).

In the endometrium the menstrual cycle takes place in three distinct phases: the menstrual phase (days 0-5), the proliferative phase (days 5-14) and the secretory phase (days 14-28). In the human endometrium estrogen and progesterone play key roles in these phases; estrogen stimulates endometrial cellular proliferation during the proliferative phase while progesterone, in the secretary phase, exerts its differentiation effects upon the estrogen-primed endometrium (Sherman and Korenman, 1975). The ovaries also undergo cyclic changes during the menstrual cycle. Unlike the endometrium the ovaries undergo two distinct phases; the follicular

phase and the luteal phase. The follicular phase takes place on days 1-14 of the cycle and the luteal phase takes place on days 14-28 (Johnson, 2012b). The changes in hormone levels during the menstrual cycle can be seen along with the different endometrial phases in Figure 1.4

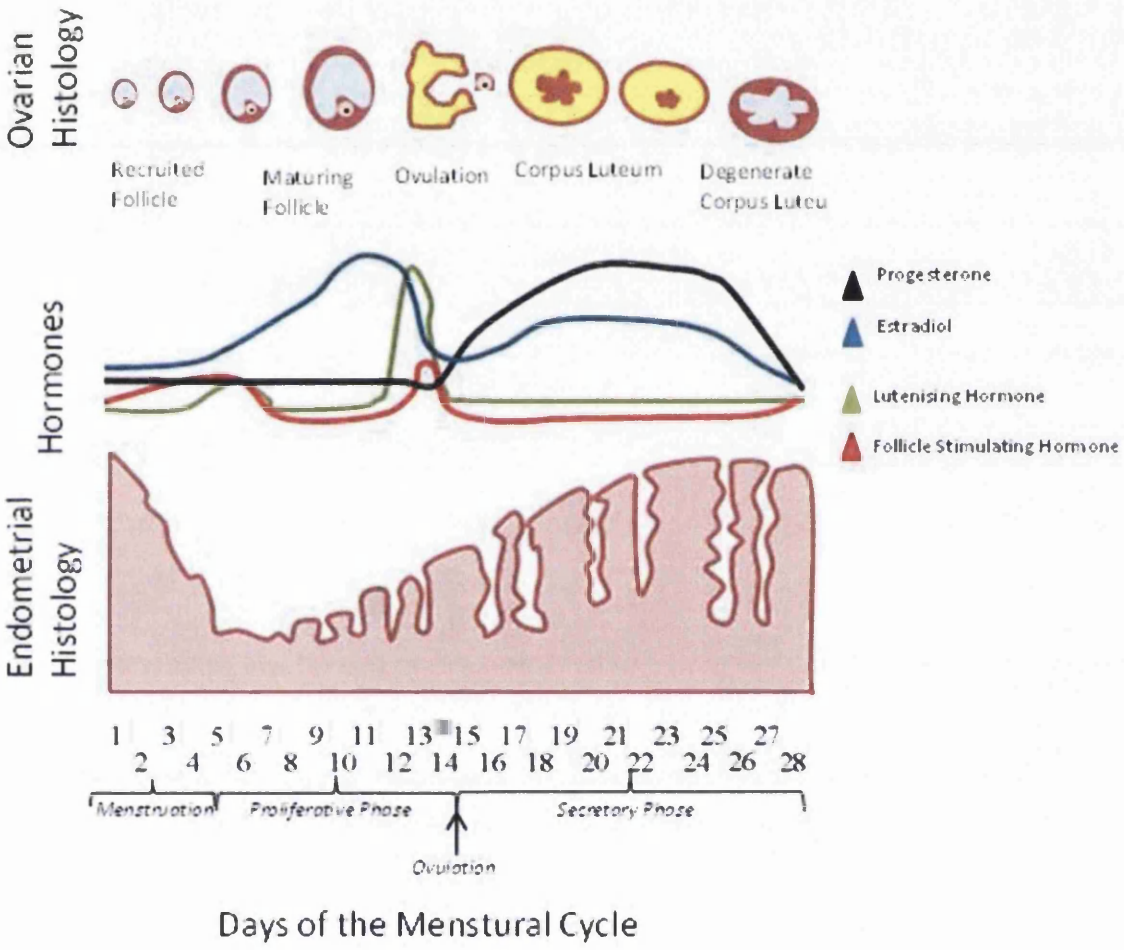


Figure 1.4: Stages of the Menstrual Cycle. During menstruation the endometrial lining is shed and the levels estrogen begin to increase. During the proliferative phase of the cycle the endometrium starts to thicken in line with increasing levels of estrogen. The ovarian follicle also begins to mature during this phase. Day 14 marks ovulation whereby the primary oocyte is released from its follicle. Ovulation correlates with a sharp increase in leutenising and follicle-stimulating hormone. Following ovulation the level of estradiol declines whereas progesterone levels increase until the released oocyte becomes the corpus luteum and begins to degenerate.

1.2.1 Menstruation

Days 1-5 of the menstrual cycle are characterised by the sloughing of the endometrial lining and the ovum. During this phase estrogen, primarily 17-β estradiol (E_2), and progesterone are at their lowest. The menstruation phase in the endometrium also marks the beginning of the follicular phase in the ovaries, during this time the gonadotropin, FSH, is released from the pituitary gland, stimulating the maturation of follicles in the ovary which bare the oocyte. At this stage the oocyte has not reached full maturation and is referred to as the primary oocyte (Sánchez and Smitz, 2012). The end of menstruation marks the beginning of the proliferative phase of the cycle.

1.2.2 The Proliferative Phase

The proliferative phase takes place on days 5-14 of the menstrual cycle. During this time the developing ovarian follicle produces estrogen, which promotes endometrial proliferation via signalling through its receptors; estrogen receptors α and β (ERα and ERβ). Proliferation of the endometrium produces a thick endometrial lining in preparation for blastocyst implantation, should conception occur. The increase in estrogen levels also stimulates the expression of ERα, ERβ and progesterone receptors (PRA and PRB) in all endometrial cell types to allow the receptor-based signalling of estrogen and progesterone (Horne and Blithe, 2007). In the normal, fertile, endometrium the highest concentration of progesterone receptors (PRs) is found in epithelial cells during the late proliferative phase of the cycle and declines throughout the late secretory phase with the concentration of PR being greatest in the glandular epithelium (Nisolle et al., 1994). Although there is a lower concentration of PR in endometrial stroma, the receptors show only minor fluctuations during the menstrual cycle, with PR remaining high in the secretory phase (Ingamells et al., 1996).

During the proliferative phase, the endometrial glands are simple and tubular and open out onto the surface of the endometrium, there is no crowding of glands within the stroma with a <50% gland to stroma ratio (Figure 1.5) (Deligdisch, 1999).

On day 14 of the cycle the rising levels of estradiol reach their peak and stimulate the pituitary gland to release the second gonadotropin, LH. The surge in levels of LH causes the mature ovarian follicle to rupture and release the oocyte (now termed the secondary oocyte) into the fallopian tube. The release of the secondary oocyte triggers the beginning of the luteal phase in ovaries and the secretary phase in the endometrium (Johnson, 2012b).

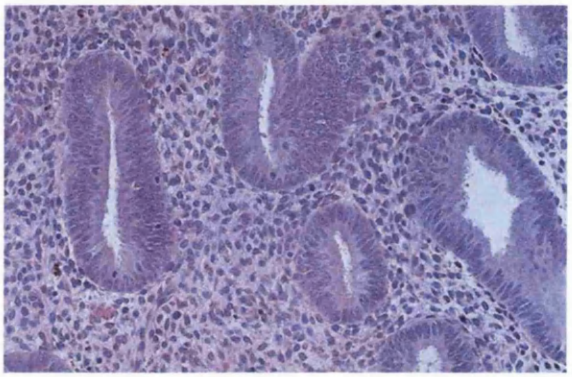


Figure 1.5: Typical biopsy of healthy proliferative endometrium

1.2.3 The Secretory phase

The secretory phase of the cycle begins with ovulation. At this time the ovaries enter the luteal phase of the cycle and cease to produce estrogen causing the levels of estrogen to drop. The released ovum makes its way down the fallopian tube becoming the corpus luteum or 'yellow body' which produces progesterone, causing progesterone levels to rise accordingly. This increase in progesterone causes a second smaller peak in estrogen during the secretory phase (Ingamells et al., 1996). The progesterone increase is also the trigger for the change in phase from the proliferative into the secretory; suitable for implantation of the blastocyst should conception occur. The rising levels in progesterone regulate genes involved in glycogenesis (Demers et al., 1977), protein synthesis and secretion, and cell cycle regulation (Savouret et al., 1990). The increase in progesterone also down-regulates its own receptor (Graham and Clarke, 1997a). Thus the expression of PR declines in epithelium and is undetectable in glandular epithelium in the late secretary phase. However, PR content within the stroma persists throughout the menstrual cycle (Mertens et al., 2001, Lessey et al., 1988). Without conception the corpus luteum

atrophies and the levels of progesterone drop triggering menstruation. During this stage the rise in progesterone levels also causes important changes in the structure of the endometrium. The endometrial glands increase in tortuosity and fill with glycogen allowing them to secrete a glycogen-rich secretion which is an important source of nutrition for the fertilised ovum (Deligdisch, 1999). The abundance of the glands also increases where the endometrium may have a more than 50% gland to stroma ratio. During this time the glands may exhibit crowding but are organised and not mitotically active (Figure 1.6). The underlying stromal cells also undergo structural changes during this time in a process known as decidualisation.

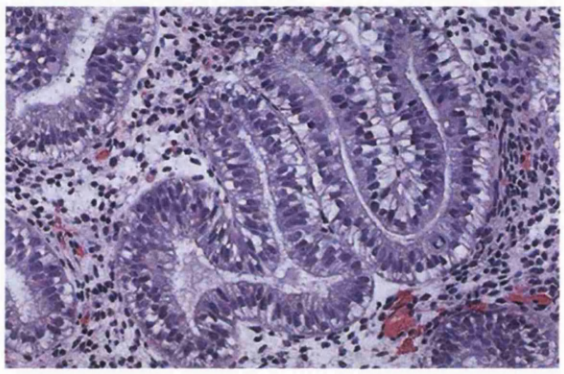


Figure 1.6: Typical biopsy of healthy secretory endometrium.

1.2.4 Decidualisation

During the late secretory phase of the menstrual cycle the proliferative activity in the glandular epithelia ceases and the epithelial cells undergo transformation into a secretory state. However, cells of the stromal compartment continue to proliferate into the mid-late secretary phase when they undergo decidualisation, a process whereby progesterone instigates functional changes throughout the stroma (Psychoyos, 1973). Decidualisation can be described as the post-ovulatory re-modelling of the uterus in preparation for pregnancy (Gellersen et al., 2007). During decidualisation differentiation and proliferation of the fibroblast-like mesenchymal cells of the stroma to a decidual cell which is morphologically and biochemically distinct (Tang et al., 1994, Salamonsen et al., 2003), (Figure 1.7). Cyclic adenosine monophosphate (cyclic AMP, cAMP) is a second messenger that initiates intracellular signal transduction through cyclic AMP dependent pathways (Rasmussen, 1970) and is absolutely paramount in the process of decidualisation

(Gellersen and Brosens, 2003). If implantation occurs the uterus remains in a state of decidualisation and is referred to as decidua, however, without implantation the structure breaks down.

The process by which stromal cells decidualise is called the mesenchymal to epithelial transition (MET) (Tang et al., 1994). During the MET the cytoskeleton is reorganised to alter interaction between cells and their surrounding tissue. Mesenchymal cells are undifferentiated cells that are loosely connected to their surroundings whereas epithelial cells are characterised by tight junctions and cell-cell adhesion markers (Kalluri and Weinberg, 2009). A key player in stromal decidualisation is decidual prolactin; a hormone known for regulating immune and endocrine functions. Prolactin is expressed in response to protein kinase A, which is induced by cyclic AMP. Progesterone works in synergy with cyclic AMP to upregulate decidual prolactin gene expression (Telgmann and Gellersen, 1998). Thus, hormone signalling and intricate communication between hormone-bound receptors and other proteins is crucial for successful completion of the menstrual cycle, for decidualisation and for blastocyst implantation.

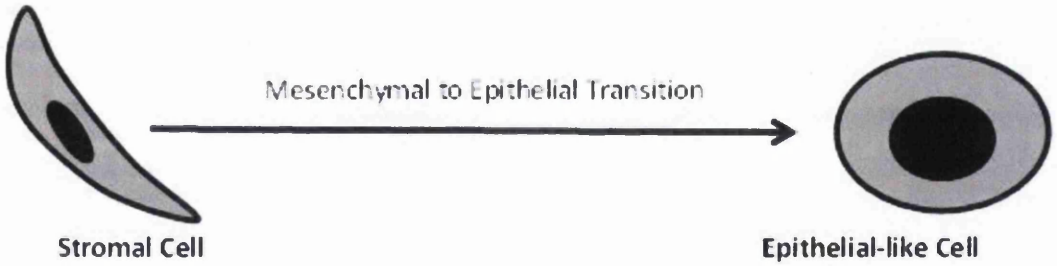


Figure 1.7: Mesenchymal to epithelial transition. During decidualisation stromal cells undergo the mesenchymal to epithelial transition whereby they transform from a stromal cell to a round cell with an epithelial like phenotype.

1.2.5 Menopause

Following the reproductive phase of life women will experience menopause. Menopause is defined as the permanent cessation of menstruation as a result of the loss of ovarian follicular activity (Utian, 1999). After menopause the endometrium undergoes gradual atrophy, starting as an inactive phase which ends up as a thin layer, often riddled with cystic cavities lined by cuboidal or flat epithelium

(Deligdisch et al., 1978)(Figure 1.8). Failure to ovulate after menopause first deprives the endometrium of progestergenic stimulation. The estrogenic stimulation may continue in some situations because of the conversion of androgens secreted by the postmenopausal ovaries and cortices of the adrenal glands, into estrogens. Obesity, diabetes and metabolic disorders may enhance the extra gonadal endogenous estrogen production by aromatisation (Kirschner et al., 1982, Friberg et al., 2007). High estrogen levels, especially estradiol, are often associated with endometrial diseases, in particular endometrial hyperplasia (Gurpide et al., 1976).

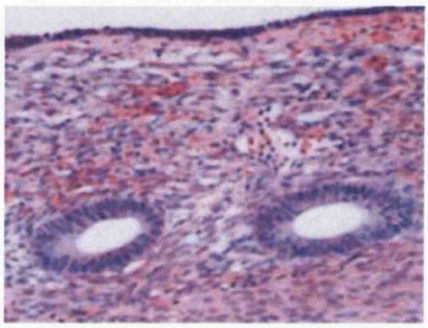


Figure 1.8: Normal post-menopausal endometrium. Adapted from Khoury 2006 (Khoury et al., 2006).

1.3 Endometrial Disease

Endometrial disease refers to a number of conditions affecting the endometrium. The most common endometrial disorders include endometriosis, polycystic ovarian syndrome (PCOS) and hyperplasia. In many incidences endometrial disease presents a heightened risk of endometrial cancer development (Lacey and Chia, 2009, Zanetta et al., 2000, Escobedo et al., 1991), which affects 1.6% of women in developed countries (Jemal et al., 2011). Endometrial disease is often linked to a hormonal imbalance, for example PCOS is linked to heightened androgen signalling (Ehrmann, 2005) and endometrial hyperplasia is linked to chronic estrogen stimulation (Gurpide et al., 1976). PCOS is most common in women aged between 18 and 44 (Teede et al., 2010) and in some cases can lead to hyperplasia making PCOS the main cause of hyperplasia in young women (Balen, 2001).

1.3.1 Endometrial Hyperplasia

Endometrial hyperplasia is a benign condition characterised by abnormal proliferation of endometrial glands whereby the glands proliferate in both size and shape with an increased gland to stroma ratio. The importance of this disease is highlighted by its association with endometrial cancer (Montgomery et al., 2004). The risk of cancer progression is dependent on the type of endometrial hyperplasia. The World Health Organisation (WHO) classification of hyperplasia is based on glandular features (stromal architectural pattern) which are described as simple and complex, and the presence or absence of nuclear atypia (Skov et al., 1997). Nuclear atypia is described as the abnormal, often pleomorphic, appearance of cell nuclei. Identification of these features allows hyperplasia to be grouped into four major categories; the simple and complex hyperplasia without atypia and the simple and complex hyperplasia with atypia. Simple hyperplasia with and without atypia can be seen in Figure 1.9A and B.

The WHO classification system correlates the hyperplasia groups with the risk of progression to endometrial carcinoma. For hyperplasia without atypia there is a less than 5% risk of cancer progression but when atypia is present the risk increases to approximately 30% (Lacey et al., 2010, Gerulath and Borth, 1977). However, these statistics may be unreliable as the finding of nuclear atypia, which is considered the best indicator of malignant potential, has a wide inter-observer variation (Zaino et al., 2006, Kendall et al., 1998).

Furthermore, the literature demonstrates that incidence of simple hyperplasia without atypia is highest in women age 50-54, whereas the rate of atypical hyperplasia is highest in women age 60-65 (Lacey et al., 2010, Rakha et al., 2012). However, regardless of hyperplasia type, an overwhelming body of evidence suggests that this disease occurs as a result of chronic estrogen stimulation unopposed by the counterbalancing effects of progesterone (Bergeron et al., 1999, Herrinton and Weiss, 1993, Smith et al., 1975).

1.3.2 Endometrial Cancer

Cancer of the endometrium can manifest as one of two pathogenetic types. The first type (Type 1) is characterised by well differentiated tumours, superficial invasion of the myometrium, sensitivity to progestin therapy and a favourable prognosis. The second endometrial cancer type (Type 2) presents with poorly differentiated tumour cells, deep myometrial invasion, a high occurrence of metastatic spread, low sensitivity to progestin treatment and a poor prognosis (Bokhman, 1983, Setiawan et al., 2013).

As with endometrial hyperplasia, type 1 endometrial cancer is often associated with over-exposure to estrogen (Weiderpass et al., 1999b, Herrinton and Weiss, 1993). Estrogen stimulation causes enhanced and long-standing endometrial proliferation leading to the gradual development of hyperplasia, followed by atypical hyperplasia and eventually type-1 cancer (Persson, 2000, Whitehead et al., 1981, Kim et al., 2013a). Type 1 tumours and endometrial hyperplasia often have positive staining for progesterone receptors making them sensitive to progestin therapy (Carcangiu et al., 1990). A typical endometrial biopsy can be seen in Figure 1.9C.

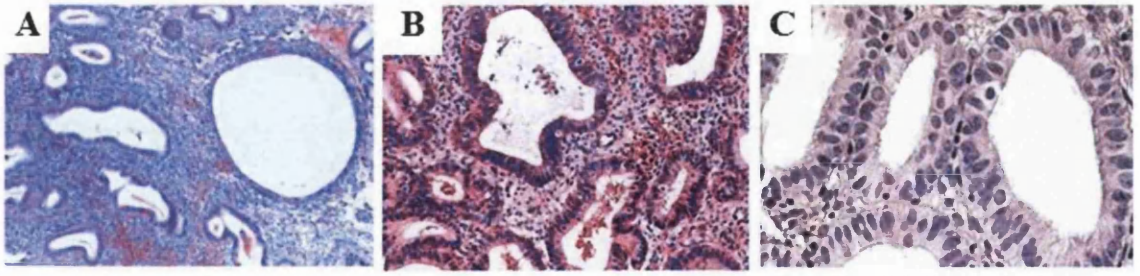


Figure 1.9: Human biopsies of endometrial hyperplasia without atypia (A), and with atypia (B) and type 1 endometrial cancer (C). Images adapted from Horn (2007)

1.4 Estrogens and Progestogens

The different roles of estrogen and progesterone in the normal and diseased endometrium are due to their ability to bind their receptors with high affinity and cause downstream regulation of target genes. Progestogens are a class of steroid hormones that bind to and activate the progesterone receptor (PR) with the most important progestogen being progesterone. Progesterone is a C-21 steroid hormone whereas the estrogens are a group of C-18 steroid hormones. The three main estrogen steroids are estrone (E_1), estradiol (E_2, 17β-estradiol) and estriol (E_3) with E_2 being the most abundant estrogen during the reproductive years. There is also a fourth estrogen called estetrol (E_4) which is only expressed during pregnancy.

The structures of natural estrogen and progesterone, along with androgen, were first determined after their isolation in the 1930s. The realisation that these steroids could be useful tools for disease treatment and contraception first came about after scientist Ludwig Haberlandt demonstrated temporary contraceptive effects in a female rabbit transplanted with the ovaries of a second pregnant rabbit (Müller-Jahncke, 1988). Soon after, it was confirmed that high levels of steroid hormones could provide inhibition of ovulation (Goldzieher, 1982, Perone, 1993).

Following this discovery, estrogens were employed by a number of investigators in an attempt to inhibit ovulation for the treatment of dysmenorrhea but it was soon realised that progesterone was a key player (Drill, 1977). The door to large scale production of progesterone was opened in the 1940s when Russell E. Marker discovered a method of extracting progesterone from diosgenin, a steroid sapogenin found in wild yam (*Dioscorea villosa*), through the removal of its side chain (Lehmann et al., 1973). This method of extraction could provide large quantities of progesterone at low-cost; allowing commercial availability of the drug.

Further investigation into the potential role of steroid hormones in the inhibition of ovulation meant attempts to develop hormone based therapies got under way. However, production of pharmacologically active estrogen and progesterone-based drugs proved difficult due to the poor bioavailability of both hormones resulting from their poor solubility and extensive metabolism.

1.4.1 Estrogen (17β-Estradiol)

17β-estradiol (E_2) has a molecular weight of 272.382 Da and an empiric formula of $C_{18}H_{24}O_2$. The chemical structure of E_2 can be seen in Figure **1.10**.

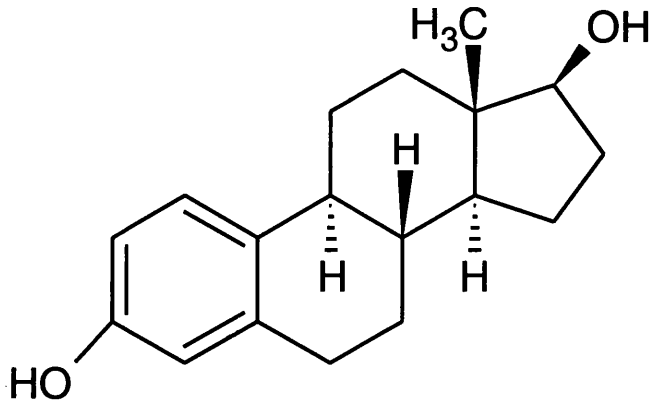


Figure 1.10: The Chemical Structure of 17β-estradiol. Produced using ACD Structure Elucidator, version 12.01 (Advanced Chemistry Development inc, 2014)

E_2 is virtually insoluble in water with an aqueous solubility of 1.51μg/mL (Shareef et al., 2006). After oral administration only 10% of estrogen is absorbed into the blood as complete estradiol with the rest being metabolised prior to absorption (Longcope et al., 1985).

As a result of its poor bioavailability estradiol is usually administered in the form of ethinyl estradiol (EE) or estradiol valerate (E2V), which are taken along with progestins for HRT and contraception (Nelson, 2007, Ahrendt et al., 2009, Endrikat et al., 2008). Synthetic estrogen is also administered alone for the treatment of HRT in women without a uterus or administered in combination with a progestin for women with a uterus. EE is produced by substituting carbon 17 of the estradiol molecule with an ethinyl group which serves to reduce the rate of metabolism of the drug in the intestinal mucosa and liver (Shoupe and Kjos, 2006). E2V on the other hand acts as a pro-drug; it is a synthetic ester and upon entry to the body, it is cleaved by esterases during intestinal absorption (Düsterberg and Nishino, 1982).

The bioavailability differs between EE and E2V. A study carried out in 2005 showed that the bioavailability of EE taken orally with a synthetic progestin as part of an oral

contraception is approximately 55% (van den Heuvel et al., 2005). The reduced amount of EE seen is mainly due to first pass metabolism in the small intestine. EE is shown to be susceptible to extensive metabolism in the human jejunal mucosa, followed by further metabolism in the liver which leads to the breakdown of estradiol into inactive metabolites. Several factors, including the patients BMI and administering EE in combination with synthetic progestins can affect the bioavailability of EE (Edelman et al., 2009).

After oral administration, E2V is rapidly and completely absorbed through the small intestine. After and during absorption the metabolic processes that occur in the GI tract, intestinal mucosa and liver rapidly cleave E2V into desired metabolites; 17β-estradiol and valeric acid. However, extensive metabolism of the resulting free estradiol also occurs in these regions. As a result only ~3% of an orally administered dose of 20mg E2V will enter the blood as unchanged E_2 (Düsterberg and Nishino, 1982).

1.4.2 Progesterone

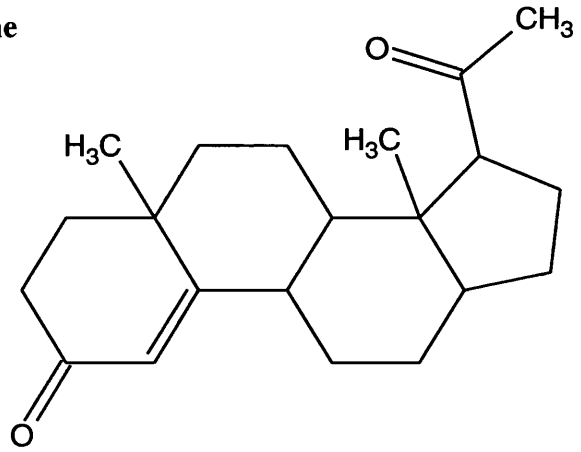


Figure 1.11: The Chemical Structure of Progesterone. Produced using ACD Structure Elucidator, version 12.01 (Advanced Chemistry Development inc, 2014)

Progesterone is also known as pregn-4-ene-3,20-dione and has a molecular weight of 314.46 Da and an empirical formula of C_{21} H_{30} O_2 (SIGMA-ALDRICH, 2010). The molecule contains two ketone and oxygenated functional groups, two methyl branches and four hydrocarbons, depicted in Figure 1.11. The presence of two ketone

groups in the A-ring makes progesterone susceptible to enzymatic degradation in the small intestine and liver leading to poor bioavailability when delivered orally (Stanczyk, 2002).

A second major problem affecting the oral bioavailability of progesterone is solubility; poorly soluble drugs have reduced intestinal absorbance hence reducing their passage to the blood stream. Progesterone is hydrophobic with a reported aqueous solubility of 16.8ug/ml (Haskins, 1949), though recent research suggests the solubility of progesterone in water is almost half this amount (8.81µg/ml) (Yalkowsky et al., 2010). It is, however, more soluble in alcohol and sparingly soluble in oil, hence micronized progesterone is often delivered in oils (Hsia et al., 2005). The low bioavailability of progesterone has been partly overcome in the form of micronized progesterone which contains natural progesterone with a considerably reduced particle size. The reduction in progesterone particle size increases its rate of dissolution within the intestinal fluids leading to increased intestinal absorption and enhanced bioavailability (Chaumeil, 1998).

In 1993 a study on healthy postmenopausal women determined that the bioavailability of progesterone, in its micronized form, was approximately 8.6% (Simon, 1993). The process of micronization drastically reduces the average diameter of particles. Micronized progesterone is absorbed rapidly with 50-60% of administered dose being successfully absorbed into the hepatic portal vein according to urinary concentrations of pregnanediol, a biologically inactive derivative of pregnane, formed by the reduction of progesterone (Bruno, 1999). The degradation of progesterone causes high levels of circulating metabolites, mainly pregnanediol, pregnanolone, allopregnanolone, pregnanedione, 20a-dihydroprogesterone, and 17-OH progesterone (de Lignières, 1999, Ottoson et al., 1984, Andréen et al., 2005).

Despite high absorbance rates, the bioavailability of progesterone remains low. Several studies have been conducted to assess the bioavailability of micronized progesterone. Norman *et al* (1991) treated healthy premenopausal women with an oral dose of 200mg of micronized progesterone. Bioavailability varied widely between individuals with peak plasma levels of progesterone ranging from 8.5 to

70.6 ng/ml 4 hours after administration. During the study orally administered progesterone was compared with vaginally administered progesterone and no significant difference in bioavailability was found between the two routes of administration (Norman et al., 1991). A study by Maxson and Hargrove (1985) also investigated the bioavailability of micronized progesterone (200mg) administered orally to 9 healthy postmenopausal women. Progesterone was rapidly absorbed with peak plasma levels of 17.0 (+/-4.9) ng/ml being achieved at approximately 2.8 hours post administration and returning to normal by 24hours. These concentrations were equivalent to progesterone levels seen during the mid-luteal phase of the cycle. Interestingly the study also showed that progesterone caused no significant change in the levels of endogenous E_2, FSH, LH, cortisol, aldosterone, lipids or hepatic enzymes within the 24hour time frame (Maxson and Hargrove, 1985).

Finally, a similar study was carried out by Nahoul *et al* (1987) whereby 200mg of micronized progesterone was given orally to 6 healthy volunteers. Although this study showed peak plasma levels were apparent at a similar time to Maxson's study (Tmax = 3.00 ± 0.44 h), they found that mean peak plasma levels were 4.70 ± 1.15ng/ml with a range of up to 10.10 ng/ml, thus further demonstrating inter-individual variability (Nahoul et al., 1987). Variation in results may also be linked to the different methodology used in the studies.

Due to the poor bioavailability and short half-life of micronized progesterone it has been limited to the treatment of certain reproductive disorders such as in HRT for the management of secondary amenorrhea (Levine and Watson, 2000a) and in assisted pregnancy as a luteal phase supplement (Friedler et al., 1999).

Despite the draw-backs with its bioavailability the development of an orally active natural progesterone formulation is still desirable. Research shows that the use of natural micronised progesterone has very few side effects and brings about benefits in lipid profiles and osteoporosis prevention (Goletiani et al., 2007, Sitruk-Ware et al., 1987, Murray, 1998).

Due to the poor bioavailability of natural P_4, a great deal of research has gone into the development of synthetic progesterone analogues which evade the detrimental

first pass effect. Early research in this area led to the development of the first commercially available oral contraceptive, Enovid, which contained the synthetic progestins mestranol and norethynodrel and was distributed in America and the UK in the 1960s (Müller-Jahncke, 1988). Like all contraceptives, Enovid worked by mimicking the effects of the ovarian steroids. The consequences of taking such drugs are primarily ovulation inhibition but changes in cervical mucus that inhibit sperm penetration also occur (Rivera et al., 1999).

Since the production and discontinuation of Enovid, a variety of hormone-based oral formulations has been made available to women nationwide. As well as concraception, such formulations are used in the treatment of diseases, particularly, endometrial hyperplasia and cancer.

1.5 Progestin Therapy for Endometrial Disease

The use of progestins for the treatment of endometrial hyperplasia and cancer serves to promote endometrial atrophy by inhibiting estrogenic proliferation and inhibiting apoptosis (Saegusa and Okayasu, 1998, Kyo et al., 2011, Amezcua et al., 2000, Syed and Ho, 2003). Such actions occur through the activation or repression of target genes. However, the synthetic progestins used in hormone therapies are often associated with adverse gene regulation as described later in this chapter. The use of man-made progestins for the treatment of endometrial disorder is often essential due to the poor bioavailability of the natural product (Simon et al., 1993) as described in section 1.4.2. At present, in the UK, natural progesterone is not prescribed for oral dosing. However, orally administered natural progesterone was previously prescribed as part of hormone replacement therapy (HRT) in order to alleviate menopausal symptoms. For the purpose of HRT, progesterone was used to offset the carcinogenic potential of estrogen. For its rare use in HRT progesterone was administered at 200mg daily under the trade name Utrogestan (Besins and Besse, 2013). However, this drug was discontinued in August 2014 due to 'low level use' (The_Parmaceutical_Journal, 2014).

Other natural, micronized, progesterone products are still prescribed in the UK, though these are delivered via different routes. For example, Gestone (Nordic

Pharma Ltd) is delivered intramuscularly, Crinone (Juniper Pharmaceuticals) is delivered as a vaginal gel and cyclogest (L.D Collins and Co) is delivered as a pessary or suppository. Each of these preparations contain micronized progesterone and is used in assisted reproduction as a luteal phase support with cyclogest being the most commonly used (Kassab et al., 2008). For assisted reproduction oral micronized progesterone is unsuitable. After oral administration the use of natural, micronized, progesterone remains limited due to its susceptibility to extensive first-pass metabolism. As a result, oral micronized progesterone is not adequate to confer decidual transformation of the endometrium which is the main goal of luteal phase support (Bourgain et al., 1990).

The reason for using natural progesterone in assisted reproductive cycles as opposed to synthetic progestins is due its low incidence of systemic side effects (de Ziegler and Fanchin, 2000). Synthetic progestins are often associated with undesirable gene regulation which is preferably avoided in the delicate process of assisted pregnancy.

However, synthetic progestins are strong PR agonists and have a high affinity for the progesterone receptor as well as a much higher bioavailability after oral administration meaning small doses of progestin can be administered with maximal progestational activity. Thus, when it comes to the treatment of most reproductive disorders synthetic progestins are the choice treatment. Two of the most important medical applications that synthetic progestins are used for are in the treatment of endometrial hyperplasia and endometrial cancer.

To date, the main progestins used to treat hyperplasia are orally administered medroxyprogesterone acetate (MPA), or levonorgestrel (LNG) delivered through an inter-uterine device. Women with complex and atypical hyperplasia tend to be treated with hysterectomy due to the increased cancer risk whereas treatment guidelines generally recommend hyperplasia without atypia is treated with progestins (Reed et al., 2009). Progestin treatment is also given to women who wish to maintain fertility or are at postmenopausal age and have other co-morbidities that make them unsuitable for surgery.

As well as being a commonly used progestin for hyperplasia treatment, MPA is also used to treat type 1 endometrial cancers. MPA is given orally at 200mg a day or given intramuscularly via 400mg twice-weekly injections (Kohorn, 2012) but this form of treatment can incur severe side-effects (Bafaloukos et al., 1999, Thigpen et al., 1999).

1.5.1 Discovery and Implementation of Hormone Treatments

The first indication that hormones could be used for the treatment of hyperplasia and cancer predates the discovery of the hormones themselves; in the 1870s Thomas Beatson noted a link between ovaries and breasts milk and went on to discover that removal of the ovaries diminished milk production in the breast. He then investigated the role of the ovaries in breast cancer and found that their removal resulted in breast cancer regression, thus he had found that the stimulating effects of the ovarian hormones could lead to cancer of the breast (Cancer.org, 2015). However, the hormones themselves were not discovered nor put to use for disease treatment until decades later (Goldzieher, 1982, Perone, 1993).

The treatment of endometrial hyperplasia and cancer came about after Wellenbach and Rakoff (1953) discovered that ovariectimised hamsters developed endometrial hyperplasia following prolonged estrogen treatment and that the hyperplasia regressed when progesterone treatment followed. The two also found that hyperplasia development was prevented when both hormones were administered in combination (Wellenbach and Rakoff, 1953).

Following this discovery, the use of progesterone for both endometrial cancer and hyperplasia treatment was suggested (Kelley and Baker, 1961, Kistner, 1959, Kistner et al., 1965). However, no suitable medication was available; the poor bioavailability of progesterone meant that painful daily intramuscular injections would be required (Kohorn, 2012). Thus the synthetic progestins came into play. Early studies into the effects of synthetic progestins on endometrial cancer showed that it was effective in over 30% of patients and that these patients had slow growing cancers and well-differentiated tumours, (Kelley and Baker, 1960) which are now referred to as type1 tumours (Bokhman, 1983).

Wellenbach and Rakoff's discovery not only instigated progesterone-based hyperplasia and cancer treatment but it also prompted investigation into the carcinogenic effects of estrogen in humans. Studies of women taking estrogen for hormone replacement therapy were carried out. The data obtained showed this regimen substantially increased both hyperplasia and cancer incidence (Weiderpass et al., 1999a, Woodruff et al., 1994). However the addition of progestins to this treatment prevented the hyperplasia and cancer risk (Rose, 1996), further elucidating the role of progestogens as inhibitors of tumorigenesis (Yang et al., 2011). This finding was further confirmed in the Million Women Study (Collaborators, 2005). Therefore, the synthetic progestins became widely used for the treatment for endometrial hyperplasia and progestin sensitive endometrial carcinoma.

Marketed progestin-based drugs are often described in generations. The generations are based on the time in which the progestin was introduced to the market with more recently manufactured progestins having the most desirable molecular profile.

As well as being classified in generations, progestins can also be grouped based on their structural derivation. Synthetic progestins can be derived from estrogen, progesterone or androgen and these are known as the estranges, pregnanes and gonanes respectively. The different structures of the progestins can lead to different selectivity for specific hormone receptors, namely the progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and estrogen receptor (ER) (Sitruk-Ware and Nath, 2010). The binding affinites of natural progesterone for these receptor are described in Table 1.1. The binding affinities of the two commonly used synthetic progestins, MPA and LNG, are also described for comparison.

	P4	MPA	LNG
PR	100	298	323
GR	11	58	7.5
AR	3	35	58
MR	1000	3.1	17
ER	0.5	<0.02, 0	<0.02

Table 1.1: Relative binding affinities of progesterone, MPA and LNG to steroid hormone receptors. Relative binding affinities are expressed as a percentage of the total specific binding of natural progesterone. Hormone receptors described include Progesterone Receptor (PR), Glucocorticoid Receptor (GR), Androgen Receptor (AR), Mineralocorticoid Receptor (MR) and Estrogen Receptor (ER). Table adapted from (Africander et al., 2011).

1.5.2 Medroxyprogesterone Acetate (MPA)

The first generation progestin, MPA, was developed in 1954 and administered to women for the treatment of endometriosis and recurrent miscarriage. It was subsequently realised that women receiving the drug late in pregnancy experienced a notable delay in fertility following birth, leading to the use of MPA as a method of contraception. Within 10 years of its development MPA was marketed as long acting injectable contraception under the name Depo-Provera and is still available today in over 90 countries worldwide (Rabe and Runnebaum, 1999). In fact, data from 2010 suggests that Depo-provera is the most widely administered progestin-only contraceptives. Moreover, MPA is the most commonly used progestin for treatment for endometrial hyperplasia and endometrial cancer (Vereide et al., 2006, Gunderson et al., 2012).

MPA is also known as 17α-hydroxy-6α-methylprogesterone acetate and is a progesterone-derived hormone with a high affinity for the PR but can also bind to the AR and GR with a substantially higher affinity than the natural product (Table 1.1) (Africander et al., 2011).

MPA has a molecular weight of 386.52Da and an empirical formula of $C_{24}H_{34}O_4$. MPA differs from progesterone by the addition of a hydroxyl group (OH) which has been acetylated ($COCH_3$) at carbon 17 and the further addition of a methyl group (CH_3) at carbon 6 which gives the molecule relatively high progestational activity (Stanczyk, 2002). Although MPA, like progesterone, is a 3-oxo-4-ene steroid it has improved biological activity when administered orally. The structure of MPA is depicted in Figure 1.12.

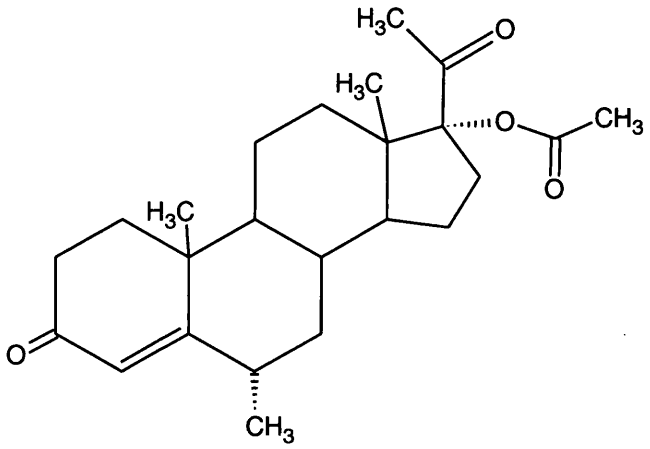


Figure 1.12: The Chemical Structure of Medroxyprogesterone Acetate. Produced using ACD Structure Elucidator, version 12.01 (Advanced Chemistry Development inc, 2014)

Unlike progesterone, the solubility of MPA presents less of an issue when attempting to deliver the drug orally. The solubility of MPA is 1mg/ml in water according to national toxicology program (1992); the increased solubility thus increases the drugs bioavailability when administered orally.

However, akin to the natural product, MPA has been shown to undergo extensive metabolism in the liver by reduction at the free oxygen molecule in A-ring, as well as hydroxylation and conjugation (Stanczyk, 2002) and yet is still active after oral dosing (Thigpen et al., 1999). There are no official figures on the oral bioavailability of MPA but it is estimated to be 15% which is still an improvement on progesterone even in its micronized form (Pannuti et al., 1982, Adlercreutz et al., 1983a, Fotherby,

1996). The reason for the slightly improved bioavailability of MPA may be down to a degree of first pass metabolism evasion through steric hindrance due to the acetate group on carbon 17 of the MPA molecule, (Stanczyk, 2003). However, the increased affinity of MPA for the progesterone receptor means that even though only a small percentage of the administered drug reaches systemic circulation it is sufficient to cause an endometrial reaction.

After intramuscular administration MPA is often well tolerated, however, a common subset of side-effects has been reported amongst users including amenorrhea and weight gain. Such side effects are commonly flagged up by users of synthetic progestins thus suggesting a requirement for developing an oral formulation containing natural progesterone with improved bioavailability.

1.5.3 Levonorgestrel (LNG)

LNG is a second generation synthetic progestin derived from nortestosterone and is widely used for the treatment of endometrial hyperplasia as well as in a variety of contraceptives including the LNG intra uterine device (Mirena Coil) and the combined oral contraceptive, Microgynon which is the most commonly prescribed combined oral contraceptive in the UK (NHS, 2010). LNG is also administered orally as a daily, progestin-only, contraceptive pill or in high doses as an emergency, post-coital contraception. It is one of the most commonly used and studied synthetic progestin in the UK and the most widely used emergency contraceptive (Brunton LL, 2006, Langston, 2010, Glasier et al.). The molecular weight of LNG is 312.4Da, its empirical formula is $C_{21}H_{28}O_2$ and its aqueous solubility is approximately 2.5µg/mL (DrugBank, 2010). LNG is derived from and structurally similar to testosterone but with the addition of an ethynyl group (HC≡C) to significantly reduce androgenic activity (Stanczyk, 2002). The chemical structure of LNG is shown in figure 1.13.

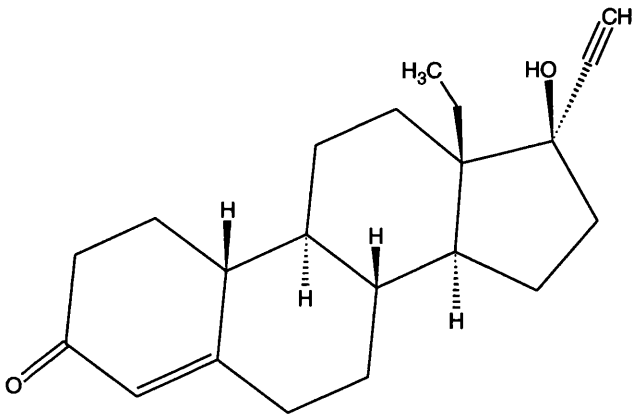


Figure 1.13: The Chemical Structure of Levonorgestrel. Produced using ACD Structure Elucidator, version 12.01 (Advanced Chemistry Development inc, 2014)

Unlike progesterone, the oral bioavailability of LNG is considered to be very high. This information is based on several studies similarly concluding that LNG is almost completely absorbed after oral administration (Hümpel et al., 1978, Grimmer et al., 1986, Back et al., 1987). The main reason for the high bioavailability of LNG is that it evades hepatic first pass metabolism and is rapidly and completely absorbed in the digestive tract (Hümpel et al., 1978). However, LNG has been shown to undergo extensive reduction at the α,β-unsaturated ketone in ring A and hydroxylation at carbons 2 and 16 with its metabolites circulating predominantly as sulphates (Stanczyk and Roy, 1990).

The general acceptance that LNG is rapidly absorbed and has high bioavailability is based on studies that quantified serum levels of circulating LNG following treatment. Initial studies in this area were carried out in the 1970s and 1980s (Back et al., 1981, Hümpel et al., 1978, Goebelsmann et al., 1986). Humpel *et al* (1978) were the first to report that LNG is rapidly and completely absorbed and not subject to first pass metabolism (Hümpel M et al., 1978). Following this, Back *et al* assessed the bioavailability of LNG in women taking 150μg and 250μg doses of the hormone combined with 30μg of ethinyl estradiol. The study reported that after the 150μg dose the bioavailability of LNG is 89% and 99% after the 250μg dose (Back et al., 1981). The final study looked at the bioavailability of LNG and ethinyl estradiol

given orally and intravenously in 24 healthy women. The study found that LNG had complete bioavailability after oral administration (Goebelsmann et al., 1986).

Although LNG has a very high bioavailability, the acting percentage of LNG found in the endometrium is low. A study conducted by Devoto *et al* looked at LNG used in emergency contraception, they concluded that the endometrial concentration of orally delivered LNG lies between 15 and 50 nmol/mg. They noted that the endometrial concentration of LNG is lower when administered orally rather than vaginally yet blood plasma concentrations are higher after oral administration (Croxatto et al., 2001). Although the endometrial concentration of progesterone is low compared to the administered dose, it is still an effective treatment due to the high binding affinity of LNG for the PR. After oral administration approximatedly 45% of LNG and its metabolites are excreted in the urine and about 32% are excreted in faeces, mostly as glucuronide conjugates (DrugBank, 2010).

1.6 Natural vs Synthetic Progestins

The use of natural progesterone is, of course, limited by its poor bioavailability. However the development of micronized progesterone products has allowed the effects of orally delivered natural progesterone to be assessed. The physical and emotional side effects of progesterone compared with MPA were analysed in a study carried out in 2002. The study concluded that neither treatment affected mood but that MPA caused increased bleeding and breast tenderness whereas no physical complaints were noted in users taking natural progesterone (Cummings and Brizendine, 2002). Natural progesterone is also said to increase quality of life relating to physiological, somatic, and vasomotor effects compared with MPA (Fitzpatrick et al., 2000).

Furthermore, natural progesterone does not influence lipid profiles and does not cause significant changes in high density lipoprotein cholesterol whereas MPA and LNG cause a significant decrease (Bruno, 1999). LNG is also known to cause side effects with the biggest complaint being abnormal menstrual bleeding (Darney et al., 1992, Berenson and Wiemann, 1993). Other side effects of LNG include weight

gain, headaches, acne and mastalgia (breast tenderness) (Berenson and Wiemann, 1993).

Patients receiving MPA to treat endometrial hyperplasia or carcinoma can experience more severe side effects; Bafaloukos *et al* described side effects such as weight-gain, hypertension and thrombophlebitis in women receiving MPA (Bafaloukos (Bafaloukos et al., 1999). Thigpen *et al* also listed thrombophlebitis as a side effect in women receiving MPA and also described the occurrence of pulmomary emboli in 1% of volunteers (Thigpen et al., 1999).

As described, during HRT, progestins are often administered in combination with estrogen to protect the endometrium against hyperplasia development. However, the protective effect of progestins seen in the endometrium is not replicated in the breast progestins can be seen to increase breast cancer risk. Yet this is not the case with micronized progesterone, Gompel (2012) showed that micronized progesterone does not heighten proliferation in breast tissue when compared to MPA (Gompel, 2012).

The physical and biological side effects of natural progesterone and its synthetic counterparts are down to their roles at the molecular level. Progesterone and its derivatives bind to and signal via hormone receptors; they bind these receptors with different affinities and differentially regulate associated target genes (Table 1.1). The predominant receptor involved in progesterone signalling is the progesterone receptor.

1.6.1 Progesterone Receptor

Both the effects of natural progesterone and synthetic progestins are mediated through binding the PR. The PR is a member of the family of ligand-activated nuclear transcription regulators. Members of this family are highly conserved between species and are characterised by organisation into specific functional domains (Scarpin.K, 2009).

Humans express three PR isoforms, PR-A (94 kDa), PR-B (116 kDa) and PR-C (60 kDa), these are encoded on the same gene on chromosome 11 at q22-23. The PR-B isoform only differs from the PR-A in that it encodes an additional 164 amino acids

in its N-terminal region (Graham and Clarke, 1997a). Both isoforms are transcribed from a single gene by alternate initiation of transcription from two distinct promoters (Gronemeyer, 1991). The more recently discovered PR-C isoform has a different conformation from the two larger isoforms, this affects the hormone-binding region of the receptor. Although unable to bind hormones, PR-C remains important as it is capable of binding to and inhibiting PR-B (Wei et al., 1997, Lange, 2008).

Both PR-A and PR-B isoforms contain three functioning domains, the N-domain, the DNA-binding domain (DBD) and the C-terminal ligand binding domain (LBD).

The structurally significant N-terminus contains transcription activation domains which are essential for transmitting a transcription activating response to receptor-bound DNA and for the binding of co-activators. The N-terminus of the PR-B isoform contains two transcription activation domains, AF-1 and AF-3, whereas the PR-A isoform contains only AF-1, allowing the binding of different coactivators to PR-A and PR-B (Giangrande et al., 2000), and causing the activation capability of PR-B to be stronger than that of PR-A (Sartorius et al., 1994) Also within the N-terminus is a PXXP region which enables ligand-bound PR to activate the mitogen activated protein kinase (MAPK) pathway, an important pathway involved in a diverse array of functions including proliferation and differentiation (Boonyaratanakornkit et al., 2001).

In the PR-A N-terminus lies an inhibitory domain (ID) which, when activated, blocks PR-As transcriptional ability but permits the receptor to suppress other steroid receptors such as PR-B and ER. The ID only becomes functional when PR binds to its hormone (Hovland et al., 1998).

Both PRA and PRB contain a DBD. The DBD contains a dimerization domain and a nuclear localisation sequence which is used to target the protein to the cell nucleus. Both receptor types also contain a LBD. Asides from its role in binding the receptor to its ligand, the LBD also contains determinants that allow the receptor to dimerise and bind heat shock proteins which are key proteins involved in stabilising the unliganed PR. Further to this, within the DBD there is a transcription activation domain (AF-2) and determinants for a nuclear localisation sequence (Leonhardt et

al., 2003b). A full annotation of the progesterone receptors can be seen in Figure 1.14.

In most human tissues PR-A and PR-B are expressed at equal ratios, however, in the endometrium, a decrease in PR-A can be seen during the secretory phase of the menstrual cycle (Scarpin.K, 2009) and more drastic changes occur during pregnancy (Taylor et al., 2009). In most tissues PR-Bs are stronger transactivaters than PR-As and the receptors regulate both overlapping and distinct genes (Richer et al., 2002). However, PR-A can act as a dominant repressor of PR-B allowing PRs to silence or induce progesterone action (Tung et al., 1993). It is unclear whether the signalling of natural progesterone through the different PR isoforms is mimicked by the synthetic progestins. For example, Schoonen and co-workers showed that the synthetic progestins, LNG and gestodene, displayed different binding affinities for PR-A and PR-B (Schoonen et al., 1998).

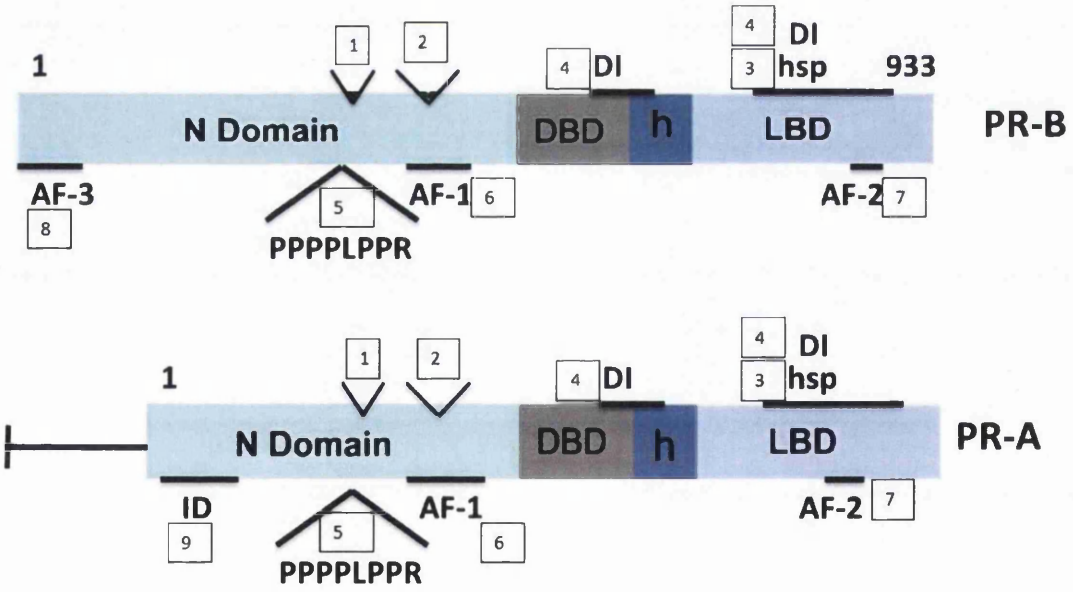


Figure 1.14: Human PR-A and PR-B Protein Structures. Figure adapted from Leonhardt 2003. Domains include N-domain, DNA Binding Domain (DBD), hinge (h), and Ligand Binding Domain (LBD). Boxed Numbers describe the following: 1-Site of MAPK phosphorylation activity, 2-Site of PR post-translational activity (sumoylation), 3-Hsp: region for binding of heat shock protien in absence of ligand, 4-Dimerisation domain (DI), 5-Region of co-regulator binding, 6-Activation function 1 (AF-1), 7-AF-2, 8-AF-3, 9-Inhibitory Domain (ID).

1.6.2 Progesterone Receptor Mechanisms of Action

Prior to ligand binding, the PR exists in the cytoplasm in an inactive state due to its association with chaperone proteins. Progesterone or progestins diffuses into the cell and binds to cytoplasmic PR at the ligand binding domain. Ligand binding causes a conformational change in the PR and allows it to dissociate from its chaperone proteins which include heat shock proteins and other protein substrates (e.g P23, P59) (Li and O'Malley, 2003). Two active, ligand-bound PRs homodimerize at the dimerization domains and are subsequently targeted to the nucleus through nuclear localisation sequences found in both the DNA binding domain and the ligand binding domain (Figure 1.15) (Tsai and O'Malley, 1994, Li and O'Malley, 2003). Once in the nucleus the PR dimers can initiate transcription to up or down-regulate target genes. Transcription initiation can occur through the direct binding of PR to progesterone response elements (PREs) in the DNA sequence or through the recruitment of protein co-regulators. Co-regulators such as members of the steroid receptor coactivator (SRC) family can bind the PR at AFs 1 and 2 (Figure 1.14) to form a stable complex and cause gene activation or repression.

The PR may be subject to a number of post-translational modifications including phosphorylation, SUMOylation, acetylation and ubiquitination (Diep et al., 2015). For example, the ligand-bound PR is phosphorylated at serine 294 by MAPK and respectively marked for ubiquitin mediated degradation (Lange et al., 2000, Qiu et al., 2003).

1.7 Regulation of Target Genes and Proteins

Ligand dependent gene regulation through the PR can cause up or down-regulation of many genes and their resulting proteins in the human endometrium. The expression level of the PR itself has been widely reported to be down-regulated in the presence of progesterone through a negative feed-back process (Graham and Clarke, 1997a, Tibbetts et al., 1998). The presence of PR in the normal endometrium is well documented (Ehrlich et al., 1981, Mylonas et al., 2004), however in the hyperplastic endometrium, the expression of PR is often different and gene regulation is affected as a result of changes in the number, abundance and type of

progesterone receptors and the abnormal levels of endogenous hormones (Nyholm et al., 1992).

The presence of PR in the endometrium and its regulation by progesterone or synthetic progestins is of great importance. Previous studies have demonstrated that the histological response to therapy with progestins is mediated by PRs and the intensity of the response correlates with receptor levels (Holinka et al., 1984, Deligdisch et al., 1978). Thus, patients with a high receptor level tend to receive progestin therapy. However, the initial success of such therapy is often short lived as the progestin treatment serves to down-regulate its own receptor which eventually leads to a reduction in the therapeutic response (Jänne et al., 1979). Therefore it is vital to assess the levels of PR in cellular research and patient therapy to establish the efficacy of treatments and studies.

As well as regulating itself, ligand binding to the PR also regulates a vast array of gene targets. Indeed, the altered expression of some PR targets has been associated with endometrial hyperplasia (Mutter et al., 2000, Gallos et al., 2013) yet, the presence and stage of this condition has not been predicted by any clinical or biological markers. Genes involved in cell secretion, adhesion, apoptosis and proliferation are often adversely regulated in such conditions and may help to distinguish hyper-proliferation with malignant potential from benign hyperplasia (Moreno-Bueno et al., 2003). As endometrial hyperplasia is often treated with synthetic progestin therapy it is of keen interest to establish if any potential markers are adversely regulated by the synthetic hormones with which they are treated. Genes of particular interest include those that are already associated with endometrial cancer as well as novel gene targets. The targets chosen for this study are described below.

1.7.1 Regulation of PR

The first gene of interest is the PR itself, which is regulated by ligand binding through a negative feedback loop in endometrial epithelium (Graham and Clarke, 1997a, Tibbetts et al., 1998). However, studies in macaque endometrial stroma, show PR is not down-regulated by P_4 in this tissue (Brenner and Slayden, 2012). In contrast, E_2

causes an induction in the expression of stromal and epithelial PR. The induction of PR by E_2 has been shown in the endometrial epithelial cancer cell line, Ishikawa (Hata and Kuramoto, 1992, Jamil et al., 1991). In this particular cell line, PR has been shown to be down-regulated by P_4 after E_2 priming (Lessey et al., 1996). Like P_4, MPA has also been reported to down-regulated PR (Tseng and Zhu, 1997), however, contradictory findings exist on the regulation of PR by LNG. Critchley (1998) demonstrated a significant reduction in PRA and B in women with an LNG intrauterine device (Critchley et al., 1998) and Vereide (2006) showed a marked reduction in PRA and PRB expression in endometrial hyperplasia following LNG treatment (Vereide (Vereide et al., 2006)2006). In contrast, a study by Meng (2010) compared biopsy samples from healthy fertile women receiving either oral or vaginal LNG and found orally delivered LNG significantly reduces PR-A and PR-B in glandular epithelial cells, but not in luminal epithelial or stromal cells, whereas vaginal delivery did not affect the expression of either PR (Meng et al., 2010).

The available data on P_4 and progestin regulation of the PR is conflicting (Critchley et al., 1998);(Vereide et al., 2006, Meng et al., 2010) but the majority of the data suggests a down-regulation of PR after progesterone based treatment. To date no direct comparison has been made between the effects of P_4 and synthetic progestins on the endometrium.

1.7.2 Forkhead box O protein 1

The second gene of interest is forkhead box O protein 1 (FOXO1). FOXO1 is a member of the FOXO subfamily of Forkhead/winged helix transcription factors and is known to be regulated through ligand-bound PR in endometrial epithelial and stromal cells (Ward et al., 2008, Labied et al., 2006) through direct signalling mechanism (Kyo et al., 2011). Proteins of the FOXO family are characterised by a distinct forkhead domain with a butterfly-like appearance and regulate diverse cellular functions, such as differentiation, metabolism and proliferation (Accili and Arden, 2004). However, FOXO1 proteins have been shown to induce the expression of genes encoding proteins involved in cell cycle inhibition and apoptosis in response

to growth factors, hormones and environmental cues (Labied et al., 2006, Kyo et al., 2011).

In the normal endometrium, FOXO1 is mainly associated with the process of decidualisation (Christian et al., 2002, Gellersen and Brosens, 2003), however, FOXO1 is a known tumour suppressor (Myatt et al., 2010) and a marked loss of the FOXO1 protein expression is associated with endometrial cancer (Goto et al., 2008). Yet the level of FOXO1 protein expression in endometrial hyperplasia has never been investigated.

Up-regulation of FOXO1 by progesterone and the synthetic progestins through the PRB in cancerous endometrium and normal endometrial stroma is well documented (Kyo et al., 2011, Wang et al., 2009, Labied et al., 2006, Ward et al., 2008). As well as regulating FOXO1 expression, the PR also cooperates directly with this transcription factor to regulate genes such as those that encode cell cycle inhibitors (Brosens and Lam, 2013).

Overall, FOXO1 serves as an excellent positive control for progesterone/progestin mediated endometrial target gene regulation and may also serve as a possible marker for endometrial hyperplasia.

1.7.3 Amphiregulin

The third gene of interest is amphiregulin (AREG) which is regulated by agonist-bound PR-A (Conneely and Jericevic, 2002, Mulac-Jericevic et al., 2000). Human AREG is an 84-amino acid glycoprotein discovered and characterized in the late 1980s (Shoyab et al., 1988). It is a member of the epidermal growth factor (EGF) family of proteins and can interact with EGF receptor to promote normal growth in epithelial cells. Although AREG does not contain a PRE site, AREG is proven to have an AP-1 (Aguiari et al., 2012)and SP1 (Lamber et al., 2010) sites which PR homodimers can bind to and regulate transcription.

AREG is expressed at varying levels in the normal endometrium throughout the menstrual cycle (Gui et al., 2008b)(Gui et al., 2008b)(Gui et al., 2008b)(Gui et al., 2008b)(Gui et al., 2008)(Gui et al., 2008)(Gui et al., 2008)(Gui et al., 2008)(Gui et

al., 2008)(Gui et al., 2008). During the secretary phase, levels of AREG are more than 30 times higher than in the proliferative phase which is down to its up-regulation by progesterone. The AREG protein is located in the cytoplasm and predominantly expressed in the epithelial layer of the endometrium (Gui, Zhang JR et al. 2008). An up-regulation of AREG by 2-fold or more has been documented in endometrial cancer (Moreno-Bueno et al., 2003, Ejskjær et al., 2007) yet its role in endometrial hyperplasia has not been thoroughly investigated. Hence, the level of AREG expression in endometrial hyperplasia warrants further research.

1.7.4 Lactoferrin

Lactoferrin (LF), also known as lactotransferrin, is an epithelial secretory glycoprotein and is a member of the transferrin family of proteins. LF has a molecular mass of 80kDa and has non-haem iron-binding properties. It was first discovered in milk and later found in surface mucosa epithelium as well as many biological secretion including tears, semen and saliva. This protein also displays antibacterial, antimicrobial and antifungal activity (Sanchez et al., 1992, Adlerova et al., 2008).

LF is expressed in the human, monkey and mouse endometrium at varying levels throughout the menstrual cycle (Teng et al., 2002, Newbold et al., 1992) and has been shown to be regulated by both estrogen and progesterone. LF is directly up-regulated by estrogen through an estrogen response element in the LF promotor (Zhang and Teng, 2000) and is down-regulated by progesterone (Walmer et al., 1992, Kurita et al., 2000), though whether this regulation is direct or requires a coregulator is unknown. Moreover elevation in LF expression has been directly correlated with a peak in endometrial proliferation. A small degree of investigation has gone into the changes in LF within endometrial hyperplasia with no changes being reported with the exception of one individual case within a small sample population (n=10) where LF was seen to be elevated (Walmer et al., 1995). LF is frequently overexpressed in PR-negative endometrial carcinomas (Moreno-Bueno et al., 2003, Walmer et al., 1995) and further research into its potential role in hyperplasia may be valid. Furthermore LF is specifically regulated through the PR-B

which may elaborate on the different roles of the individual PR types in hyperplasia progression.

1.7.5 Gene Regulated in Breast Cancer 1 (GREB1)

As discussed the regulation of genes by the different progestins used in the treatment of hyperplasia is important. Therefore, as well as looking at potential markers of hyperplasia progression, a further subset of genes will also be assessed to observe any differences in gene regulation by the synthetic compounds.

The first gene to be looked at will be the gene regulated in breast cancer 1 (GREB1). The GREB1 gene codes for the GREB1 protein which is a single-pass membrane protein that has been suggested to play an important role in hormone responsive tissue and cancer (Ghosh et al., 2000). GREB1 is present in the human endometrium at both RNA and protein level. The GREB1 gene is an early response gene in the estrogen receptor-regulated pathway (Pellegrini et al., 2012). Not only is Greb1 a direct target of estrogen but it is also an androgen target and contains a progesterone response element in its promoter (Rae et al., 2006). Furthermore GREB1 expression has been shown to be induced by estrogen in endometrial epithelial cells (Gori et al., 2011). Therefore, GREB1 will be used as a positive control for estrogen treatment and its potential novel regulation by progesterone and the synthetic progestins will also be investigated.

1.7.6 Prolactin

Prolactin (PRL) is a highly conserved polypeptide hormone present in all vertebrates examined. It is synthesized in, and secreted from, specialized cells of the anterior pituitary gland called lactotrophs (Freeman et al., 2000). This multifaceted hormone has more roles than any other hormone secreted by the pituitary with more than 300 functions described and carries out its actions through binding the prolactin receptor (Bole-Feysot et al., 1998). Although the role of PRL is predominantly associated with milk production it also plays an important role in endometrial decidualisation and is temporally expressed in the endometrium at the window of implantation (Jones et al., 1998). PRL expression has been shown to be regulated through the PR

in differentiating human endometrial stromal cells. However, progestins are very weak inducers of PRL and require more than 8 days of stimulation before enhancing PRL expression, yet, the role of progestins in PRL regulation is important in amplifying the decidual response. The ability of progestins to synergistically enhance cAMP-induced PRL expression has been demonstrated in several studies (Gellersen et al., 1994, Telgmann et al., 1997, Brosens et al., 1996, Brar et al., 1997, Tang and Gurpide, 1993). As PRL is a well-established marker of stromal decidualisation, its expression in this process after exposure to different progestins will be examined.

1.7.7 Insulin-like Growth Factor 1

The final gene target of interest is insulin-like growth Factor 1 (IGF-1). IGF-1 is a 70 amino acid, single chain polypeptide (Rinderknecht and Humbel, 1978) and is the only gene described herein that is not regulated by estrogen or progesterone (Weihua et al., 2002, Ace and Okulicz, 1995). However, IGF is upregulated by androgens via signalling through the androgen receptor (Zhu and Kyprianou, 2008). It is therefore possible for synthetic progestins, capable of binding the AR, to upregulate this gene and such differences will be assessed during this thesis.

The regulation of the described PR target genes is depicted in Figure 1.15.

Differences in gene regulation between progesterone and synthetic progestins coupled with the increased prevalence of side effects after progestin consumption highlights the benefits of delivering a the natural compound over its artificial counterparts. However, the difficulty in delivering natural progesterone has meant no such drug has been made available for long term oral delivery.

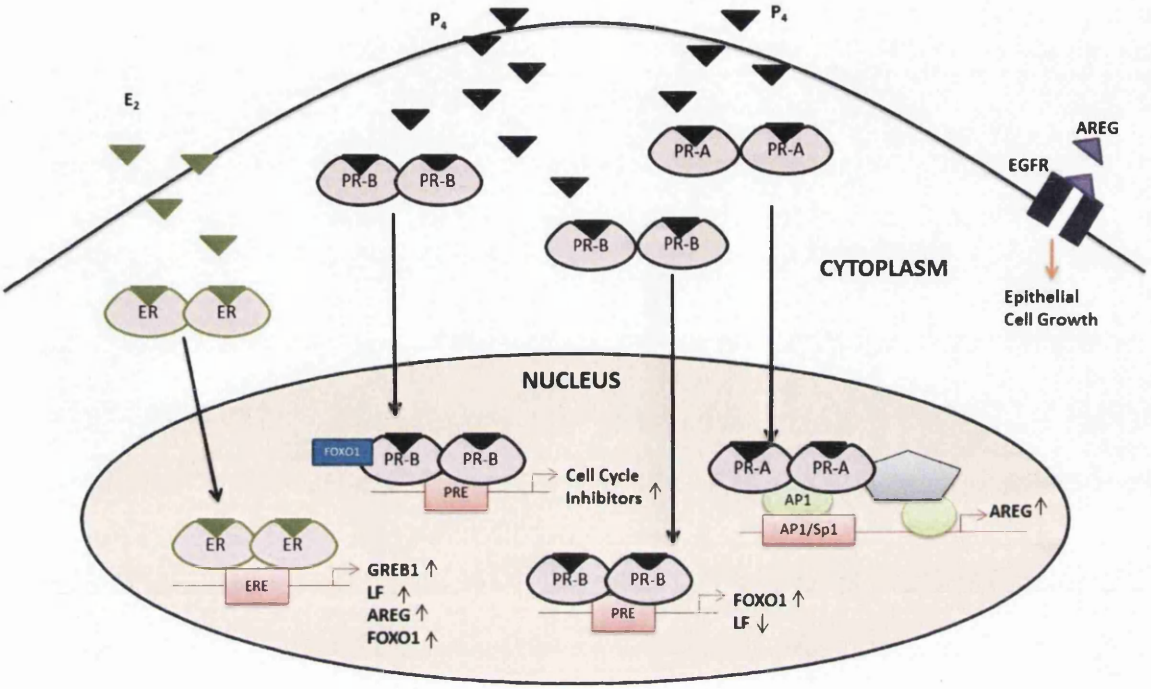


Figure 1.15: Gene Regulation by Ligand-bound Progesterone and Estrogen Receptors. Progesterone (P_4, black) and Estrogen (E_2, Green) bind to their receptors and enter the nucleus where they activate or repress target genes by either directly binding to response elements in the DNA (ERE, PRE) or recruiting co-regulators such as AP-1 and SP-1.

1.8 The Gastrointestinal Tract and Liver

To understand the underlying mechanisms involved in the poor oral bioavailability of progesterone it is important to first understand the physiology of the gastrointestinal (GI) tract and moreover the problems associated with progesterone absorption through, and metabolism within this organ system. Once absorbed through the intestinal wall progesterone travels through the hepatic portal vein to the liver where it undergoes further metabolism and can enter the enterohepatic circulation (Symonds et al., 1994) Thus the roles of the GI tract, the liver and enterohepatic circulation will be discussed herein.

1.8.1 The GI Tract

The components of the GI tract consist of the oral cavity, oesophagus, stomach and small and large intestines (Figure 1.16). The histology and physiology of each region varies depending on their particular functions (Lee and Yang, 2001). Consumed items enter the oral cavity and are passed into the oesophagus as a bolus where they are moved toward the stomach through peristalsis. Entry of the bolus into the stomach is governed by the cardiac orifice; it is then processed into chyme before exiting the stomach through the pyloric region into the duodenum. Stomach juices are highly acidic consisting of mainly hydrochloric acid, mucus and several enzymes (Johnson, 2012b). The chime produced in the stomach is also highly acidic (pH 3.5) and needs to be neutralised upon entry to the small intestine. The duodenum represents the first 25cm of the small intestine and receives the opening to the bile and pancreatic duct. Bile is highly alkaline and serves to neutralise the acidic chyme from the stomach causing the pH to rise to approximately pH 6 (Fallingborg, 1999). Bile is also involved in emulsifying fats and aiding digestion. The entry of pancreatic juices into the duodenum provides enzymes such as peptidases and lipases to further aid in the digestion of food. The main role of the duodenum is thus in the breakdown of food but the presence of enzymes in the duodenal mucosa and fluid also have a major influence on the metabolism of many drugs (Lee and Yang, 2001).

Upon exiting the duodenum semi-digested food enters the jejunum; which is approximately 2.5m long. The jejunum is the major site of food and drug absorption able to absorb small nutrient particles that have been enzymatically broken down in the duodenum. After absorbtion nutrients and drug enter the enterohepatic circulation and are delivered to the liver through the hepatic portal vein for further metabolism (Johnson, 2012a).

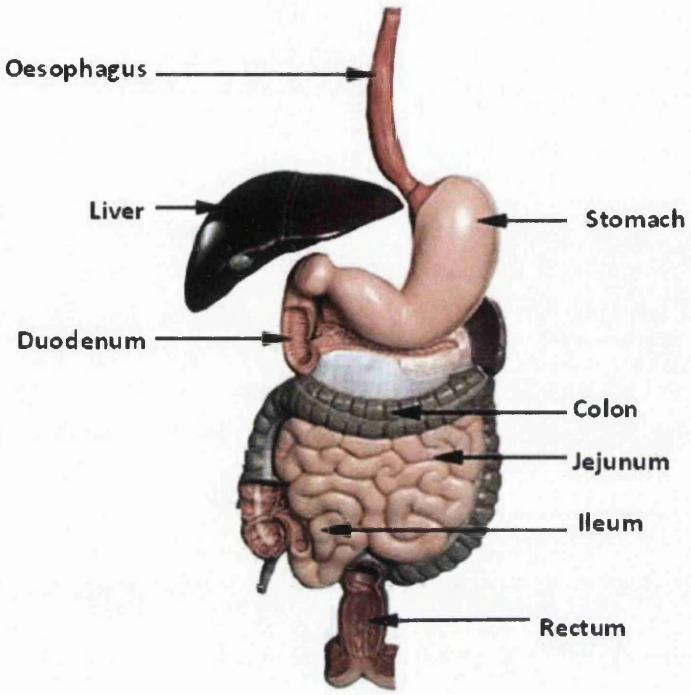


Figure 1.16: Anatomy of the Gastrointestinal tract. Adapted from http://www.nvasi.com/anatomy-of-gastrointestinal-tract/

Enterohepatic circulation is the process by which bile, bilirubin, and other substances including many drugs leave the small intestine and are transported to the liver via the hepatic portal vein, from here they are returned to the duodenum through the bile duct and can repeat the cycle again (Figure 1.17). It has been suggested that progesterone is able to undergo entherohepatic circulation which can cause a second peak in blood plasma levels of this drug (Symonds et al., 1994). Enterohepatic circulation is important to consider when administering drugs as some drugs which are not considered toxic can accumulate at high levels in the liver becoming extremely hepatotoxic (Zimmerman, 1999).

The remaining jejunal contents enter the final and longest region of the small intestine; the ileum which lies between the jejunum and large intestine. This is the longest region of the small intestine; reaching up to 4m in length in some adults. The main role of the ileum is in the absorption of vitamin B and bile salts as well as any small undigested products not absorbed in the jejunum. The pH of the small intestine gradually rises from pH6 in the duodenum to about pH 7.4 in the terminal ileum (Fallingborg, 1999). After passing through the ileum any unabsorbed content enters

the large intestine; the ileum is separated from the large intestine by the ileocaecal sphincter.

The large intestine is approximately 1.5m long and consists of the caecum, colon and rectum. The large intestine has four major functions; storage and elimination of faeces, metabolism of proteins, absorption of water and electrolytes and production of certain vitamins such as vitamins K and B (Johnson, 2012a).

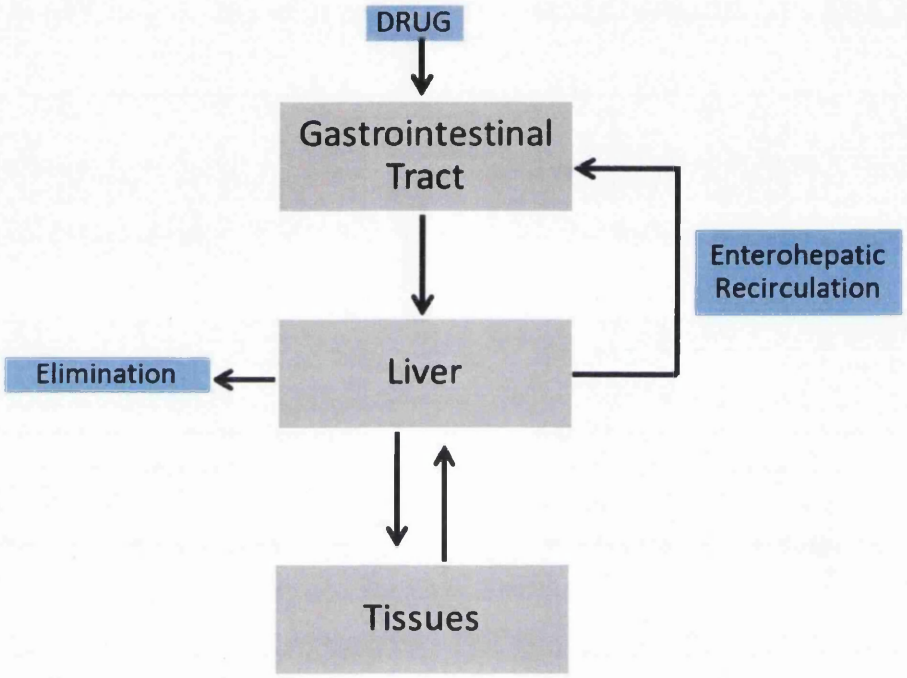


Figure 1.17: Schematic Diagram of Enterohepatic Circulation. Drugs administered orally are absorbed through the GI tract and transported to the liver via the hepatic portal vein before entering body tissues. From the liver some drugs are returned to the GI tract through the bile duct and can repeat the cycle again

1.8.2 The Liver

The liver is the largest internal organ in humans and rests in the abdomen below the diaphragm. The main function of the liver is to provide metabolic control which responds to the changing needs of the body. To achieve this precise metabolic control, the liver produces proteins and enzymes at a specific and changing rate through regulation of gene transcription (LeBouton, 1993). The liver also has a wide

variety of functions outside of protein synthesis including detoxification, glycogen storage, decomposition of erythrocytes, and producing chemicals involved in digestion such as bile. This series of liver functions are carried out by the liver cells, the hepatocytes. The most important function of the liver in terms of this project is its major role in steroid hormone metabolism (Cameron, 1957). The liver contains a number of enzymes capable of metabolising steroid hormones, in particular enzymes of the cytochrome P450 (CYP) pathway (Ogu and Maxa, 2000) and aldo-keto reductases (AKRs) (Jez and Penning, 2001). The main route of steroid hormone degradation by such enzymes is through reduction at the ketone structure in ring A (Yates et al., 1958) followed by glucuronide conjugation (Rao and Taylor, 1965).

1.9 Progesterone Metabolism

The significance of liver metabolism on the oral bioavailability of drugs, including hormones, is widely recognised (Obach, 2001, Kuhnz and Gieschen, 1998). This first pass effect has been shown to have a huge impact on the bioavailability of key hormones used in contraception and hormone therapies. Natural progesterone undergoes extensive metabolism in the liver, predominantly by cytochrome P450 (CYP) enzymes (Yamazaki and Shimada. 1997) but also by reductases and hydroxysteroid dehydrogenases and has a low oral bioavailability (<10%) (Stanczyk, 2002).

A great deal of research has shown that the liver is responsible for a large percentage of progesterone first pass metabolism; many studies have observed the extent of progesterone metabolism by CYP enzymes in the liver (Yamazaki and Shimada, 1997, Williams et al., 2004, Nebert and Russell, 2002, Szklarz and Halpert, 1997b)

Nicotinamide adenine dinucleotide phosphate (NADPH) is a cofactor required for several anabolic reactions. In particular it acts as a source of electrons for redox reactions of several enzymes implicated in progesterone metabolism including CYPs and aldo-keto reductases (AKRs) (Lemley and Wilson, 2010). Such enzymes utilise NADPH by reducing hydrogen atoms to water through the addition of an oxygen molecule; this reaction is dependent on a supply of electrons which are derived from NADPH (Nelson, 2005).

The role of CYPs and AKRs in the reduction and oxidation of progesterone is well established (Penning et al., 2000, Yamazaki and Shimada, 1997, Williams et al., 2004, Swart et al., 1993a, Szklarz and Halpert, 1997b, Nebert and Russell, 2002). Moreover, a high rate of NADPH dependent progesterone metabolism in liver homogenates of common laboratory animals has been described (Rao and Taylor, 1965, Cooke and Vallance, 1965).

Even though the hepatic metabolism of progesterone is well characterised there is very little information on progesterone metabolism in the intestine. The only data that appears to be available is in early studies by Nienstedt and colleges (Nienstedt et al., 1980). These authors reported the metabolism of progesterone in ileum and colonic tissue samples of two adult male subjects and pooled intestinal homogenates of 13 human foetuses. The same group also published an earlier paper describing the metabolism of progesterone in the intestine of mongrel dogs, they suggested that the main routes of progesterone metabolism in the intestine was through reduction at keto groups or the a-ring double bond and through the addition of new functional groups to the molecule (Nienstedt and Hartiala, 1969, Harri et al., 1969).

Interest in the metabolising capabilities of the small intestinal mucosa has been increasing since the realisation that it is likely to be the most significant site of drug biotransformation outside of the liver, which is due to the presence of metabolising enzymes such as CYPs and AKRs (Barski et al., 2008, Thelen and Dressman, 2009). The levels of CYP and AKR enzymes have been shown to vary along the intestinal tract and are both generally higher in the proximal regions of the small intestine. Moreover the expression of different subtypes of these enzymes differs between regions of the small and large intestine (Barski et al., 2008, Thelen and Dressman, 2009, Mitschke et al., 2008). The reduction in enzymatic activity in the distal small intestine and colon may improve drug stability in these regions; for instance the HIV inhibitor UC-781 has been shown to be significantly more stable in the colon and ileum compared to the duodenum and jejunum (Van den Mooter et al., 1998).

The mucosa of the human small intestine has been found to contain CYP enzymes known to metabolise progesterone, namely CYP3A and CYP2C (Thörn et al., 2005,

Paine et al., 2006, Zhang et al., 1999). However in the rat intestine different CYPs have been identified; CYPs 1A1, 2B1, and 3A1 can all be detected and CYP 2C6 and 2C11 can also be found at low levels (Zhang et al., 1996, Kaminsky and Fasco, 1992). Of these, CYP2B1 and CYP2C11 are known catalysers of progesterone (Borlakoglu et al., 1993, Kitareewan and Walz, 1994). Further down the intestinal tract the expression levels of CYP enzymes change in type and abundance with but all are expressed at a reduced level compared to the proximal intestine (Thelen and Dressman, 2009). Enzymes of the AKR1C family have all been shown to be involved in the oxidation of progesterone, these can be found at varying levels in the rat small intestine with the exception of AKR1C4 which is found almost exclusively in the liver (Penning et al., 2000). Other members of the AKR1C family of enzymes are also highly expressed in the human liver but can also be found throughout the human gastrointestinal tract with the highest levels detectable in the duodenum (Palackal et al., 2002).

Differences between rat and human cytochromes have also been observed in the liver, for instance the progesterone metabolising CYPs, CYPs 3A4, 2C9 and 2C19, are all present in the human liver yet these homologs are not found in the rat liver (Martignoni et al., 2006). Furthermore the metabolising enzymes present in the liver differ from those found in the intestine of the same individual in both type and abundance (Martignoni et al., 2006, Chhabra et al., 1974).

Interspecies differences in the metabolising capabilities of drugs can often make animal models poor surrogates for humans; for instance the bioavailability of several progestins have been shown to differ significantly between human and animal models (Düsterberg et al., 1981), and many more such discrepancies have been described (Musther et al., 2014). It is therefore important to compare differences between human and animal models as such differences could have considerable implications on human risk.

Although liver metabolism clearly has a large impact on the oral bioavailability of progesterone, the role of large intestinal bacteria and enzymes on the stability and overall bioavailability of drugs is often ignored. Progesterone, mainly as metabolites,

has been recovered from the faeces and urine of primates after oral administration with over 40% of the total drug recovered reportedly being found in faeces (Wasser et al., 1994, Ziegler et al., 1989). This suggests that the ileo-colonic region of the GI tract may play a role in progesterone transformation. Contents of the large intestine consist of several enzymes and many different to those found in the liver. These enzymes are secreted by the large amount of bacterial species present in the colon (10^{11}-10^{12} cfu/mL) leading to different mechanisms of drug metabolism in the colon compared to the liver (Jung and Kim, 2010, Sousa et al., 2008a). The available information on progesterone, stability to colonic bacterial metabolism is insufficient. In fact the available studies provide no quantitative information regarding the stability of progesterone to colonic bacteria but only highlight the metabolites formed as a result of metabolism. Furthermore these studies were all carried out before 1980.

The limited information on progesterone stability in intestinal tissues and fluids makes it difficult to understand what proportion of metabolism is owed to the liver and what is owed to the intestines. In order for a natural progesterone drug to be produced understanding such concepts is vital as the metabolic capabilities of the intestine could have a huge impact on oral bioavailability.

1.10 Progesterone transporters

Like metabolising enzymes, membrane transporters can also be major determinants of the pharmacokinetic profiles of drugs. It is now considered more as a rule, than an exception, that a given pharmacological agent will interact with a membrane transporter at some point after oral administration. Although progesterone is a highly lipophilic molecule, able to diffuse through cells across a concentrations gradient, transporters are still likely to affect progesterone uptake. For example, the uptake of progesterone by adrenal cells might be at least partially accounted for by a specific carrier-mediated transport mechanism generated by sodium ions and an electrochemical mechanism (Ogihara et al., 2004). Furthermore, a number of specific transporters have been implicated in progesterone transport. ATP-binding cassette (ABC) transporter 2 is able to transport a number of steroids including progesterone (Vasiliou et al., 2009) and intracerebral progesterone concentrations have been

shown to be influenced by MDR1-type P-gp function; however, the effects are only small (Uhr et al., 2002). Although detailed investigation into the role of transporters in progesterone metabolism will not be investigated during the course of this project, the possibility of progesterone active transportation should be taken into consideration when investigating the rate of progesterone metabolism.

1.11 Overcoming Poor Bioavailability

When it comes to enhancing the bioavailability of lipophilic drugs, improving their solubility is of great importance. When administered orally, drugs will only fully permeate the intestinal wall if they have good solubility in the surrounding medium and drugs with these properties tend to have better oral bioavailability (Savjani et al., 2012).

Solubility can be defined as the maximum amount of a substance/solute that can be fully dissolved in a solvent to form a homogenous solution (Gong et al., 2007). This process involves the active breakdown of chemical bonds in the solute and the separation of molecules in the solvent to create space for the dissolving substance (Figure 1.18) (Gong et al., 2007). Thus the chemical structure of the solute affects its ability to dissolve within the given solvent and vice versa. The energy required to breakdown bonds within the solute comes from the formation of new bonds between the solute and solvent. When taking the example of hydrophilic and lipophilic drugs in water, the hydrophilic compound would readily form bonds with the water molecules which would, in turn, release energy to breakdown the drug framework causing the drug to dissolve. However, lipophilic drugs do not readily form bonds with the water molecules and thus no energy is produced to break down the drug lattice (Silberberg, 2009).

Another factor affecting intestinal permeability is dissolution. Dissolution is a kinetic process quantified by the rate at which the solute dissolves in the solvent. Drug particle size can strongly influence the rate of dissolution whereby small particles dissolve more readily as they have an increased surface area to volume ratio (Jinno et al., 2006). Micronization of this progesterone can significantly enhance the rate of dissolution. Indeed, micronized progesterone with a particle size of <25μm has been

shown to improve progesterone oral bioavailability (Hargrove et al., 1989, Salin-Drouin, 2000, Levine and Watson, 2000b). However, micronized progesterone is only administered in high doses for the short-term treatment of certain reproductive disorders and has significant intra- and inter-patient variability (Levine and Watson, 2000b). Other patents have also been written for natural progesterone with improved bioavailability and all contain progesterone in its micronized form (Besins and Besse, 2013, Maxson et al., 1990, Chandler et al., 1997, Liu et al., 2010a), yet none are mainstream, commercially available, products (Liu et al., 2010a). Thus, there still exists a need for a progesterone composition that has a higher oral bioavailability and reduced inter-patient and intra-patient variability than the currently available products. It is also important that the formulation does not contain unsuitable components which could lead to adverse reactions after consumption. One method for producing such a formulation is with solid dispersions.

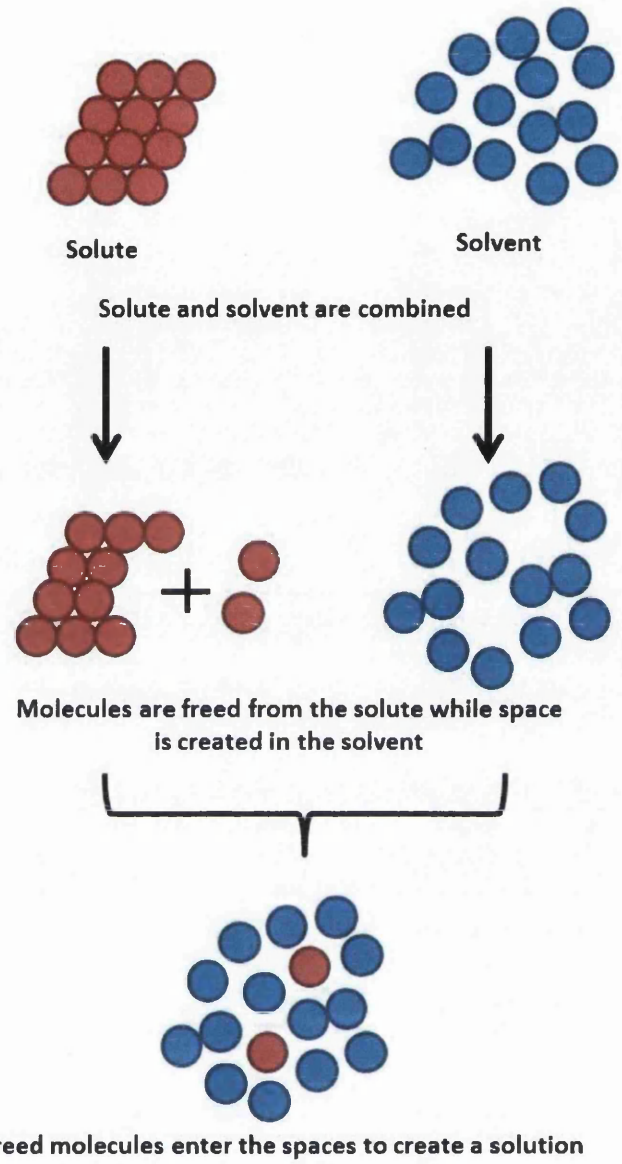


Figure 1.18 The Solution Formation Process.

1.11.1 Solid Dispersions

A review paper by Dhirendra *et al* (2009) defined solid dispersions as "a group of solid products consisting of at least two different components, generally a hydrophilic matrix and a hydrophobic drug. The matrix can be either crystalline or amorphous. The drug can be dispersed molecularly, in amorphous particles (clusters) or in crystalline particles" (Dhirendra et al., 2009).

Solid dispersions were first proposed in 1961 by Sekiguchi and Obi; they described the melting of sulfathiazole and urea followed by rapid cooling. The resulting eutectic mixture displayed faster dissolution and improved bioavailability compared

to conventional formulations (Sekiguchi and Obi, 1961). Since this discovery, a great deal of research has led to improvements and enhancements of this type of formulation. The next development in solid dispersion production contained molecularly dispersed particles in a carrier system as opposed to eutectic mixtures. These systems demonstrated faster dissolution and improved bioavailability (Kanig, 1964).

The next step in solid dispersion development came in the shape of amorphous solid dispersions (ASDs) which improve dissolution as they are less thermodynamically stable (Chiou and Riegelman, 1969). With ASDs the chosen compound is dispersed molecularly in an irregular form in an amorphous carrier. The term amorphous refers to a typically crystalline solid in a non-crystalline state (Figure 1.19).

The main advantages of using ASDs have been described by Vasconcelos (2007) and can be summarised in a few main points. Firstly, the preparation of ASDs leads to a reduction in particle size which results in increased surface area improving dissolution and therefore increased bioavailability. Secondly, the wettability of the particles is improved meaning the surface tension of surrounding fluids is reduced allowing the particles to become 'wet', causing them to dissolve more rapidly. And finally, ASDs tend to have a higher apparent solubility than crystalline solid dispersions as they contain the drug in a supersaturated state due to the forced solubilisation in the carrier (Vasconcelos et al., 2007).

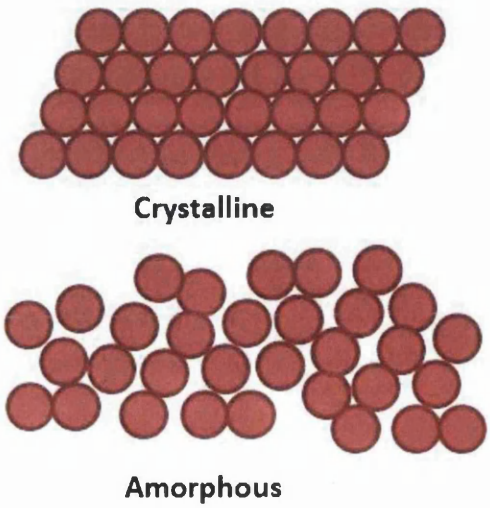


Figure 1.19: The Atomic Structure of Crystalline and Amorphous Solids.

1.11.2 Progesterone Amorphous Solid Dispersions

Saffoon *et al* (2011) discussed suitable candidate drugs for improved solubility with solid dispersions. They reported that poorly water-soluble drugs with high permeability are the best options as dissolution is the rate limiting step to intestinal absorption (Saffoon et al., 2011). Progesterone, therefore, appears to be an ideal candidate.

There are several methods which could be utilised to produce progesterone ASDs and each has its advantages and disadvantages. The most commonly used methods include hot-melt extrusion, spray-drying and emulsion solvent evaporation and these are discussed herein. Hot-melt extrusion is an anhydrous process whereby a drug and polymer are melted and forced through an orifice to form fine particles in a fast and continuous manner (Breitenbach, 2002, Chokshi and Zia, 2010). However, hot-melt extrusion requires high temperatures which may inactivate sensitive products and the machinery is often expensive and difficult to maintain (Madana and Madanb, 2012). The spray drying technique is very different from hot-melt extrusion. It involves dissolving a drug and polymer in a solvent then spraying this solution into a stream of heated air to rapidly evaporate the solvent. Spray drying possesses many advantages which make it an attractive option for industrial applications; the precise control of production parameters results in good reproducibility and particle size can be reduced to as little as 1μM (Vasconcelos et al., 2007). The draw-backs of the spray-drying technique include the cost at lab scale and the use of high temperatures which, once again, may inactivate sensitive products (Nandiyanto and Okuyama, 2011).

The final method to be discussed is emulsion solvent evaporation. During this technique the polymer and drug are both dissolved in a solvent which is added to a liquid with which the solution is immiscible. A surfactant is also added to the mixture. The mixture is stirred continuously until the solvent is removed by evaporation, which usually takes several hours. Eventually, a suspension of amorphous microparticles containing the drug and polymer is formed and can be separated from the solution by filtration (Watts et al., 1989). The key advantages to

using the emulsion solvent evaporation method is its simplicity and low cost making it a useful technique for assessing new drug candidates for ASDs. However, conductivity to industrial scale-up is difficult and solvent residues may be an issue if evaporation is not fully complete (Freitas et al., 2005) Watts (Watts et al., 1989). Nonetheless, emulsion solvent evaporation is a good option for early investigation into the production of progesterone ASDs.

As well as considering the method for progesterone ASD production, it is also important to consider the type of polymer to be used. Polymers play two main roles; first of all, they stabilise against crystallisation. ASDs are thermodynamically unstable and can recrystallize within a pharmaceutically relevant time-scale (Lee et al., 2014); such occurrences would negate any solubility advantages. Secondly, polymers can enhance dissolution by preventing crystallisation of the supersaturated solution produced as a result of the dissolved ASD.

The mode by which polymers prevent crystallization has been ascribed to various mechanisms including interactions between the drug and polymer (Khougaz and Clas, 2000), an increase in activation energy for nucleation (Van den Mooter et al., 2001) and a glass-transition (Tg)-dependent mobility reduction (Kumavat et al., 2013). Tg refers to the temperature region at which the polymer transitions from a hard, glassy material to a soft, rubbery material (Gibbs and DiMarzio, 1958). When the drug and carrier form a homogenous mixture the solid dispersion is in one amorphous phase. The high Tg of the polymer causes an increase in the Tg of the drug, thus reducing the drugs mobility. This type of reduced mobility is often referred to as anti-plasticisation (Kumavat et al., 2013).

There are a variety of polymers available for the production of ASDs including synthetic polymers and natural derivatives. Examples of synthetic polymers are povidone, polyethyleneglycol and polymethacrylates. Natural product based polymers are primarily composed of cellulose derivatives including ethylcellulose, hydroxypropylcellulose and hydroxypropyl methylcellulose (hypromellose, HPMC) or starch derivatives such as cyclodextrins (Leuner and Dressman, 2000).

One example of a well-known synthetic polymer is Eudragit, a polymethacrylate. Eudragit is commonly used for the production of amorphous solid dispersions (Nollenberger et al., 2009). Eudragit is trademark of Rohm GmbH & Co. KG. Darmstadt in Germany and was first marketed in the 1950s. It is prepared by the polymerization of acrylic and methacrylic acids or their esters (Nikam V .K 2011). An example of a naturally derived polymer is the cellulosic enteric coating agent, HPMC acetate succinate (HPMCAS) which is an esterified derivative of hypermellose, a semi-synthetic, inert polymer. HPMCAS has been shown to be an attractive candidate for use as a carrier in solid dispersions (Tanno et al., 2004). There are a variety of Eudragit and HPMCAS polymers avialble on the market which differ by their dissolution profiles. Eudragit S for example is already in use for the targeted delivery of mesalazine to the colon for the treatment of ulcerative colitis and is marketed as Asacol®.

1.12 Thesis Aims

The aim of this thesis is to first investigate the differences in target gene and protein regulation between natural progesterone and the synthetic progestins, MPA and LNG which are commonly used in the treatment of hyperplasia. These differences will be assessed through a series of *in vitro* stimulation experiments on endometrial epithelial and stromal cell lines to observe changes at the RNA and protein level. Furthermore, biopsies will be taken from patients receiving synthetic progestin treatment before and during therapy to assess the effects of the two named progestins on protein targets in an *in vivo* situation. Moreover, several of the targets will be assessed for their potential role as markers of hyperplasia presence and progression.

To probe the reasons behind the poor biavailability of natural progeserone the stability of progesterone throughout the GI tract will be established using tissues and fluids from rat and human intestine and liver, the results of this data will be used to determine an area of the intestine where progesterone is most stable with a view to developing an oral formulation targeted to this region.

The production of a natural progesterone targeted delivery system will be attained through the use of amorphous solid dispersions (ASD). Once produced, the

efficiency of the progesterone loaded ASDs will be characterised through a series of experiments to determine particle topology, dissolution profiles and chemical stability. The progesterone amorphous powder will then be subjected to the same series of *in vitro* experiments as the synthetic progestins to establish whether progesterone ASDs differs from progesterone at the molecular level.

In summary the aims of this thesis are: a) to investigate the effect of natural and synthetic progestins on the regulation of PR targets in endometrium using *in vitro* models and patient samples, b) to better understand the stability of progesterone in the GI tract and liver and c) to produce a natural progesterone formulation for targeted oral delivery with improved bioavailability.

Chapter 2

Materials and Methods

2. Materials and Methods

During this chapter materials and methods are grouped into the chapters within which they are used.

2.1 Materials and Methods Used in Chapter 3

2.1.1 Immunohistochemistry Analysis of key Progestin Target Proteins

2.1.1.1 Clinical Data and Patient Demographics

Sixty patients were enrolled in this study. Patient recruitment and consent was carried out at Gynaecology clinics in Singleton, Neath Port Talbot and Bridgend Hospitals, ABMUHB. Ethical approval was obtained from the South Wales Research Ethics Committee. Formal written consent was obtained from all patients. The patients were pre or postmenopausal. The study groups contained patients with hyperplasia without atypia (20 patients) or with atypia (20 patients). Study groups were matched by age and BMI with the control group (20 patients) (Table 2.1). The endometrial samples were obtained either by Pipelle endometrial sampling in clinic, or by Dilatation and Curettage for patients undergoing Hysteroscopy assessment under general anaesthetic. The groups were established after the histological examination, which was routinely performed for all patients as part of their care. From 18 patients in the non-atypia group, samples were investigated for the expression of biomarkers pre and post endogenous hormone treatment with the synthetic progestin, Medroxyprogesterone Acetate (MPA) 20mg/day for 3 months. The histology of all these samples reverted back to atrophic endometrium after MPA treatment.

	Controls	Hyperplasia	Hyperplasia with atypia
AGE	50 ± 9.76	51±12.56	53±10.6
BMI	28 ± 6.75	27± 4.78	28± 2.3

Table 2.1: Age and BMI of Patients from Control, Hyperplasia and Hyperplasia with Atypia Groups. Values are given as mean ± standard deviation

2.1.1.2 Immunohistochemistry Analysis of Patient Samples from Control and Study Groups

Immunohistochemistry (IHC) is a method used to demonstrate the presence and location of proteins within tissue sections. For IHC analysis, control and study samples were fixed in 10% buffered formaldehyde for 24hours at the time of endometrial biopsy. The fixed samples were embedded in paraffin and cut into sections between 3 and 4 μM in thickness. Sections were removed of paraffin (dewaxed) with xylene, incubated through a series of methanol grades and fixed onto Superfrost slides for staining. Routine Haematoxylin and Eosin staining was performed on each slide and the sections examined for morphometric dating of the endometrial biopsy according to Noyes criteria modified by Accosta and Lessey (Acosta et al., 2000).

For the detection of proteins using specific antibodies, barcode labels for each antibody were printed and attached to the slides; the Ventana machine (Ventana Biotek Solutions, Tucson, AZ, USA) at Singleton Hospital is able to recognise the barcode and was used for the IHC experiment. First, slides were heated in citrate buffer CC1 (Ventana) on a benchmark XT processor to 100°C for 1hour for antigen retrieval. Next, the slides were incubated with 100μL of primary antibody (Table 2.2). The length of time and the temperature used for this incubation was optimal for the specific antibody used.

Antibody name	Catalogue number	Species
FKHR (FOXO1) (H128)	Sc-11350	Rabbit
Progesterone Receptor	Novo-Castra NCL-PGR	Mouse
Amphiregulin (H155)	Santa Cruz SC25436	Rabbit
Lactoferrin (B97)	Santa Cruz Sc-53498	Mouse

Table 2.2: Antibody Name, Manufacturer/ Catalogue Number and Species of origin used for Immunohistochemical Analysis

Positive immunostaining was detected using Ventana I View DAB detection kit (Ventana Biotek Solutions Tucson AZ). Staining was visualised by detection of the interaction between Avidin-Biotin peroxidase complex solution and the secondary biotinated antibody.

Finally, slides were counter-stained with 100µL Haematoxylin stain for 1 minute at room temperature, incubated in xylene for 5 minutes then left to air-dry and mounted in DPX to be examined by light microscopy.

2.1.1.3 Scoring of IHC slides

Fixed slides were visualised under Zeiss light microscope at 40x objective. The presence of proteins within the samples was detected by evidence of brown staining. Each slide was scored by three independent observers for both intensity and distribution of proteins in glandular and luminal epithelium and also in Stroma. The cellular location of the staining was also noted (nuclear or cytoplasmic). Each scorer used an immunohistochemical scoring system previously described by Lai *et al*, 2005 (Lai et al., 2005). Positive red-brown stain was scored for intensity and distribution of staining (H-score). Intensity was scored from 0 to 4 where 0 reflects the absence of a stain and 4 is very strong staining. Distribution of stain throughout the tissue section was scored as follows: 0 - absent, 1 – less than 30%, 2 – 30-60%, 3 – more than 60% and 4 – 100%. Positive and negative controls were also scored; positive control was from human colon and negative control was endometrial tissue without primary antibody. Scoring of controls allowed for more accurate scoring of tissue samples. The observers were blinded to the patients' diagnosis and demographics prior to scoring the slides.

2.1.1.4 Statistical Analysis of IHC Scores

Data distribution was assessed for normality using the Kolmogorov-Smirnoff test. Non-normally distributed data were then analysed with the non-parametric Kruskal Wallis test, followed by a Mann-Whitney U test applied post hoc to determine statistical significance. All IHC data was deemed non-normal and analysed using this method. Statistical analyses were performed using SPSS version 10.0 (SPSS,

Chicago IL). A *P* value of less than 0.05 was considered statistically significant and was expressed by one star (*), a *P* value of >0.01 was expressed by two stars (**) and a *P* value of >less than 0.001 was considered highly significant and expressed by three stars (***).

2.1.2 Cell Culture

2.1.2.1 Introduction to Endometrial Cell Lines

Two endometrial cell lines were utilised during this study; one representing the epithelial compartment of the endometrium and one representing the stromal compartment. A brief background on the cell lines is given.

The well-differentiated endometrial adenocarcinoma cell line, Ishikawa, was isolated from a 39 year old female of Asian ethnicity in 1982. The presence of estrogen, progesterone and androgen receptors have been documented in *in vitro* culture cells from this cell line (Lessey et al., 1996, Lovely et al., 2000, Nishida et al., 1985). Furthermore, the fact that cell growth can be maintained in an estrogen-free medium shows a lack of estrogen dependency (Nishida et al., 1985). Extensive research has been carried out on this cell line and estrogen and progesterone receptors have been shown to be regulated in a similar manner to the normal endometrial epithelium (Lessey et al., 1996), thus making this cell line adequate for the experiments carried out in this study.

The second cell line to be used was the human endometrial stromal cell (HESC) cell line. HESCs were cultured from a woman of reproductive age; the cells were obtained during a hysterectomy for a benign condition. The cells were immortalised using human telomerase reverse transcription (hTERT); hTERT was used to add TTAGGG repeats to telomeres to compensate for their shortening. The HESC cell line contains no chromosomal, structural or numerical abnormalities and is shown to display similar outcomes to primary, unpassaged, stromal cells after treatment with ovarian hormones. HESCs are karyotypically, morphologically and phenotypically comparable to primary parent cells and are a credible cell line for work in this project

(Krikun et al., 2004). Both the Ishikawa and HESC cell lines were purchased from the European Collection of Cell Cultures.

2.1.2.2 Culture of Cell lines

The cell lines described each have adherent properties and were cultured in plastic culture vessels (Falcon T25, 75, 125) in Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (DMEM/F-12) supplemented with 10% foetal bovine serum (FBS), 1.5mM glutamine, 1mM sodium bicarbonate, 1mM sodium pyruvate and 1% pen-strep. The cells were maintained at 37°C in a 5% CO_2 humidified incubator. Cells were sub-cultured according to guidelines set by distributor; Ishikawa cells were split at subcultivation ratio of 1:5 at 70-80% sub-confluency and HESC cells were split at a subcultivation ratio of 1:3 at 70-80% sub-confluency.

In order to sub-culture cells, cells were first washed in calcium and magnesium free phosphate buffered saline (PBS) (Gibco) in order to maintain pH, osmotic balance and to promote cell detachment. The PBS wash serves to promote detachment as it removes DMEM/F12 residue from the cells. The supplemented DMEM/F12 contains calcium, magnesium and bovine pancreatic trypsin inhibitor which block the anti-adherent action of trypsin (Borjigin and Nathans, 1993). Following the wash step, cells were detached from the culture vessel by incubation with 0.25% trypsin 1mM Ethylenediaminetetraacetic acid (EDTA) (pH 8) in Hanks' Balanced Salt Solution (HBSS) containing phenol red and excluding $CaCl_2$, $MgCl_2$ and $MgSO_4$ (Gibco Invitrogen Cat No: 25200-056). The cells were incubated in the trypsin solution for no more than 5 minutes at 37°C. To confirm cell detachment, cells were observed using an inverted light microscope (Zeiss). On confirmation of detachment 10mL of supplemented DMEM/F12 media was added to the suspension to neutralise the trypsin and prevent further tryptic activity which could damage the cells. The suspended cells were transferred to a sterile 15mL centrifuge tube and spun at 1200 rotations per minute (RPM) for 5minutes in an Eppendorf 5810 centrifuge. The excess media was decanted and the resultant cell pellet was re-suspended by physical disruption and immersed with 10mL of supplemented DMEM/F12 media. The desired ratio of cells was taken from the suspension and incubated in a T75 flask in

fresh media at 37°C in a limited 5% CO_2 atmosphere. All materials in this section were sourced from Gibco Invitrogen unless otherwise stated.

2.1.2.3 Plating Cell Lines for RNA, Western Blot and InCell Analysis

In preparation for *in vitro* incubation with steroid hormones, Ishikawa and HESC cell lines were plated into either 6 or 12 well plates. For RNA, Western Blot and InCell analysis cells were handled as described in Section 2.1.2.2. However, when re-suspended in 10mL of supplemented media, cells were counted using TC10 automated cell counter (biorad). To count cells, 50µl of cell suspension was diluted in 1:1 with trypan blue; trypan blue can enter the nucleus of dead cells allowing these cells to be excluded from the cell count. 10µl of the cell suspention with trypan blue was pipetted onto a dual chamber counting slide (Biorad) and placed in the cell counter; the cell counter then automatically estimated the amount of cells per mL excluding cells with trypan blue in the nucleus. Cells were counted 3 times using this method and an average cell count was taken. The amount of cells was then adjusted appropriately by adding supplemented DMEM/F12 media. For western blot and RNA analysis 1 x 10^5 Ishikawa cells were added to 6 well plates (well volume 9.6cm^2) (CellStar Cat no. 657160). For HESCs 2 x 10^5 cells were added to 6-well plates. The use of an increased concentration is necessary for HESC cells as the growth rate of these cells is reduced in comparison to the Ishikawa cell line. For InCell Analysis 1x10^4 Ishikawa cells or 2x10^4 HESC cells were counted and seeded into a 24-well plate (CellStar Cat no. 662160). After seeding, cells were placed in a 5% CO_2 atmosphere at 37°C for 24h. At this time cells were approximately 70% confluent.

After the 24h incubation cells were rinsed in PBS. Following this, cell media was replaced with DMEM F12 media supplemented with 10% dextran-coated, charcoal treated FBS (Gibco), 1.5mM glutamine, 1mM sodium bicarbonate, 1mM sodium pyruvate and 1% pen-strep (stripped media) and incubated for a further 24h.

After the second incubation period, media was replaced with fresh stripped media and treatment preparations were added to the cells for 48h.

Ishikawa and HESC cells to be harvested for PCR or InCell analysis were treated with MPA, P_4 or Levonorgestrel (LNG). Ishikawa cells were also treated with 17β-estradiol (E_2) alone or combined with each of the progestogens (MPA, P_4 or LNG) while HESC cells were treated with 8-Bromodadenosine 3', 5' Cyclic Monophosphate (cyclic AMP, cAMP) alone or combination with progestegens.

2.1.2.4 Preparation of Cell Treatment

E_2 was prepared by adding 0.00272g of powdered E_2 (Cat: E8875 >98% Sigma Aldrich) to 1ml of absolute ethanol (100%) (Fisher Scientific) to give a concentration of 10mM. This was then serial diluted 1:10 until a 100μM stock was reached. 1μL of the 100μM stock was added to each 1mL of cell media to give a 100nM treatment concentration.

P_4, MPA and LNG were prepared as follows: 0.00314g of Progesterone (PO130-25g) or 0.00386g of MPA (Cat: M1629, SIGMA) or 0.00312g of LNG (Cat: N2260, SIGMA) were added to 1ml of absolute ethanol (100%) (Fisher Scientific) to give a concentration of 10mM. This was then diluted 1:10 to give a 1mM stock. 1μL of stock was diluted in 1mL of media to give a treatment concentration of 1μM of hormone.

For the preparation of cyclic AMP 1.16mL of distilled water was added directly to 25mg of cyclic AMP powder (Cat number B7880 25MG) to give a stock concentration of 50mM. This was diluted 1:100 into cell media to give a final concentration of 0.5mM.

2.1.3 Western Blot Analysis

Ishikawa or HESC cells were grown to confluency in a six-well plate as described in Section 2.1.2.3. Cells were then scraped directly into 250μl of extraction buffer consisting of 0.05M Tris (pH7), 8M Urea, 1% sodium dodecyl sulphate (SDS) and 0.01% phenylmethanesulfonylfluoride (PMSF) in distilled water. The samples were then ready for immediate protein quantification using the Bradford assay.

2.1.3.1 Protein quantification by Bradford Assay for Western Blot Analysis

Prior to Western Blot analysis protein was quantified with the Bradford assay using Bradford Reagent from Sigma (B6916) and following manufacturer's guidelines; a brief description of the procedure is given. Due to the large amount of urea in the samples, samples were diluted 1:10 in water in order to be compatible with the Bradford Reagent. 100µl of diluted sample was then added to 3mL of Bradford Reagent in 15mL centrifuge tube (fisher scientific) and vortexed gently for thorough mixing. The samples were incubated for 10-15minutes and 1mL of sample was transferred to 1.5mL cuvettes (fisher scientific). The absorbance was measured using the nanodrop 2000c (Thermo scientific) at an absorbance of λ595nm. A standard curve was generated using known concentrations of bovine serum albumin (BSA) in diluted extraction buffer to give concentrations between 0 and 0.25mg/ml protein.

The concentration of protein in the samples was then calculated using the straight line equation (Y=mX+c) from the BSA standards and inputting the values obtained for the protein within this equation. The quantified protein was adjusted to 10µg for analysis by Western Blot.

2.1.3.2 Protein Separation by Gel Electrophoresis

Separation of proteins was achieved through sodium doecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). A 7.5% polyacrylamide gel was prepared for SDS-PAGE using the components listed in Table 2.3. The resolving gel was made first and pipetted into a 1.5mm space between two glass plates (biorad); 2-propanol (Sigma Cat no. 109827) was pipetted on top of the gel to create a flat surface for the application of the stacking gel. 2-propanol was removed using distilled water (millipore) and blotting paper (Whattman). Once the resolving gel had set the stacking gel was added and a 1.5mm comb containing 10 wells was rested in the solution until it set.

Reagent	Stacking Gel (4%)	Resolving Gel (7.5%)
MilliQ Distilled Water (Millipore)	3mL	7.25mL
30% Acrylamide solution (Bio-Rad, UK)	650µL	3.75mL
1.5M Tris (pH 8.8)	-	3.75mL
1.0M Tris (pH 6.8)	1.25mL	-
10% SDS (Fisher Scientific)	50µL	150µL
TEMED (SIGMA)	5µL	15µL
10% APS (SIGMA)	25µL	75µL

Table 2.3 Reagents required for the production of Stacking and Resolving Gels for Gel Electrophoresis.

To prepare the protein samples for gel electrophoresis, the samples in extraction buffer were added in a 1:1 ratio to 2x laemmli buffer (Sigma Cat no. 53401-1VL). Laemmli buffer contains glycerol and bromophenol blue. Glycerol causes the samples to sink to the bottom of the well and bromophenol blue is a tracking dye which migrates through the gel first and provides a good indication of how far the proteins have travelled. The laemmli buffer also contains SDS detergent which fully denatures the protein and removes all higher order structures as well as causing the protein to become uniformly negatively charged allowing it to migrate towards the positive electrode during the electrophoresis process. A colour-stained molecular weight marker (Sigma) was also diluted in laemmli buffer at the same ratio allowing it to travel at the same rate as the samples.

After the addition of laemmli buffer, samples were incubated at 95°C for 5 minutes in a T100 thermal icycler (Biorad) to denature the proteins and 10µg of protein was loaded into the required wells. 5µl of protein ladder (Biorad) was also added to the first well. Samples were run on a 7.5% SDS-polyacrylamide gel for 80minutes at 120volts in 1% SDS electrophoresis buffer (pH 8.3). 1% SDS buffer was produced by adding 15.1g Tris Base, 72g Glycine and 5g SDS to 1L of distilled water, this was then pH tested and diluted 1:5 in water before use.

2.1.3.3 Protein Transfer to Membrane

Separated proteins were transferred to polyvinylidene (PVDF) membrane with 0.2mM pores and a protein binding capacity of 150-160 mg/cm2 (Bio-Rad). The membrane was cut to a similar size to the electrophoresis gel, activated for 15s in absolute ethanol (fisher scientific), transferred to distilled water (millipore) for 5mins then allowed to equilibrate in transfer buffer (Table 2.4) for 10minutes. The activated membrane was loaded into a biorad cassette against the SDS gel enveloped in filter paper and sponges as depicted in Figure 2.5.

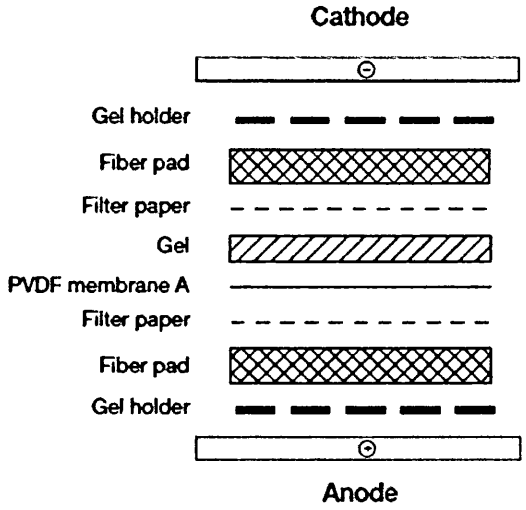


Figure 2.1: Layout of Components for Protein Transfer from Electrophoresis Gel to PVDF membrane.

The transfer was carried out in Transfer buffer and subsequent wash steps carried out in 1x Tris Buffered Saline with tween (TBS tween_Ethylenediaminetetraacetic acid) (Table 2.4). The transfer was carried out for 60minutes at 100volts and the appearance of the molecular weight marker on the transfer paper was used to confirm the transfer of proteins.

Reagent Name	Component	Quantity
Transfer Buffer	Tris-Base	3.63g
	Glycine	16.8g
	Methanol	300mL
	dH_2O	1200mL
5xTBS buffer	Tris	12.1g
	Sodium Chloride	40g
	dH_2O	1000ml
1xTBS Tween	5xTBS buffer	100mL
	dH_2O	400mL
	Tween20	0.5mL

Table 2.4: Buffer Reagents, Components and Quantities use in Western Blot Analysis.

2.1.3.4 Protein Detection

Following transfer, the membrane was removed from the cassette and submerged in 2mL of blocking buffer containing 5% BSA in 1xTBS tween (Table 2.4) for 1 hour on a see-saw rocker (Stuart Ltd) at 20osc/min. The membrane was washed 5 times for 5minutes each time in 1xTBS tween then submerged in primary antibody in blocking buffer overnight at 4°C on a rocking table at 40osc/min. The antibodies and concentrations used are described in Table 2.5. Following incubation the membrane was washed again 5times for 5minutes each time in 1xTBS tween. It was then incubated in secondary antibody (Ant-rabbit IgG-HRP, Table 2.5) for 1hour at room temperature on rocker at 20osc/min. A final 5 washes were carried out before the membrane was incubated for 3 minutes at room temperature in Biorad enhanced chemiluminescence (ECL) stain. The secondary antibody used in the procedure is coupled to a horseradish peroxidise (HRP) label, the ECL stain contains the substrate luminol which is oxidized by HRP to produce light. The light emitted from this reaction produces a protein band, the band was visualised using a chemidoc™ MP imaging system (Biorad).

Antibody name	Manufacturer	Species	Final conc. (µg/mL)
GAPDH (FL335)	Santa Cruz sc-25778,	Rabbit	1
PR (H-10)	Santa Cruz sc-7208	Rabbit	2
ERα (HC-20)	Santa Cruz sc-542	Rabbit	2
ERβ	Pierce Ltd PA1-311	Rabbit	2
AR	Santa Cruz N-20	Rabbit	2
Ant-rabbit IgG-HRP	GE Healthcare, NA934V	Donkey	1

Table 2.5 Antibody Name, Manufacturer, Species of origin and Concentration used for Western Blot Reactions

2.1.3.5 Western Blot Data Analysis

Fluorescent bands were analysed using Quantity One Imaging softoware (Biorad). The volume rectangle tool was used to draw around bands and background and quantify the band density by calculating signal intensity/area mm^2. The housekeeping protein, GAPDH, was also run against all samples and quantified in the same way. The density of GAPDH was used as a reference to normalise the concentration of protein. The volumes were then plotted on a graph in Microsoft excel.

2.1.4 Real Time Polymerase Chain Reaction (RT-PCR)

2.1.4.1 Isolation and Quantification of Ribonucleic Acid

The RNeasy kit from quiagen was used for the Isolation of ribonucleic acid (RNA); all components discussed were supplied by the kit unless otherwise stated and the full manufacturers protocol, 'total RNA isolation from animal tissues' can be found in Appendix A. In short, after 48 hours of treatment, 350µl of RLT buffer was added to the confluent monolayer of cells. Cells were scraped using cell scrapers (greiner bio-one) and the cell lysate was pipetted directly into qia-shredders for homogenisation. The homogenised lysate was combined with ethanol and transferred to RNeasy spin columns which bind RNA strands more than 200 base pairs in length.

To eradicate any unwanted genomic DNA bound to the spin columns, DNAse 1 digest kit (quiagen) was used in combination with the RNeasy kit by adding DNAse to RDD buffer in a 1:7 ratio. The solution was added directly to the spin column membrane and incubated for 15 minutes.

Finally RNA was eluted in 30µl of RNase free water. The spin column was centrifuged between each step as described, all centrifugation steps were carried out in eppendorf centrifuge 5424. The eluted RNA was quantified using the nanodrop 2000c (thermo sceientific) spectrophotometer and the reading taken in µg/µl of RNA.

2.1.4.2 cDNA Synthesis with Reverse Transcription (RT) Enzyme

RNA was converted to cDNA using the Applied Biosystems high capacity reverse transcription kit. First RNA was adjusted to a final concentration of 100ug/mL in a total volume of 12µL of RNAse free water in microfuge tubes. 2µL of this solution was isolated and added to a further 38µL of RNAse free water and kept for PCR untranscribed negative control. The remaining 10µL was added to components from the reverse transcription kit in the following concentrations; 2µL of 10x RT–buffer, 0.8µL of 25x dNTP mix (100mM), 2µL of 10x RT-random primers, 1µL of Multiscribe Reverse Transcriptase and 3.2µL of Nuclease-free water to give a total volume of 20µL. The components were mixed gently by pippetting up and down and centrifuged briefly to collect residual liquid to the bottom of the tube and to eliminate any air bubbles.

The samples were subsequently placed in a T100 Thermal Cycler (Biorad) and heated to 25^{O}C for 10 minutes, 37^{O}C for 2hours and 85^{O}C for 5minutes, the cycle was then held at 4^{O}C until the samples were collected from the machine. The long incubation at 37^{O}C allows for cDNA synthesis and the short incubation at 85^{O}C is required for the inactivation of the reverse transcriptase enzyme. cDNA was stored at -20^{O}C until used in the RT-PCR reaction. Furthermore, cDNA was diluted in nuclease free water (Ambion) in ratios of 1:5 1:10, 1:100 and 1:1000 (cDNA: water). The 1:10 dilution served as the working template for RT-PCR and the remainder of the dilutions were used to produce a standard curve.

2.1.4.3 Primers used in Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to amplify cDNA against specific primer pairs. The primers used are described in table 2.6.

Each primer set was designed using beacon designer software and ordered through Sigma Aldrich. Primers were designed to span a region of 75-150bp and were ordered with a synthesis scale of 0.025µmol and purification desalt. Primers were ordered dry in a powder constitution and diluted to a 100µM stock upon arrival. For use in the qRT-PCR reaction primers were converted to 4µM by diluting in distilled water.

Gene	Primer	Primer Sequence (F)	Amplicon Length (bp)	Ta (°C)
GAPDH	Sense	GTCCACTGGCGTCTTCAC	145	54.7
	Antisense	CTTGAGGCTGTTGTCATACTTC		
PR	Sense	CTGCACTCGGCCTCAACGGG	119	66.2
	Antisense	TGTGGGCTCTGGSCTGGCTTC		
FOXO1	Sense	TCCTACGCCGACCTCATCAC	93	63.0
	Antisense	CACGCTCTTGACCATCCACT		
AREG	Sense	TGGACCTCAATGACACCTACTC	150	53.5
	Antisense	AGGACGGTTCACTACTAGAAGG		
LF	Sense	GCTACTTCACRGCCATCC	128	48.7
	Antisense	ACTCCACTGGTTACACTTG		
PRL	Sense	CCACTACATCCATAACCTCT	140	60.0
	Antisense	GGGCTTGCTCCTTGTCTTC		
GREB1	Sense	ATCAGCTGCTCGACTTGCTG	134	62.0
	Antisense	TGAGCTCCGGTCCTGACAGAT		
IGF1	Sense	CTTCAGTTCGTGTGTGGAG	75	55.0
	Antisense	CGCCCTCCGACTGCT		

Table 2.6: qRT-PCR primer pairs for mRNA expression levels

2.1.4.4 Quantitative Real-Time Polymerase Chain Reaction

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to amplify cDNA against specific primers (described previously) in order to obtain an amplicon of 75-150bps in length. All results were normalised against an internal reference gene (GAPDH) and primers were also run against RNA template negative control. For the PCR reaction 2.5µl of cDNA (diluted 1:10 in nuclease free water), 2.5µl of forward and reverse primer premix (4µM) and 5µl of ssofast evagreen supermix (Biorad, 172-5203) were pipetted, in triplicate, into a clear 96-well plate (Biorad). The plate was sealed with Biorad PCR microseal plate sealers then vortexed briefly and centrifuged for 1 minute at 1000 rpm to collect residual liquid. Dilutions of 1:5, 1:100 and 1:1000 of the untreated control sample were also added to the plate in

order to generate a standard curve. The plate was heated and cooled using a CFX96 PCR thermal Cycler (Biorad). Plates were heated to 98°C for 2minutes, then underwent 40 cycles of heating and cooling as follows; the plate was heated to 98°C for 2 seconds to denature the DNA then cooled for 5 seconds to the annealing temperature of the primer pair being utilised, the plate was then heated to 72°C for 10seconds for elongation and the cycle was repeated (Figure 2.2). Following the PCR reaction a melt curve was generated by holding the plate at, at least, 1°C below the primer annealing temperature for 10 seconds then steadily raising the temperature in increments of 0.2°C until a temperature of 95°C was reached (Figure 2.2).

Data obtained from the PCR and High Resolution Melt analysis was automatically saved to BioRad CFX manager 3.0, further analysis of the data was carried out on an excel spreadsheet.

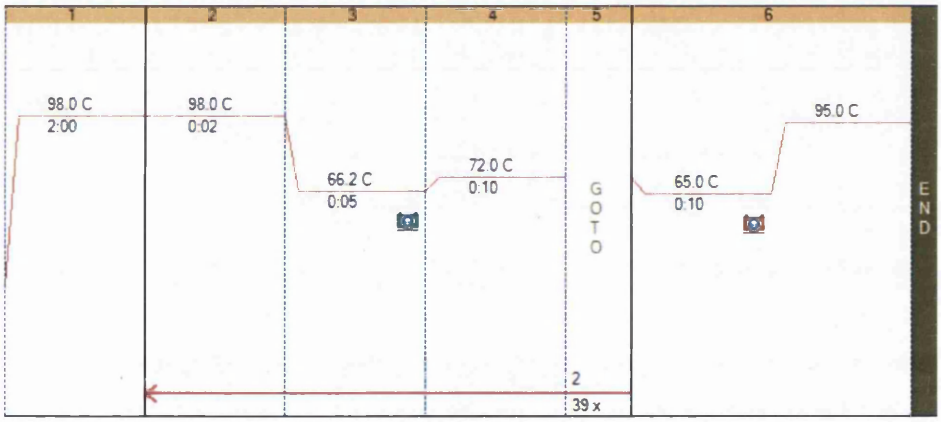


Figure 2.2: Typical qRT-PCR protocol layout. The current example is based on PR primers.

2.1.4.5 qRT-PCR Data Output (CFX manager)

The PCR reaction mix consists of primer, sample and Sybr green. Sybr green is a cyanine dye that binds double-stranded DNA and emits green light at λ520nm upon binding. During the elongation step the light emitted increases as the dye binds more DNA, thus the light emitted is proportional to the amount of DNA amplified by the primers. The amount of light emitted is recorded in relative fluorescent units (RFU) and is plotted against the number of cycles on a logarithmic scale. From this data a quantification cycle (Cq) value is determined as a cycle number at which

fluorescence has increased above background (Jordan and Kurtz, 2010). The point at which signal is no longer background is determined by the program (See Figure 2.4). Fluorescence detected below the background level is from non-specific signal from inefficient binding of sybr green. The possibility of it being from primer dimers, incorrect annealing or contamination is ruled out by the production of a melt curve. Melt curves were generated during each run and the presence of only one peak confirms the absence of possible contaminants. An example of a typical melt curve can be seen in Figure 2.3.

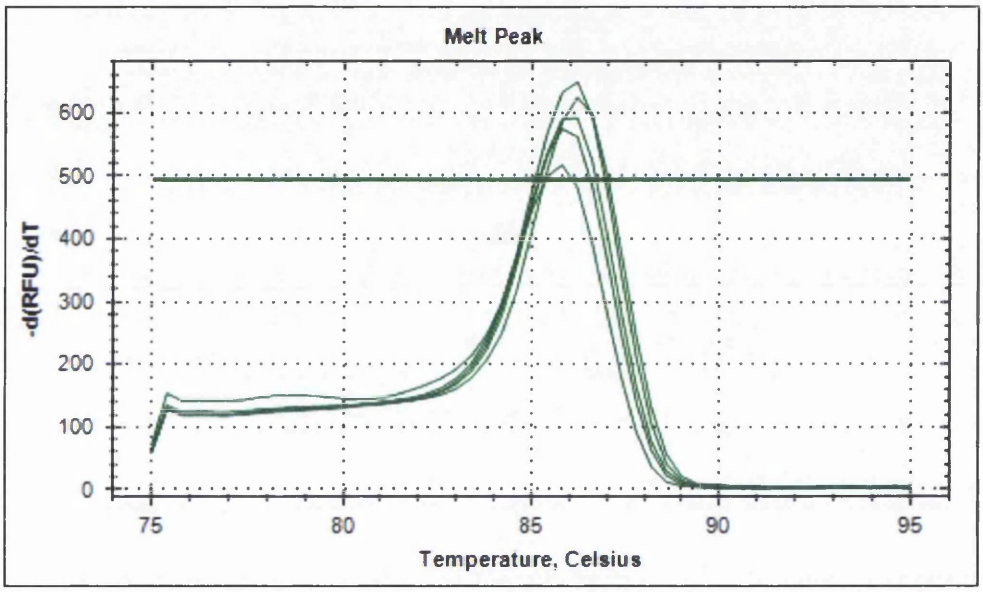


Figure 2.3: Melt curve from a typical Quantitative PCR reaction. The example shows a melt curve generated with PR primers against Ishikawa cDNA.

2.1.4.6 Generation of a standard curve

In order to effectively analyse PCR data and check the efficiency of primers, standard curves were generated. The control sample for each replicate of each experiment was serial diluted as previously described (1:10, 1:100 and 1:1000 cDNA: water), 2.5µls of these dilutions was added to 2.5µl of reverse and forward primer premix and 5µl of sybr green. The samples were run with the PCR reaction and cT values were generated and plotted against the logarithm of the input amount of standard. An example of the standard curve generated for GAPDH on Ishikawa cells can be seen in Figure 2.4.

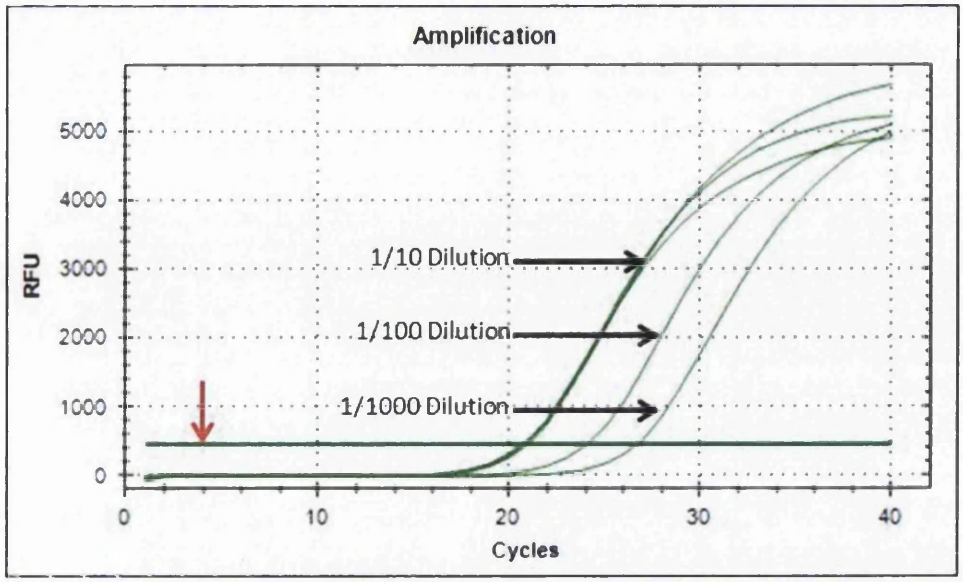


Figure 2.4: Typical qRT-PCR amplification plot. The example shows a standard curve generated with GAPDH primers against Ishikawa cDNA. Red Arrow indicates point at which signal overtakes background noise.

A new standard curve was produced on each PCR run. Each standard curve was checked for validity with the slope falling between -3.3 and -3.8, standard curves that varied greatly from these values were discarded and new standard curves were generated. The slope of the standard curve is a good indicator of the efficiency of the PCR, a slope of -3.322 is a 100% efficient but due to experimental limitations this is rarely possible and a slope with an R^2 value of between 0.95 and 1.00 is acceptable. Figure 2.4 shows an example of a standard curve obtained from the reference gene GAPDH.

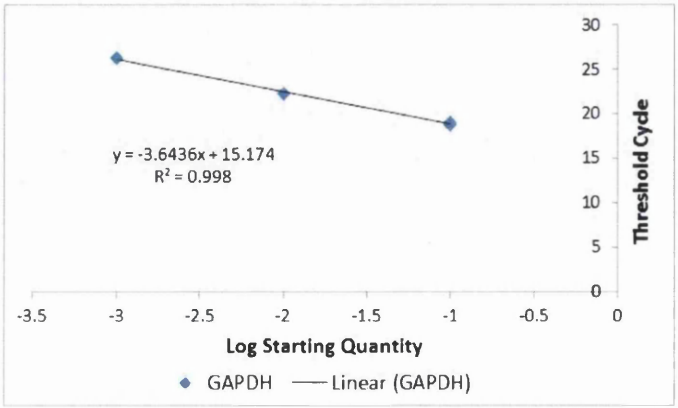


Figure 2.5: Typical Standard curve obtained from reference gene GAPDH.

2.1.4.7 qRT-PCR Data Analyses using Microsoft Excel

The triplicate cT values generated by the biorad (CFX manager 3) were copy-pasted to Microsoft excel. An average of the three values was calculated and one value was excluded where necessary. Next the standard curve was generated and data from the linear equation was used in calculating the log of the starting quantity. The log of the starting quantity was calculated using the following equation:

$$Y = \frac{X - Z}{M}$$

Where Y is the log of the starting quantity, X is the average cT, Z is the slope of the standard curve and M is the curve intercept on the Y axis. To calculate the actual starting quantity the log of the starting quantity was calculated to the power of 10 using the excel function Power (10,Y). The starting quantity was then normalised to the constitutively expressed genes, GAPDH, by dividing the starting quantity of the sample by the starting quantity of the reference gene.

All experiments were run in triplicate and subject to statistical analyses was based on the three repeats. Normalised starting quantity of hormone-treated samples was compared to control and to each other using two-tailed students T-Test in excel. As with all experiments, a *P* value of less than 0.05 was considered statistically significant and was expressed by one star (*), a *P* value of >0.01 was expressed by two stars (**) and a *P* value of >less than 0.001 was considered highly significant and expressed by three stars (***).

2.1.5 High Content Screening using InCell technology

Cells were cultured as previously described (Section 2.1.2.2). After 48h of incubation with hormone treatments Ishikawa or HESC cell monolayers were washed with PBS 5 times for 5 minutes each time and fixed with paraformaldehyde (4% w/v) for 10 minutes at 4°C. For the remainder of the procedure three 5-minute PBS washes followed each incubation step unless otherwise stated. Following fixation, cells were permaeblised by incubating with Triton X-100 (0.01%v/v) for 5 minutes at 25°C. Cells were incubated in blocking buffer (5% goat serum and 1% BSA in PBS) for 2

hours at 25°C. Next, cells were incubated at 4°C overnight with primary antibody. The primary antibodies used and the concentrations used for incubation are described in Table 2.5. PBS containing 1% BSA and 0.01% Tween20 was used for all washes from this point onwards. For fluorescent detection of the primary antibodies, an incubation of 1 hour with a secondary anti-body conjugated to a fluorescent label was required. The corresponding secondary antibodies used against each protein can also be seen in Table 2.7. The cell nuclei in all preparations were counter stained with a NucBlue™ (Life Technologies #R37605) 4', 6-diamidino-2-phenylindole (DAPI) preparation (2 µl/ml PBS for 30-60s at 25°C). Following the final washes cell were imaged in excess PBS.

Antibody name	Santa Cruz Catalogue number	Species	Final conc. (µg/mL)
FOXO1 (FKHR) (H-128)	SC-11350	Rabbit	1
PR (H-190)	SC-7208	Rabbit	2
Amphiregulin (H-155)	SC-25436	Rabbit	1
Lactoferrrin (B97)	SC-53489	Mouse	4
GREB1 (N-13)	SC-138794	Rabbit	1
Anti-Rabbit IGG Texas Red	SC-2780	Goat	1
Anti-Mouse IGG Texas Red	SC-2781	Goat	1

Table 2.7 Antibody Name, Manufacturer, Species of origin and Concentration used for InCell Analysis

2.1.5.1 Image Acquisition

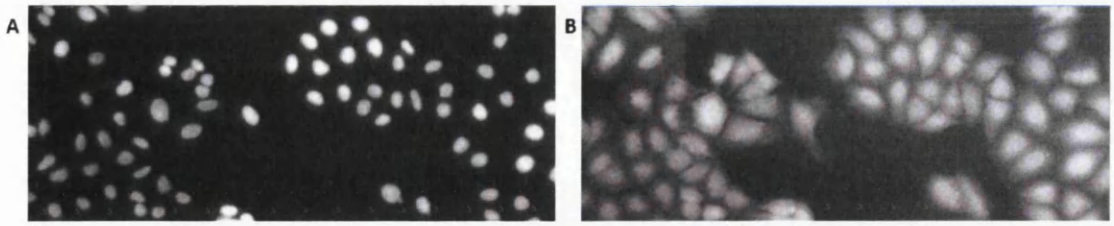
High content screening of all proteins using immuno-fluoresce was undertaken using the InCell Analyzer 2000 (GE Healthcare). Image data was recorded in two emission spectra channels; DAPI nuclear staining (Channel 1 λ 470 nm for 0.1s) and Texas Red protein staining (Channel 2 λ __nm for up to 5s). The nuclear stain was a requirement to separate individual cells in subsequent analysis. Five low

magnification images (10x objective) were obtained for control and treated samples. Four images were obtained per well and each image contained approximately 500 cells giving a total of 2000 cells imaged per well. All experiments were carried out in triplicate.

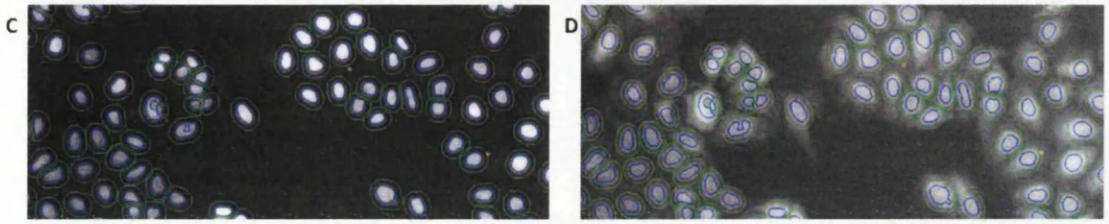
The InCell analyser acquisition software allows for a random distribution of fields across the well surface and regions of the centre or edge of the well may be excluded from field selection. The regions close to the well boundary were excluded from field selection as they were not representative of other areas of the well. The exposure time for each channel was kept constant across all experiments; therefore changes in fluorescence were directly attributable to protein expression levels. The output file is termed XDCE, which is a folder containing compiled images from all channels and all fields of view screened.

2.1.5.2 High Content Screening Image Analysis

XDCE files were analysed using InCell workstation software suite (GE Healthcare Analysis) to quantify protein immuno-fluorescence. The protocol used on InCell workstation was able to detect individual cells as the high contrast between DAPI stain and background facilitated efficient nuclear identification. To analyse fluorescent stain across the whole cell, a 10μM collar was drawn around the nucleus representative of the cytoplasmic region (Figure 2.6). The protein fluorescence was then determined for the cell nucleus based solely on the area overlying the DAPI stain whereas whole cell intensity was calculated based on the area covering the whole cell, inclusive of the area overlying the nucleus. The intensity was calculated by the software on a cell by cell basis and assigned a value for protein expression given in arbitrary units. The intensity of fluorescence was normalised against a negative control which involved subtracting the cell intensity of a no-antibody negative control from the experimental value to account for any background staining meaning the remaining signal was due to specific protein-bound primary antibody. All analysis was subject to three independent repeats and data was transferred to Spotfire Decision Tree for further analysis.



Cell images aquired from InCell analyser 2000. **A.** DAPI channel (Nucleus). **B.** Texas Red Channel (Protein Stain)



Flourescent Intensity determined using InCell Workstation. **C.** DAPI channel (Nucleus). **D.** Texas Red Channel (Protein Stain)

Figure 2.6: Nuclear and Cellular Identification using InCell Analyser Workstation. Nuclear staining was identified by the high-contrast intensity between DAPI stain and background as determined by the software whereas cellular intensity was identified by the application of a 10µm collar around the nuclear circumference.

2.1.5.3 InCell Data Analysis in Spotfire DecisionSite and Microsoft Excel

After running InCell Workstation analysis on a cell-cell basis, data was compiled using Spotfire DecisionSite analytics software (Tibco), which is able to condense the vast amounts of data from the Workstation output.

Data obtained in Spotfire was then transferred to excel in order to graph data efficiently and carry out statistical analysis. The well values were tested for significance using a two-tailed Student's T-test conducted on average values. Treated samples were compared against the untreated controls where P values of ≤ 0.05, ≤ 0.01 and ≤ 0.001 were considered statistically significant and denoted with a *, ** and *** respectively.

2.1.6 Prolactin Enzyme-linked immunosorbent assay (ELISA)

After incubation and treatment of HESCs for RNA extraction as described (Section 2.1.2.2), cell supernatant was collected and stored at -20°C in 1.5mL eppendorf tubes until utilised in an enzyme-linked immunosorbent assay (ELISA). The amount of

secreted prolactin was determined using commercially available ELISA kit (R&D systems, cat no.DY682) following the manufacturers standard protocol. All antibodies described were provided in the kit.

Prolactin monoclonal capture antibody was incubated overnight in a 96-well ELISA microplate (greiner) at a concentration of 0.8µg/ml. The long incubation allowed the antibody to bind to the plate. The plate was next incubated with blocking buffer, consisting of BSA (1% w/v) in PBS, to block all unbound sites on the plate and prevent false positive results. The plate was then ready to perform the assay.

To quantify the amount of secreted prolactin, known concentrations of prolactin were used to generate a 7-point standard curve. A standard was provided with the ELISA kit, containing 150ng/ml of recombinant human prolactin. The standard was serial diluted to concentrations of 1000, 500, 250, 125, 62.5, 31.25 and 15.63pg/ml for use in the assay. Reagent diluent without standard was prepared to account for background and subtracted from each reading.

Samples or standards were incubated in the ELISA plate for 2hours allowing prolactin protein to bind the capture antibody, after the incubation excess supernatant was decanted and the plate was washed 3 times in a wash buffer consisting of 0.05% Tween® 20 in PBS to remove any unbound protein.

Next biotinylated secondary antibody containing goat anti-human Prolactin was added to each well for 2 hours to bind adhered prolactin protein. Next, after 3 washes, Streptavadin-horseradish peroxidase (HRP) was added to the plate for 20 minutes out of direct sunlight. Streptavadin binds to biotin in the secondary antibody while the conjugated HRP provides enzyme activity for the detection of prolactin. After washing away excess streptavidin HRP, a substrate solution was added to the plate. The substrate contained hydrogen peroxide and Tetramethylbenzidine which reacted with HRP to caus a blue colour to develop in proportion to the amount of prolactin present in the sample or standard. Sulphuric acid (1M) was then added directly to the plate to stop this reaction; the addition of sulphuric acid also causes the solution to turn yellow. The optical density of each well was then determined immediately using a microplate reader set to 450 nm. The standards were used to

prepare a standard curve and all optical densities from unknown samples were compared to this.

2.1.7 Cell Imaging and Image J

HESC cells were imaged with Zeiss light microscope using Zeiss imaging system. Still images were taken of live cells at 20x magnification in 6 well plates at 48hours after treatments.

Images were then subjected to image J analysis which quantifies cell roundness based on the equation:

$$R = 4 * pi * A/P\text{^}2$$

Where R is roundness, A is area in pixels and P is Perimeter

This equation generates a value from 0-1 where 1 is a perfect circle. Image analysis was carried out on three independent repeats and displayed as a bar graph showing the average of 3 repeats. The data was then subject to students t-test to establish significance where *P* values of ≤ 0.05, ≤ 0.01 and ≤ 0.001 were considered statistically significant and denoted with a *, ** and *** respectively.

2.2 Materials and Methods Used within Chapter 4

2.2.1 Stability of Progesterone in Tissue Homogenates

The stability of progesterone was assessed in homogenates of the human and rat liver and pooled intestinal mucosa and in the rat proximal and distal small intestinal mucosa and colonic mucosa. Stability assays were carried out in the presence and absence of the co-factor NADPH. The stability of progesterone was also assessed in human liver and intestinal cytosol in the presence and absence of several enzyme inhibitors.

2.2.1.1 Preparation of Stock and Working Solutions for Homogenate Stability Assay

Fresh 10mM stock solution of progesterone or midazolam positive control (Abcam Biochemicals) was prepared in Dimethyl Sulphoxide (DMSO). The 10mM stock solutions were then further diluted in DMSO to give a final working solution of 0.4mM. Fresh stock solution of NADPH was prepared by adding NADPH tetrasodium salt (Roche Applied Science) to PBS to a final concentration of 100mM. Stock solutions were prepared fresh before each experiment.

2.2.1.2 Preparation of Liver and Intestinal Homogenates

Pooled samples of human and rat intestinal homogenates and rat liver homogenates were purchased from biopredic international and arrived pre-prepared in a pH 7.4 buffer containing 100mM Tris-HCl, 1mM EDTA and 250mM Sucrose. Rat Intestinal and liver homogenates were obtained from a pool of 3 male Sprague Dawley rats. The rats from which intestinal homogenates were produced weighed between 250 and 260 grams and the rats from which liver homogenates were produced weighed between 190 and 230g and were from 6-8weeks old. The human intestine homogenate was obtained from a 75 year old deceased male with peritonitis. Human livers were obtained from the UK human tissue bank and contained pooled samples from 9male and 3 female donors (donor details can be seen in Appendix B). Liver homogenates were prepared by removing any excess fat and weighing 50g from each liver sample. The samples were then suspended in ice cold SET buffer (50 mM Tris-HCl and 50 mM EDTA in distilled water adjusted to pH7.4) at a final volume of approximately 2.5 times the weight of the liver. The liver was minced and poured into a pre-cooled bead-milling homogenisation vessel (Precellys) and homogenised at 11,000 rpm. Any unhomogenised tissue was discarded and the total weight of homogenised tissue was determined. The samples from individual sections of rat intestine approximating duodenum, jejunum and ileum were obtained from 6 male Sprague Dawley rats between 6-10weeks old, the rats weighed between 200-270g. After sacrifice by lethal injection the intestines were immediately excised and placed in oxygenated KREBs buffer (1.26 M NaCl, 25 mM KCl, 250 mM $NaHCO_3$, 12 mM

NaH_2PO_4, 12 mM $MgCl_2$ and 25 mM $CaCl_2$). The intestines were washed in KREBs buffer to remove any part-digested food contents. The muscle layer of the intestinal wall was removed by scraping away with a scalpel and the remaining mucosa was weighed then added to 2.5 times its weight in KREBs buffer. The homogenate was minced then homogenised in a Precellys bead-milling homogeniser. Any unhomogenised tissue was discarded and the total weight of homogenised tissue was determined.

The protein concentration for liver and pooled intestinal samples was determined using the method described by Lowry *et al* (1951) with BSA as a standard. The Lowry assay is based on 2 steps which lead to colour development; the first is a reaction between protein and copper and the second is the reduction of a folin reagent by the copper treated protein. The absorbance of the colorimetric change within the BSA standards and the samples was measured at 540nm on a SPECTRAFluor Plus (TECAN) spectrophotometer. The protein concentration was adjusted to 5mg/ml for these homogenates by diluting in phosphate buffer (pH7.4). Phosphate buffer (PB) was produced by dissolving 7.1g of Na2HPO4 anhydrous in 500ml of distilled water, this solution was warmed to 37°C and the pH was adjusted to 7.4 using the a solution containing 6.8g KH2PO4 anhydrous in 500ml of distilled water.

The protein concentration of rat duodenum, jejunum and ileum samples was determined using the detergent compatible (DC) protein assay kit (Biorad) with BSA standards. This assay is based on the Lowry method and uses copper and folin to develop colour. Briefly, 5 µl of sample or standard, 25µl alkaline copper tartrate and 200µl of dilute folin reagent were pipetted into a 96-well microplate. The solution was incubated at room temperature for 15 minutes and the absorbance was measured at 750nm on SPECTRAFluor Plus (TECAN) spectrophotometer.

2.2.1.3 Homogenate Stability Assay

Homogenates of the human and rat liver and pooled intestine or PB control were incubated in wells of a 1ml deepwell RNAse/DNAse 96 well plate (fisher) for 5 minutes in thermomixer comfort (eppendorf) until reaching a temperature of 37°C.

Progesterone or midazolam stock solution was diluted into the homogenate to give a final concentration of 1μM. A final concentration of 1μM of progesterone was also added to the PB control.

After the short incubation of up to 5 minutes, either a final concentration of 1mM NADPH stock solution or PB was added to the mixtures to start the reaction and samples were taken immediately and diluted 1:3 in ice cold Acetonitrile (t-0). The samples were then continuously incubated at 37°C with shaking (700rpm) and further samples were taken at 5, 10, 20, 30, 60 and 120minutes, these were also immediately added to ice-cold Acetonitrile upon sampling to stop any enzymatic reaction. The samples were centrifuged in Heraeus Multifuge centrifuge (thermo scientific) for 30 minutes at 3000rpm to pellet the homogenates.

After centrifugation supernatant was removed and added diluted 1:1 with water, a final concentration of 0.2μM of Metoprolol (Sigma Aldrich, UK) was included in this solution as an internal standard for LC-MS/MS.

2.2.2 Stability of Progesterone in Human Liver and Intestinal Cytosol

2.2.2.1 Preparation of Human Liver and Intestinal cytosol

Human liver and intestinal cytosol were purchased from In Vitro technologies and stored at -80°C until use. Human liver cytosol came prepared in 0.1mM EDTA in phosphate buffer with a protein concentration was 24.1mg/ml. Details of the donors were not made available. Human intestinal cytosol came with a predetermined protein concentration of 11.7mg/ml. The cytosol consisted of a pooled sample obtained from 3 male and 3 female donors (donor details can be seen in Appendix C). The protein concentration was adjusted to 0.5mg/ml for both intestinal and liver cytosol by diluting the preparation in phosphate buffer with 0.1mM EDTA (From here on this buffer will be referred to as PB).

2.2.2.2 Cytosol Stability Assay

The stability of progesterone was assessed in liver and intestinal cytosol in the presence or absence of specific enzyme inhibitors and NADPH cofactor.

Progesterone was also incubated in phosphate buffer (PB) alone as a control. The stability of two as positive control substrates, pthalazine and dolasetron, were also assessed.

Human liver cytosol, human intestinal cytosol or PB control (with 0.1mM EDTA) were pre-incubated in wells of a 1ml deepwell RNAse/DNAse 96 well plate for 10 minutes in thermomixer comfort (eppendorf) until reaching a temperature of 37°C. The enzyme inhibitors listed in Table 2.8 were prepared in PB with 0.2% DMSO and added to the cytosol or PB at the final concentrations also seen in Table 2.8. The resulting mixtures were then incubated for a further 5 minutes. Next, a final concentration of 100μM NADPH or PB was added to the mixtures. Finally progesterone or control substrates (pthalazine or dolasetron) were added to the mixture at a final concentration of 1μM and mixed by pipetting to initiate the reaction. The final concentrations of each component in the mixture were: 100μM NADPH, 50-100μM enzyme inhibitor and 1μM of substrate in cytosol containing 0.5mg/ml protein.

Upon initiation a 50μl sample was immediately taken followed by sample removal at 10, 20, 30, 60 and 120 minutes. Enzymatic reaction was terminated by immediately diluting the samples in 100 μL of acetonitrile. Samples were centrifuged for 30 minutes at 4°C and 2500 rpm. Supernatant was removed and diluted in a 1:1 ratio with milli-Q water. Metoprolol internal standard was added to this solution at a final concentration of 0.2μM.

Inhibitor Name	Molecular Weight (kDa)	Enzyme Inhibited	Final Concentration (μM)
Epalrestat	319.40	AKR, particularly AKR1B1	100μM
Finasteride	372.54	AKR, particularly AKR1D1	100μM
Diazepam	284.74	AKR1C	100μM
AKR cocktail			
Epalrestat	319.40	AKR	100μM
Finasteride	372.54		100μM
Diazapam	284.74		100μM
Hydralazine	196.64	Aldehyde oxidase	50 μM
Menadione	172.18	Aldehyde Oxidase, cytochrome P450 reductase	100 μM
Chlorpromazine	355.33	Aldehyde Oxidase	100 μM
Allopurinol	136.11	Xanthine Oxidase	100 μM
AKR1C cocktail			
Flufenamic Acid	281.23	AKR1C	100μM
Diazapam	284.74		100μM

Table 2.8: Enzyme Inhibitors incubated with progesterone in human liver or intestinal cytosol.

2.2.2.3 Sample Analysis by Liquid Chromatography Tandem Mass Spectrometry

All samples obtained from the homogenate and cytosol stability assays were analysed by liquid chromatography with tandem mass spectrometry (LC MS/MS). The LC MS/MS system used for all homogenate analyses consisted of an Acquity Binary Solvent Manager (BSM), Acquity 4-position heated column manager, 2777 Ultra high pressure autosampler and a Xevo-TQ MS Triple Quadrupole mass

spectrometer (Water Ltd, Herts, UK). The analysis was performed using a solvent system and gradient (parameters can be seen in Table 2.9). Mobile phase A consisted of 10mM ammonium formate +0.1% v/v formic acid in water and mobile phase B contained acetonitrile only. The column used was Aquity HSS T3 (1.8um) 2.1x50mm (Water Ltd, Herts, UK). The samples were run at 70^{0}C with an injection volume of 8µl. Mass Spectrometry specifications can be seen in Table 2.10.

Time (min)	Flow Rate (uL/min)	% Mobile Phase A	% Mobile Phase B	Gradient Profile
0.00	600	100	0	6
0.05	600	100	0	6
1.00	600	5	95	5
1.40	600	100	0	11
1.80	600	100	0	6

Table 2.9: LC parameters for the analysis of progesterone in homogenate and cytosol stability assays.

Ionisation	Positive
Source Temp	600°C
Needle Current	500µA
Aquistion	Multiple Reaction Monitoring (MRM)
Collision Gas	High Purity Nitrogen

Table 2.10 Mass Spectrometry Parameters for analysis of progesterone in homogenate and cytosol stability assays.

2.2.2.4 Stability Assay Data Analysis

After LC-MS MS analysis peak area of substrates was determined using Labsys (Setaram) through auto-integration. The stability of progesterone and control substrates to all homogenates was assessed by taking the average peak area of three

repeats. The percentage degradation was assessed by taking the concentration of drug at the first time point as 100%. Rate constants (k) were determined by fitting the percent drug remaining vs. incubation time curves to a first-order kinetic model by least squares minimization. Half-life ($t_{1/2}$) was determined by plotting the peak area against time for each drug and plotting the point at which half the drug had degraded.

2.2.3 Solubility of Progesterone in Relevant Solutions

In order to assess the stability of progesterone in intestinal fluids it was first important to ensure that progesterone was fully dissolved in these solutions. The main component of the intestinal fluids is Phosphate Buffered Saline (PBS). PBS was prepared according to the British Pharmacopeia by adding 1 gram of KH_2PO_4, 2 grams of K_2HPO_4 and 7.5 grams of NaCl into 1 L of distilled water and stirring until completely dissolved. The pH of the obtained buffer was adjusted to 6.80 with diluted NaOH. The solubility of progesterone was determined in this solution alone and in combination with solubility enhancers in order to obtain a maximal concentration of fully dissolved progesterone for sufficient analysis of progesterone depletion in intestinal fluids. The solubility of progesterone was determined in triplicate in the PBS solutions described in Table 2.11.

Solution	Components
1	PBS (99%), DMSO (1%)
2	PBS (99%), Etoh (1%)
3	PBS (98%), DMSO (1%), Tween80 (1%)
4	PBS 98%, Etoh (1%), Tween80 (1%)
5	PBS (99%), DMSO (1%) Cremophor (1%)
6	PBS 98%, Etoh (1%), Cremophor (1%)

Table 2.11: Components of 6 Solutions in which Progesterone Solubility was Determined.

To assess the solubility of progesterone in these solutions the 'shake flask' method was used. To assess the solubility of progesterone in Solutions 1 and 2 (Table 2.10), 65μM of progesterone was first dissolved in the solvent in triplicate and added to

PBS with the final concentration of solvent being 1% in each solution. The solutions were then tightly closed in vials and placed in a heated shaking block (BarnsteadStem RS900) at 37°C and 300rpm. The solutions were left to shake for 2 hours then a 100µl sample was carefully removed, filtered through a 0.2µm filter (VWR international) and analysed by HPLC (Agilent 1100 series). To calculate the solubility of progesterone standards containing known amounts of progesterone in 80:20 methanol: water were again used to obtain a linear equation. This equation was then used to determine the solubility.

The solubility of progesterone in Solutions 3-6 (Table 2.10) was determined by dissolving progesterone first in the solvent and then further diluting this solution in a ratio of 1:4 in 25% Tween80 or 25% Cremophor in water. This solution was then added to PBS at a ratio of 1:19. The final concentration of solvent was 1% and the final concentration of Tween80 or Cremophor was 1% in all solutions. The final concentration of progesterone in these samples was 65µM (0.02mg/ml). The solutions were again incubated in a heated shaking block and at 2 hours, 100µl of solution was removed and filtered through a 0.2µm filter (VWR international). The samples were then analysed by HPLC and the solubility calculated as previously described. Separate standard curves were used for each experiment (R^2>0.98).

For the solubility experiments the following isocratic HPLC parameters were maintained: The column used was Phase Seperations 250 x 4.6mm licrosphere column with 5uM particle size, the detector wavelength was 242 nm, the flow rate used 1ml/min and the mobile phase consisted of 80:20 methanol water. The thermostat temperature was maintained at 26°C. The injection volume used for the all solubility experiments was 10µl with one exception. The injection volume was increased to 100µl for experiments containing PBS 2 as the concentration of progesterone was too low to detect with a smaller injection volume.

As the aqueous solubility of MPA and LNG is higher than that of progesterone, the solubility of these compounds was not assessed in the relevant fluids.

2.2.4 Stability of Progesterone in Simulated Intestinal Fluid

2.2.4.1 Preparation of Drug Stock Solution for Simulated Intestinal Fluid Studies

Drug stock solutions were prepared on the day of the experiment. Stock solutions of progesterone, MPA and LNG were prepared in DMSO (50%), tween80 (12.5%) and water (37.5%), at a concentration of 1.3 mM. On dilution with the human simulated intestinal or colonic fluid, the concentrations of tween 80 (Sigma Aldrich) and DMSO was 1% w/w as to minimize any potential effects on colonic enzymes. All three drugs were shown to be stable for at least 24 hours in the stock solution.

2.2.4.2 Preparation of Simulated Small Intestinal Fluid

Simulated intestinal fluid was prepared according to USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). 0.68 g of monobasic potassium phosphate was dissolved in 75 ml water, then 7.7 ml of 0.2 N NaOH was added to adjust the pH to 6.8. To this 1g of pancreatin (Sigma Aldrich) was added and shaken gently until dissolved and the volume adjusted to 100ml with water. Pancreatin was added after adjusting the pH of the solution to 6.8 to avoid precipitation of the enzyme.

2.2.4.3 Stability Testing in Human Simulated Small Intestinal Fluids

Simulated intestinal fluid was prepared as described. The drug stock solution at a concentration of 1.3 mM was added to simulated human colonic fluid or PBS control to achieve a final concentration of 65 μM and mixed on a horizontal shaker at 100 rpm (VXR basic Vibrax®, Leicestershire, UK). Samples were withdrawn at 0, 2, 6 and 24 hours and a final sample was taken at 24 hours and added to ice-cold acetonitrile as a stop reagent to inactivate the degrading enzymes and precipitate the interfering proteins during analysis. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected and stored at 4^0C until analysed by UV-HPLC

2.2.4.4 Preparation of Basal Medium and Phosphate Buffer Saline

A Basal medium was first prepared to support anaerobic bacterial growth in the faecal slurry, by dissolving Peptone water and yeast extract (3g) (Oxoid Limited) in 1.3 L of distilled water was and autoclaving at 125 °C for 20 minutes. In a 200 ml volumetric flask, sodium chloride (0.1g) (Fisher Chemical), dipotassium hydrogen orthophosphate (0.06g) (Fisher Chemical), of magnesium sulphate heptahydrate (0.015g) and calcium chloride hexahydrate (0.01g) (VWR) were weighed and dissolved in distilled water (*ca.* 150mL) under stirring. Following that, tween 80 (3mL) was added to the solution and was stirred until completely dissolved. L-cysteine and bile salts (0.75g) (Sigma Life Sciences) were added and stirred until completely dissolved. To this solution, vitamin K (15 µL), haemin (0.0075g, in two drops of 1 N sodium hydroxide) and 0.025% resazurin (6mL) in deionised water solution were added under stirring. Sodium bicarbonate (3g)(Sigma Aldrich) was added at the end and the final volume was made up to 200 ml with distilled water. The flask was stoppered during stirring to avoid dissolution of oxygen. The resulting solution was filtered (0.45µm, Millex GP syringe-driven filter units, Millipore, Ireland) into the peptone water and yeast extract solution. This step was performed aseptically in a Laminar Flow Cabinet. The bottle was kept tightly closed and at room temperature until used.

Phosphate buffer saline was prepared according to British Pharmacopoeia specifications by adding potassium dihydrogen orthophosphate (0.014M), dipotassium hydrogen orthophosphate trihydrate (0.017M) and sodium chloride (0.256M) in distilled water (2L) and stirring until dissolved. The pH was adjusted to 6.8 using 0.1N HCl or 1N NaOH solutions.

2.2.4.5 Preparation of Human Simulated Colonic Fluid

Two healthy male volunteers were given a plastic receptacle into which the samples were provided. The receptacle containing freshly voided human faeces was placed into an anaerobic workstation (Electrotek 500TG workstation, Electrotek, UK) maintained at 37°C and 70% relative humidity. The faecal material was weighed and diluted with PBS pH 6.8 to obtain 20% (w/w) slurry (60g of faecal material was

diluted with 240g of PBS 6.80) by homogenization using an Ultra Turrax (IKA T18 Basic) homogenizer at a speed of 18,000 rpm until no large solid agglomerates were observed. The homogenized faecal slurry was sieved through 350µm nylon mesh (Sefar NitexTM, Heiden, Switzerland) to remove any unhomogenised fibrous material. The basal medium prepared was then added to the sieved homogenized faecal slurry to achieve a 1:1 dilution (300g of the sieved homogenized faecal slurry was diluted with 300g of basal medium). Hence, the final concentration of faecal material in the slurry used for stability studies was 10% (w/w).

2.2.4.6 Stability Testing in Human Simulated Colonic Fluids

The stability of all drugs in simulated human colonic fluid was carried out in an anaerobic workstation maintained at 37°C and 70% relative humidity. Control studies were carried out in PBS pH 6.8 in the absence of colonic bacteria. The drug stock solution at a concentration of 1.3mM was added to simulated human colonic fluid to achieve a final concentration of 65 µM and mixed on a horizontal shaker at 100rpm (VXR basic Vibrax®, Leicestershire, UK). Samples were withdrawn at 0, 10, 20, 30, 60, 120, 240 and 360 minutes and a final sample was taken at 24 hours and added to ice-cold acetonitrile as stop reagent to inactivate the degrading enzymes and precipitate the interfering proteins during analysis. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected and stored at 4^0C until analysed by UV-HPLC.

2.2.4.7 Simulated Intestinal Fluid Sample Analysis by UV-HPLC

All UV-HPLC analysis were performed using Hewlett Packard 1100 series HPLC Value System with G1312A binary pump, G1315A diode array detector and G1313A Autosampler. The equipment was connected to and subsequently analysed on Dell Dimension Dim 300 PC with Chemstation for LC 3D systems software (Agilent).

The HPLC method described by Maliwal *et al* was used to analyse progesterone, and adapted for MPA and LNG. The hormones were eluted isocratically using a mobile phase consisting of 80:20 methanol and water, the column used was Phase Separations 250 x 4.6 mm licrosphere column,5µM particle size, at 40^0C and the UV

detector wavelength was set at 242 nm with a flow rate of 1ml/min. 100µl of sample was injected for each run (Maliwal et al., 2009). The samples were allowed to run for 60 minutes to reduce run-over. The retention time for progesterone, MPA and LNG was 8.6minutes, 6.8 minutes and 7 minutes respectively.

2.2.4.8 Simulated Intestinal Fluid UV-HPLC Data Analysis

The stability of the three drugs was assessed by taking the average peak area of three repeats. The percentage degradation was assessed by taking the concentration of drug at the first time point as 100%. Rate constants (k) were determined by fitting the percent drug remaining vs. incubation time curves to a first-order kinetic model by least squares minimization. Half-life ($t_{1/2}$) was determined by plotting the peak area against time for each drug and plotting the point at which half the drug had degraded.

2.3 Materials and Methods Used within Chapter 5

2.3.1 Preparation and Characterisation of Progesterone Amorphous Solid Dispersions

2.3.1.1 Production of Progesterone Amorphous Solid Dispersions

Two polymer types were utilised for the production of progesterone microparticles; Eudragit and HPMCAS. Eudragit L100 is an anionic copolymer of methacrylic acid and methyl methacrylate with pH dissolution threshold of pH 6.0 and was used in the initial production of progesterone loaded microparticles. HPMCAS HF, MF and LF are cellulosic enteric coating agents with dissolution thresholds of pH6.8, 6 and 5.5 respectively.

Progesterone loaded microparticles were produced using the emulsion solvent evapouration method. This method involves a preparation containing two immiscible liquids where one liquid (the internal phase) is dispersed in the other (the external phase) (O'Donnell and McGinity, 1997). Figure 2.7 shows the steps involved in the preparation of microparticles using this method.

Progesterone loaded Eudragit L100 microparticles were prepared using the oil-in-oil emulsion solvent evaporation method based on previous work by Kendall (2006, 2009) whereas microparticles with HPMCAS as a polymer were prepared using the oil-in-water emulsion solvent evaporation method. The emulsion solvent evaporation process comprises of two mass transfer processes i) the diffusion of the solvent from inner phase droplets to the external phase and ii) The solvent evaporation from the external phase to the environment.

For the preparation of progesterone microparticles with Eudragit L100, 300mg of progesterone and 2.7g of Eudragit L100 were dissolved in 30ml of absolute ethanol (the internal phase). The internal phase was slowly added to liquid paraffin (165ml), containing 1% (w/w) sorbitan sesquioleate as a dispersing agent, under stirring at 1000rpm. The stirring speed was obtained using Heidolph RZR1 mechanical overhead stirrer (Heidolph Instruments, Schwabach, Germany). The solution was left to stir overnight at room temperature allowing the solvent to fully evaporate and the microparticles to form. The solidified microparticles were separated from solution and washed 3 times with 50mL of *n*-hexane (95%) via vaccum filtration through a pyrex sintered glass filter (pore size 4; 5-15µm). This method is subject to patent number US 20130101646 A1 (Basit et al., 2013) and available to use within this study through collaborative work with Kuecept LtD who are licenced to use this method.

For the preparation of progesterone microparticles with HPMCAS polymers, 200mg of progesterone and 1.8g of HPMCAS were dissolved in 30ml of ethyl acetate: dichloromethane (1:1 v/v). The resulting clear solution was emulsified into 200ml deionised water containing 1% (w/w) polyvinyl alcohol as a dispersing agent, under stirring at 1000rpm. The stirring speed was obtained using Heidolph RZR1 mechanical overhead stirrer. The solution was left to stir overnight at room temperature allowing the solvent to fully evapourate and microparticles to form. The solidified microparticle were separated from solution and washed 3 times with 50mL of deionised water through vaccum filtration using a pyrex sintered glass filter (pore size 4; 5-15µm). Post filtration, the sample was stored under desiccation prior to further use.

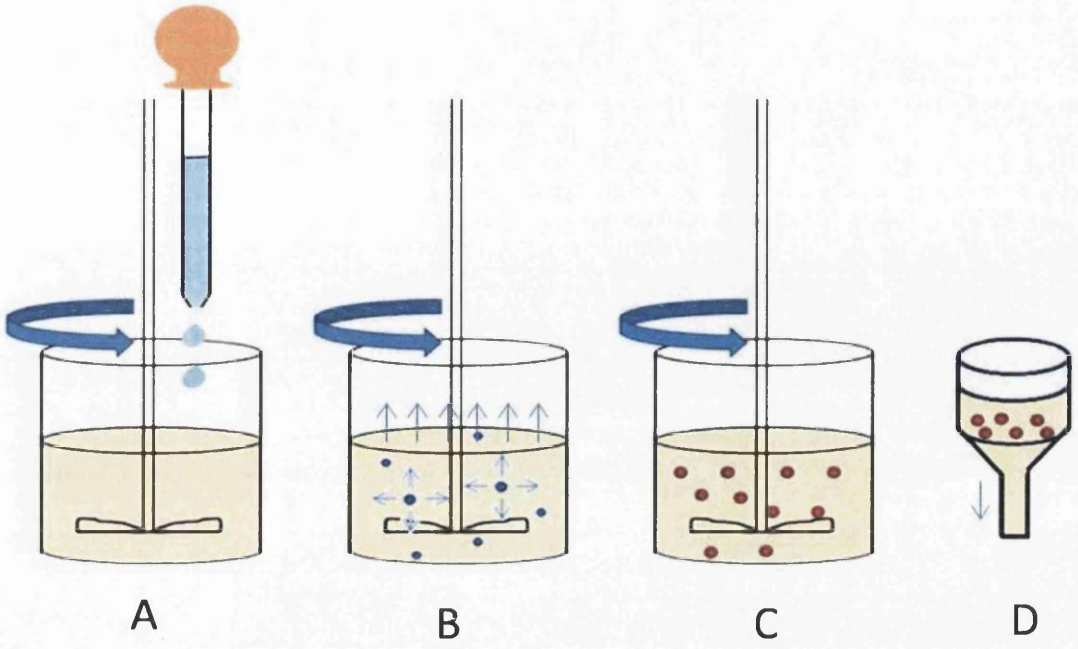


Figure 2.7: Emulsion Solvent Evaporation. A) The drug and polymer are both dissolved in the solvent and then added gradually to the immiscible phase containing a surfactant under propeller stirring. B) The solvent is then removed by a long period of evaporation at room temperature under continuous propeller stirring (solvent evaporation). C) Eventually a suspension of microparticles containing the drug and polymer forms within the external phase and D) are removed from solution through filtration, washed 3 times with an appropriate solution and allowed to dry.

2.3.1.2 Microparticle Morphology and Size Estimate

Microparticle morphology and approximate size were determined using scanning electron microscopy (SEM). Microparticles were fastened on SEM adhesion pads and coated with gold using a gold sputter module in a high vacuum evaporator (Emitech, K550). A Philips/FEI XL30 TMP SEM was used to observe the samples at 5kV to provide details of the surface characteristics as well as the morphology of the microparticles. Images were obtained 100x, 500x, 1000x, 2500x and 5000x magnification. Images obtained at 500x magnification were used to estimate microparticle size.

2.3.1.3 Powder X-ray Diffraction

Powder X-ray diffraction (PXRD) is a technique often used in the pharmaceutical industry to assess the physical form of samples; particularly crystal structure and atomic spacing. X-rays are electromagnetic radiation that can be measured in wavelengths. If an X-ray beam impacts on a crystal, the beam will be scattered in all directions by the atoms within the crystal. Although some beams will be disguised by destructive interference others will be made visible through constructive interference causing the X-ray scattering to be concentrated into a few sharp diffraction peaks which are then measured in wavelengths. However if the X-ray hits a non-crystalline (amorphous) structure the X-ray scattering is spread throughout reciprocal space and therefore not detected as peaks (Guinier, 1994). Therefore PXRD was used to assess the crystallinity/amorphicity of natural progesterone powder and progesterone in solid dispersions.

The X-ray diffractometer is composed of three major parts: an X-ray tube, a sample holder and a diffracted x-ray detector where electromagnetic radiation is applied to the sample. The sample and the detector are rotated and the intensity of the reflected x-ray beam is measured by the detector. A filter slit focuses the X-ray beam and the intensity peak of the diffracted X-ray is recorded. The angle of diffraction (the theta angle) is measured in degrees (^{o}). For practical reasons the diffractometer measures an angle two times the theta angle, this is referred to as 2theta.

A Bruker D8 scanning X-ray diffractometer was used for the analysis of progesterone and progesterone amorphous solid dispersions. The samples were placed in a 9mm silicon wafer and gently smoothed and compressed with a Perspex block. Samples were scanned on a 2-theta scale ranging from from 2^{o} to 40^{o} at 0.039 ^{o}/sec. The peak was calculated using Diffrac.Suite data analysis software (Bruker).

2.3.1.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a relatively fast technique that requires only limited amount of sample and can provide high levels of sensitivity. DSC is the measurement of changes in a uniform heat flow rate passed to a sample and a

reference sample whilst they are subjected to a controlled temperature program (Höhne et al., 2003). As the temperature of the samples changes the differences of heat flow between them is recorded and can be visualised on a thermogram. Crystalline materials have a melting endotherm, ie. a temperature at which the lattice of the structure breaks down, which can be visualised as sharp dip in heat flow on the thermogram. Amorphous materials on the other hand have a reduced melting point due to the absence of a structured lattice. As a result, this technique can be used to distinguish between amorphous and crystalline materials. A DSC Q1000 V9 differential scanning calorimeter (TA instruments) was used for the analysis of progesterone and progesterone ASDs. Reference and samples were accurately weighed to weights between 3 and 5mg and placed in hermetic aluminium pans. An isothermal period of 100°C was applied to the samples for 5 minutes to eliminate any residual content on the pan. The samples were then cooled and scanned from 40°C to 150°C at rate of 20°C/min. Data was subsequently analysed on TA Instruments Universal Thermal Analysis Sofware (version 4.5a).

2.3.1.5 Drug Encapsulation Efficiency in Microparticles

Progesterone microparticles were prepared with a 1:9 drug to polymer ratio. To assess the actual concentration of progesterone within the microparticles, 50mg of microparticles were dissolved in 50ml of 100% methanol to give a final concentration of 1mg/ml of microparticles and therefore a final concentration of 0.1mg/ml progesterone. The efficiency of the microparticle loading was determined by running samples on HPLC. The HPLC system parameters are described in Section 2.2.3.8. All samples were eluted isocratically using a mobile phase consisting of 0.1% Trifluoroacetic acid (TFA) in 50:50 acetonitrile and water. The column used was Zorbax 150 x 4.6 mm with 3.5µM particle size at 25°C and the UV detector wavelength was set at 242 nm with a flow rate of 1ml/min. 10µl of sample was injected for each run. The samples were allowed to run for 20 minutes. Standards of known concentrations were used to produce a standard curve and the straight line equation was used against peak area to quantify drug present in the sample. The method was validated for system suitability and linearity.

System suitability was evaluated by replicate (n= 6) injection of the same standard solution containing progesterone at a concentration of 1mg/ml. The retention time, peak area and tailing were consistently replicable between repeats indicating system suitability. Linearity was tested in the concentration range of 0.1mg/ml-2mg/ml.

Furthermore, progesterone standards (1mg/ml) were injected intermittently throughout the procedure (every 6^{th} injection) and in triplicate at the beginning and end of each HPLC analysis and and no change was observed in retention time, peak area or tailing.

2.3.2 In Vitro drug release profiles of progesterone-loaded HPMCAS microparticles

Dissolution of HPMCAS HF and MF coated progesterone ASDs was carried out using the shake flask method with manual pH switch. An important requirement when carrying out a dissolution test is to ensure the procedure is carried out in sink conditions whereby the amount of medium should be sufficient enough to dissolve the expected amount of drug released. To ensure the experiment was under sink conditions the amount of sample added was 10% that of the solubility in the media used. The dissolution medium used for this experiment was fed-state simulated gastric fluid (FeSSGF). FeSSGF was prepared according to manufacturer guidelines (Biorelevant). To produce 500mL of FeSSGF, 1g of NaCl was dissolved in 450mL of distilled water and the pH was adjusted to 1.6 with HCl. The volume was made up to 500mL with distilled water. Next 0.03g of SIF Powder Original was added to 250 mL of this buffer and stirred until completely dissolved; the volume was then made up to 500mL using the same buffer.

The solubility of progesterone in FeSSGF was calculated as described in Section 2.3.4. The encapsulation efficiency of the progesterone ASDs was taken into account when carrying out dissolution to further ensure sink conditions and accurately measure the amount of progesterone being added to the dissolution buffer.

To determine the dissolution of progesterone ASDs in relevant media, 4mL of FeSSGF (pH 1.6) was warmed to 37°C in a heated shaking block (Barnstead Stem

RS900) with a shaking rate of 100rpm. Predetermined amounts of accurately weighed progesterone ASDs were added to the mixture and a 500µL sample was taken and filtered through a 0.2µm filter. A further 500µL sample was taken at 2hours. After taking the second sample the dissolution media was manually adjusted to pH 6.5 for HPMCAS MF ASDs or pH7 for HPMCAS HF ASDs with a 4N NaOH solution and the volume of liquid was brought to 4mL with the addition of FeSSIF buffer. Following pH switch 500µL samples were taken immediately and another sample was taken after 2 hours. Dissolution media without ASDs was also sampled at the same time-points and taken as a blank sample. All experiments were carried out in triplicate and analysed by HPLC.

2.3.2.1 Drug Release Data Analysis

To determine the dissolution rate of progesterone ASDs, the quantity of progesterone in the samples was determined by HPLC. HPLC analysis was performed using the system described in Section 2.2.3.8. All samples were eluted isocratically using a mobile phase consisting of 0.1% TFA in 50:50 acetonitrile and water. The column used was Zorbax 150 x 4.6 mm with 3.5µM particle size at 25^{0}C and the UV detector wavelength was set at 242 nm with a flow rate of 1ml/min. 100µl of sample was injected for each run. The samples were allowed to run for 20 minutes. The retention time for progesterone was 7minutes. Peak area determined using Chemstation (Agilent) auto-integration software. Peak area for each sample was subsequently entered into an excel spreadsheet and the concentration of progesterone in each sample was determined based on progesterone standards of known concentration. The removal of 500µl samples and the increase in media volume were taken into account during the analysis.

2.3.3 CACO-2 Cell Culture and Assay

2.3.3.1 Introduction to the CACO-2 cell line

In 1975 Fogh et al established a collection of cell lines from gastrointestinal tumors in order to carry out work on cancer mechanisms (Fogh and Trempe, 1975). In 1983 attention was turned to such cell lines as models for differentiated intestinal cells due

to their specific intestinal properties. It was then discovered that most of the cell lines established could be induced to partially differentiate after treatment with certain biological factors. However, one of the cell lines, the CACO-2 cell line, showed spontaneous differentiation after long-standing culture. Studies carried out on these cells showed that upon differentiation they express the same biochemical and morphological characteristics as enterocytes of the small intestine including transporter proteins, efflux proteins, and Phase II conjugation enzymes (Pinto et al., 1983). As a result of its intestinal properties CACO-2 cells serve as a model of paracellular movement of compounds across the monolayer.

To assess the permeability of compounds using the CACO-2 model cell line, monolayers are cultured on a semi-permeable membranes inserted into wells of multi-well culture plates. Test compounds are then added to either the apical or basolateral sides of the monolayer and their permeability assessed (Breemen and Li, 2005).

2.3.3.2 Routine Subculture of Caco-2 Cells

CACO-2 cells were grown until passage 59 in a 175cm^2 cell culture flask (Falcon). Cells were cultured in Dulbecco's modified eagle medium (DMEM; +L-glut., +D-glucose, +Na pyr.) containing foetal bovine serum (10%) L-glutamine (2mM) non-essential amino acids (1%) v/v, penicillin (50units/mL) and streptomycin (50µg/mL).

All sub-culture techniques were carried out aseptically in a lamina flow hood.

For the sub-culture of CACO-2 cells, cells were grown to between 70 and 100% confluent. PBS, Trypsin/EDTA and growth medium were warmed to 37°C using a beaded water bath (Thermoscientific). Cell media was removed from the flask and cells were washed two times in 10mL warm PBS. Cells were incubated at 37°C in 2ml of trypsin EDTA in a shaking incubator (Stuart Scientific). After 5-7 minutes >90% of the cells detached and were diluted in 10mL of warmed media to stop the action of Trypsin/EDTA on the cells. The cell suspension was transferred to a 15ml sterile centrifuge tube and pelleted by centrifugation for 4 minutes at 800rpm. Supernatant was decanted and the resulting cell pellet was re-suspended in fresh

media and de-aggregated by aspiration. CACO-2 cells were split at a subcultivation ratio of 1:5 into 15ml of fresh media in a sterile cell culture flask.

2.3.3.3 Seeding 12-well inserts with CACO-2 cells

In order to seed CACO-2 cells into a 12-well transwell plate, the cells were sub-cultured until reaching passage number 59 and grown until >90% confluent in a T-175 flask. Cells were sub-cultured as described until a cell pellet was obtained. At this point cells were re-suspended in 10ml of culture medium and de-aggregated by aspiration. Immediately after aspiration cells were counted with a haemocytometer using the following procedure:

- 50µl of cell suspension was added to 50µl of 0.4% trypan solution in PBS. Trypan blue was used to determine cell viability; non-viable cells take-up Trypan blue and become dark blue in colour whereas blue dye is seen only around the edge of viable cells.
- A cover slip was secured to the haemocytometer and 10µL of cell/trypan blue solution was pipetted directly into one of the counting chambers. The preparation was observed microscopically at 10x objective. A click counter was used to count cells in each of 5 squares of the haemocytometer as depicted in Figure 2.8. To avoid counting the same cells twice, cells that were positioned over the bottom and right border were not counted. The number of viable cells was recorded.

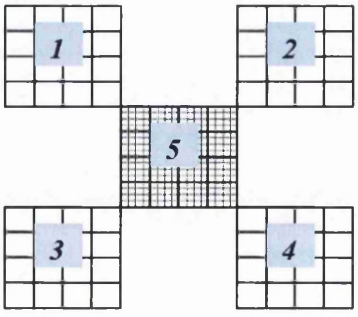


Figure 2.8: Haemocytometer grid. When counting CACO-2 cells using the haemocytometer the finer grid lines shown in square 5 were ignored. Cells in the main squares numbered 1 to 5 were counted and the average taken.

After determining the number of viable cells per square of the haemocytometer the following equation was used to calculate the number of cells per mL:

Number of cells per mL = average cell count x 10^4 x dilution factor

Based on the calculation cells were diluted appropriately to a final concentration of $2x10^5$ cells/mL. 500µl of the cell dilution was then added to the apical chambers of the CACO-2 transwell. The transwell inserts used for this procedure were of 12mm in diameter with a 0.4µM pore size polycarbonate membrane in a 12-well culture plate. The following procedure was used when plating the CACO-2 transwell:

- Prior to seeding, the semi–permeable insert membranes were pre-wetted with culture medium for approximately 30min at 37°C by aseptically adding 1.5mL of warmed growth media into the basolateral plate wells and adding 500µL of warmed media to the transwell insert. Transepithelial electrical resistance (TEER) measurements were taken before and after incubation using Evom Epithelial Voltohmmeter.
- Following the incubation step, media was removed from the transwell inserts and replaced with 500µL of diluted cell suspension to give a final concentration of 1 x 10^5cells per transwell.

- The lid was replaced and the assembly was placed in a 37°C, 5% CO_2 incubator. TEER measurements were taken again after 1hour of incubation and cells were replaced in the incubator.

2.3.3.4 Replacing Media in 12-well Cell Inserts and Feeder Plates

Replacement of CACO-2 cell media was carried out every two to three days for 28 days; at this time the cells were used for the permeability experiment. To replace the growth media, the media was first removed from the basolateral compartment of the transwell membrane then carefully removed from the apical compartment using a vacuum suction pipette. 500µL of fresh media was added to the apical side of the membrane followed by 1.5mL of fresh media to the baso-lateral side. Cells were placed in a 37°C, 5% CO_2 incubator for 1 hour and TEER measurements were recorded in triplicate.

2.3.3.5 Preparation of Assay Solutions

The CACO-2permeability assay was carried out in HBSS (GIBCO) supplemented with 1% DMSO, 1%HEPES (GIBCO), 1%FBS and 0.1% Vitamin E (pH 6.8). Progesterone and progesterone ASDs were added directly to this media to give a final concentration of 5µg/ml (16µM) of progesterone. The concentration of progesterone in the ASDs was calculated prior to the experiment in order to ensure equal concentrations of progesterone were added to wells containing the natural drug and ASD.

2.3.3.6 CACO-2 Permeability Assay

Before carrying out the CACO-2 permeability assay TEER measurements were taken and recorded in triplicate.

The culture media was removed from both the apical and basolateral side of the transwell plate and replaced three times with Hanks Balanced Salt Solution substituted with 1% N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1% FBS and 0.1% vitamin E. Cells were incubated in the supplemented HBSS solution for 30 minutes at 37°C. After incubation the TEER measures were taken again in triplicated and recorded. The HBSS was removed from the donor compartment and replaced with the pre-prepared progesterone and progesterone-loaded HPMC-AS solutions. 100µL of sample was taken immediately from the basolateral compartment of each well and

Samples (100 µL) were taken from the basolateral compartment at regular time intervals over a 120 minute period and replaced with the same volume of fresh buffer. At the end of the experiment, 100 µL was also taken from the upper compartment. All experiments were conducted in triplicate at pH 6.0, 37 °C, 5% CO2 and 95% relative humidity.

All samples were diluted with acetonitrile immediately after removal to obtain a sample-to-acetonitrile volume ratio of 1:4 and then analysed for progesterone content using HPLC.

2.3.3.7 Sample analysis by HPLC

HPLC analysis was performed using the system described in Section 2.2.3.8. All samples were eluted isocratically using a mobile phase consisting of 80% Methanol and 20% distilled water. The column used was Zorbax 150 x 4.6 mm with 3.5µM particle size at 40^{0}C and the UV detector wavelength was set at 242 nm with a flow rate of 1ml/min. 100µl of sample was injected for each run. The samples were allowed to run for 20 minutes. The retention time for progesterone was 7minutes. Peak area was determined using Chemstation (Agilent) auto-integration software. Peak area for each sample was subsequently entered into an excel spreadsheet and the concentration of progesterone in each sample was determined based on progesterone standards of known concentration. The removal of 100µl of sample and was taken into account during the analysis.

System suitability was evaluated by replicate (n= 6) injection of the same standard solution containing progesterone at a concentration of 5µg/ml. The retention time, peak area and tailing were consistently replicable between repeats indicating system suitability. Linearity was tested in the concentration range of 1-10µg/ml.

Furthermore, progesterone standards (5µg/ml) were injected intermittently throughout the procedure (every 6th injection) and in triplicate at the beginning and end of each HPLC analysis and and no change was observed in retention time, peak area or tailing.

2.3.3.8 CACO-2 Data analysis

Data was analysed based on percent disappearance/ appearance of progesterone in the apical and basolateral compartments of the transwell. This was calculated by running samples of known concentrations of progesterone to obtain a standard curve. From this, the concentration of progesterone was determined in the apical and basolateral compartments and presented as percent of the initial treatment concentration.

The apparent permeability coefficient (P_{app}) was also calculated. P_{app} was calculated using the following equation:

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{AC_0}$$

where ($\partial Q/\partial t$) is the permeability rate, A is the diffusion area of the monolayer and C_0 is the initial concentration of progesterone in the apical compartment (Sadeghi et al., 2008).

2.3.4 Solubility of Progesterone and Progesterone loaded HPMCAS microparticles

The shake flask method (Baka et al., 2008) was used to determine the solubility of progesterone versus progesterone ASDs in simulated fasted and fed intestinal fluids (FaSSIF and FeSSIF). Both fluids were prepared according to manufacturer guidelines (Biorelevant). To produce 500mL of FeSSIF, 2.02g of NaOH pellets, 4.325g of glacial acetic acid and 5.937g of NaCl were dissolved in 450mL of distilled water. The pH was adjusted to 5 with 1N HCl and the volume was made up to 500mL with distilled water. Next 5.600g of SIF Powder Original (Biorelevant) was added to 250 mL of this buffer and stirred on a magnetic stirrer until completely dissolved. This volume was made up to 500ml with buffer and used within 48hours of production. FaSSIF was produced in the similar manner to FeSSIF. To produce 500mL of FaSSIF, 0.210g of NaOH pellets, 1.719 g of NaH_2PO_4 Anhydrous, and 3.093g of NaCl were dissolved in 450mL of distilled water. The pH was adjusted to 6.5 with 1N HCl and the volume was made up to 500mL with distilled water. Next, 1.120g of SIF Powder Original (Biorelevant) was added to 250 mL of this buffer and stirred on a magnetic stirrer until completely dissolved. This volume was made up to 500ml with buffer and allowed to stand at room temperature for 2hours until the colour became opalescent. The solution was used within 48 hours of production.

The solubility of progesterone was determined by adding saturated amounts of drug to the relevant solution in 3 separate screw-top vials (VWR). The vials were tightly

closed and placed in a heated shaking block (BarnsteadStem RS900) at 37°C and 300rpm. The solutions were left to shake for 24 hours and 100µl of supernatant was carefully withdrawn at 2 and 24h, filtered through a 0.2um filter (VWR international) and analysed by HPLC. Solution without API was taken as a blank.

To calculate solubility, standards containing known amounts of progesterone in 80:20 methanol: water were used to obtain a linear equation. This equation was used to determine the solubility of progesterone in both buffers. Solubility was determined as µg/mL based on the average of 3 repeats using the same HPLC method described in Section 2.2.3.8.

2.3.5 Gene and Protein Analysis of Progesterone Targets

The regulation of progesterone target genes and proteins was compared between natural progesterone and the progesterone ASD in Ishikawa and HESC cell lines. The methods used for this comparison were qRT-PCR, InCell Analysis, Protein ELISA and cell image analysis using image j. These methods are described in detail in Section 2.1 with the only difference being the preparation of hormone treatments.

To produce stock concentrations of P_4 ASD, the encapsulation efficiency was taken into account. A stock solution of 1mM of P_4 ASD was then prepared in DMSO. For comparison of unformulated P_4 and P_4 ASD, fresh stock of P_4 was prepared in a final concentration of 1mM in DMSO as opposed to ethanol which was used in earlier studies. Both DMSO and ethanol were shown not to effect gene expression at the given concentrations.

CHAPTER 3

Gene and Protein Regulation by Progesterone and Synthetic Progestins in the Human Endometrium

3. Gene and Protein Regulation by Progesterone and Synthetic Progestins in the Human Endometrium

3.1 Introduction

Natural progesterone has poor bioavailability limiting its medicinal use. In fact in the UK, natural progesterone is only prescribed for luteal support in IVF protocols and is delivered vaginally in this circumstance (Kassab et al., 2008). Synthetic progestins are man-made versions of progesterone with progestogenic effects similar to that of the natural compound but have a vastly improved oral bioavailability. As a result, progestins are commonly used in contraception and hormone replacement therapy and also used for the treatment of endometrial hyperplasia and cancer. Endometrial hyperplasia is a condition characterised by a benign proliferation of endometrial glands and can be seen to have simple or complex glandular features with or without the presence of nuclear atypia (Montgomery et al., 2004). Synthetic progestins are routinely administered for the treatment of this condition and serve to antagonise estrogen driven growth, slowing disease progression (Gunderson et al., 2012, Kim et al., 2013a). There are two main progestins used to treat endometrial hyperplasia, these are medroxyprogesterone acetate (MPA) and levonorgestrel (LNG) (Vereide et al., 2006, Zaino et al., 2014, Kim et al., 2013b). MPA is progesterone-derived hormone that is given orally at 20mg/day for hyperplasia treatment. LNG on the other hand is derived from, and structurally similar to, testosterone. It is used for the treatment of endometrial hyperplasia and cancer either alone (Orbo et al., 2014, Pal, 2012) or in combination with MPA (Kim et al., 2013b, Kim et al., 2012) and is typically delivered via an intrauterine device.

However, the use of these progestins is associated with the occurrence of side-effects. Minor side-effects are common and include abnormal menstrual bleeding and breast tenderness (Cummings and Brizendine, 2002, Berenson and Wiemann, 1993); though more serious side-effects have been reported in hyperplasia patients (Bafaloukos et al., 1999, Thigpen et al., 1999). Side-effects are most often attributed to adverse progestin signalling within the target tissue; the main receptor through which progestin signalling occurs is the progesterone receptor (PR), however,

synthetic progestins may bind and activate other hormone receptor with high affinity (Sitruk-Ware and Nath, 2010). Furthermore, the regulation of targets through the PR itself may also differ. Alterations in signalling patterns can possibly lead to the differential regulation of target genes and their resulting proteins including targets that are linked to cancer; indeed increased incidence of breast and endometrial cancer have been linked to hormone exposure (Fournier et al., 2005, Stanford et al., 1995, Seeger et al., 2003, Kelley et al., 1976, Kaaks et al., 2002). Thus, the aim of this chapter is to establish whether progesterone target genes and proteins are adversely regulated by MPA and LNG compared to the natural compound.

To help determine this, a number of target genes and proteins were selected and their control compared between the progestogens. The expression of four main targets of interest will first be assessed in healthy and hyperplastic endometrium in order to see if they are adversely regulated in this condition. Following this, target expression will be assessed before and after progestin (MPA) therapy to determine whether their expression is restored. The targets to be analysed are PR, Forkhead Box O Protein 1 (FOXO1), Amphiregulin (AREG) and Lactoferrin (LF) which are described in Section 1.7.

As natural progesterone is not currently prescribed to patients with endometrial hyperplasia it is impossible to compare gene regulation between natural and synthetic progestogens *in vivo* in patients with this condition. However, it is possible to make a direct comparison of natural progesterone with MPA and LNG in well-established endometrial cell lines. Therefore, gene and protein regulation by progesterone, MPA and LNG, will be assessed in two model cell lines representing the endometrial epithelium (Ishikawa) and stroma (HESC).

These cell lines will be treated with natural progesterone, MPA and LNG in order to determine how each progestogen regulates specifically selected target genes and proteins (listed in Table 3.1). As the available cell lines are not hyperplastic in origin, to make a direct comparison of protein regulation between the cell lines and the patient biopsies may be misleading. Nonetheless, if the regulation of the targets by MPA in the cell lines is in line with that seen *in vivo* it may be a good indication that

any differential gene alteration by natural progesterone seen in the cell lines may also occur in patients.

As well as directly regulating target genes, progesterone also works in synergy with the second messenger cyclic AMP. Cyclic AMP and progesterone are involved in the regulation of genes associated with a phenotypic change in the endometrial stroma known as decidualisation (Gellersen and Brosens, 2003) (Section 1.2.4). Therefore, in addition to assessing progestogen target regulation, the role of natural and synthetic progesterone in stromal decidualisation will also be compared.

Furthermore, as endometrial hyperplasia is most often caused by an excess of estrogen, particularly estradiol (Ziel and Finkle, 1975, MacDonald et al., 1976), and many progestin therapies are administered in combination with synthetic estradiol (Bono et al., 2014), progesterone and progestin signalling will also be studied in the presence of this hormone.

For the purpose of this study, target regulation by the progestogens will be assessed in the epithelial carcinoma cell line, Ishikawa, in the presence or absence of estradiol and in the stromal cell line, HESC, in the presence or absence of cyclic AMP. The specific target genes and proteins to be analysed are listed in Table 3.1 along with a summary of their regulation through relevant hormone receptors.

A direct comparison of the effects of MPA, LNG and progesterone on a specific subset of progesterone-sensitive genes and proteins has yet to be carried out in human endometrial cells making much of this work novel.

In sum, the main aim of this chapter is to determine if synthetic progestins differentially regulate a number of specifically selected target genes compared to the natural compound. This will be determined *in vivo* endometrial hyperplasia patients and *in vivo* in endometrial cell lines.

Target	Location within the Endometrium	Regulation through Ligand-bound Receptors		
		PR	ER	AR
FOXO1	Epithelium and Stroma	↑ (Ward, Hoekstra et al. 2008)	↑ (Schuur, Loktev et al. 2001)	↓ (Huang, Muddiman et al. 2004)
PR	Epithelium and Stroma	↓ (Mote, Balleine et al. 1999)	↑ (Aronica and Katzenellenbogen 1991)	Unknown
AREG	Epithelium	↑ (Mulac-Jericevic, Mullinax et al. 2000)	↑ (Gielen, Hanekamp et al. 2006)	↑ (Sehgal, Bailey et al. 1994)
GREB1	Epithelium and Stroma	Unknown	↑ (Pellegrini, Gori et al. 2012)	↑ (Rae, Johnson et al. 2006)
LF	Epithelium and Stroma	↓ (Kurita, Lee et al. 2000)	↑ (Kelver, Kaul et al. 1996)	- (Yu and Chen 1993)
PRL	Stroma	↑* (Brosens, Hayashi et al. 1999)	↑ (DeVito, Avakian et al. 1992)	- (Nevalainen, Valve et al. 1997)
IGF1	Unnknown	- (Ace and Okulicz 1995)	- (Ace and Okulicz 1995)	↑ (Weihua, Ekman et al. 2002)

Table 3.1: Regulation of target genes through ligand bound PR, ER and AR. ↑ indicates upregulation, ↓ indicates downregulation, - indicates no change.

***Weakly induced but enhances cAMP-induction**

3.2 Materials and Methods

Materials and methods used within this chapter are described in detail in Section 2.1. Briefly, IHC was used for protein detection in slides produced from patient biopsies. Western Blot and PCR were used to assess the basal protein and gene levels of PR, AR and ER within the Ishikawa and HESC cell lines. PCR was also used to determine regulation of PR, AREG, FOXO1, LF, and IGF1 by progesterone and the synthetic progestins in the Ishikawa cell line. PCR was further used to assess the

regulation of PR, FOXO1, LF, GREB1 and PRL in the HESC cell line. All primers used in these reactions are detailed in Table 2.6. Furthermore, InCELL analysis was used to determine regulation of target proteins by progesterone and the progestins in both cell lines. Antibodies used for InCELL analysis can be seen in Table 2.7. Images of HESC cells were obtained using a light microscope and cell roundness was determined using imageJ to establish the extent of cellular decidualisation. Decidualisation was further confirmed by the detection of secreted prolactin using ELISA.

3.3 Results and Discussion

3.3.1 Expression of Progestogen-Regulated Proteins in Endometrial Biopsies of the Healthy and Hyperplastic Endometrium.

In order to determine differences in protein regulation by natural and synthetic progestins in endometrial hyperplasia, a number of proteins (PR, FOXO1, AREG and LF) were selected. These were chosen due to their potential adverse expression in this disease in order to see if their expression was restored after synthetic progestin treatment in an equivalent manner to natural progesterone. Thus, during the first section of this chapter, the expression of the chosen targets was determined in the normal human endometrium (control) and hyperplastic endometrium without atypia (H) and with atypia (HA). Moreover, there is currently no single gene or protein marker available to assess the presence or stage of this condition. Therefore, these targets will also be assessed as potential biomarkers for this condition.

To determine protein level, immunohistochemistry (IHC) was used and the intensity and distribution of staining was calculated using H-score (min 0, max 4) (Section 2.1.1).

3.3.1.1 Differential Expression of PR in Endometrium of Women with Hyperplasia Comparing to Controls

The first protein to be investigated was PR. The presence of PR is essential if progesterone regulated targets are to be analysed; the hormonal milieu of the endometrium and its potential response to the action of progestins is represented by

PR concentration. Therefore, the expression of PR was determined in glands and stroma of hyperplastic endometrial samples with and without atypia, and controls. The intensity and distribution of staining was calculated using H-score (min 0, max 4) (Section 2.1.1) and the total score was compared between the groups (Figure 3.1).

PR expression in the glands at nuclear and cytosol level was significantly different between study and control groups. At nuclear level, there was a higher concentration of PR in the H ($p<0.05$) and HA ($p<0.05$) groups compared to controls. A similar pattern was also observed at cytosol level, again with higher expression of PR in the H ($p=0.001$) and HA ($p>0.01$) groups compared to controls (Figure 3.1). Contradictory to this study, PR has been shown to decrease from control to hyperplasia to atypical hyperplasia in glandular nucleus (Pieczyńska et al., 2011). Yet the presence of PR in patients with hyperplasia is well documented in both nucleus and cytoplasm (Vereide et al., 2006, Ehrlich et al., 1981, Wildemeersch et al., 2007, Bozdogan et al., 2002). However, estrogen is a known inducer of PR (Hata 1992 and Jamil 1991) and hyperplasia patients are known to have an increase serum estradiol levels (Baskin et al., 2002, Mutter et al., 2007) so it is not wholly surprising that such an induction in PR was observed. A study by Nunobiki *et al* also reported high levels of PR in patients with simple hyperplasia, though his findings showed a stepwise decrease with disease progression (Nunobiki et al., 2003).

In the stroma, the expression of PR was significantly lower in the HA group comparing to control at nuclear ($p=0.04$) and cytosol level ($p=0.02$). However, there was no significant difference in PR expression between the H group and control (Figure 3.1). A reduction in cytosolic and nuclear PR in the HA group compared to the H group was also apparent but not statistically significant. The overall reduction of PR in HA group compared to both H and control is in line with the literature. Research carried out by Snijders *et al* (1992) described a loss of stromal PR expression in atypical hyperplasia compared to hyperplasia without atypia (Snijders et al., 1992) and a loss in stromal PR expression has been well correlated with endometrial hyperplasia disease progression (Ehrlich et al., 1981, Bozdogan et al., 2002). The presence of stromal PR is important in women who go on to receive progestin therapy, as progesterone-induced paracrine signaling can affect

endometrial epithelium meaning loss of PR in this tissue can affect surrounding endometrial compartments (Kurita et al., 2000).

Although changes in PR level were seen with disease presence, these were not in line with previous findings, making PR a poor marker for the presence and progression of endometrial hyperplasia. However, the high level of PR expression in these tissues suggests that progesterone therapy would be a suitable treatment option for these patients, and given the adverse regulation seen with the synthetic progestins, a natural progesterone treatment may be a better option for these patients.

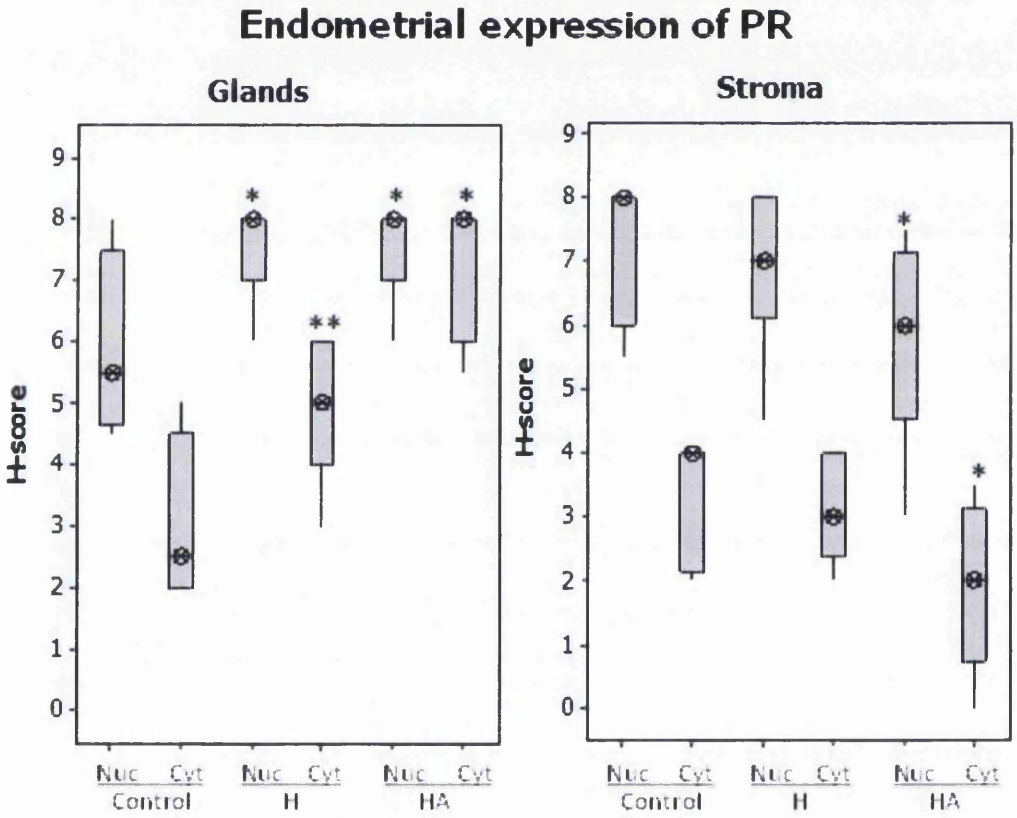


Figure 3.1: Expression of PR in endometrial samples from normal human endometrium (Control), hyperplasia without atypia (H) and hyperplasia with atypia (HA). Staining was assessed in the nuclear (Nuc) and cytosolic compartments (Cyt) using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix D. (n=20)

3.3.1.2 Differential Expression of FOXO1 in Endometrium of Women with Hyperplasia Comparing to Controls

FOXO1 is a transcription factor involved in cell cycle regulation and differentiation in the endometrium (Takano et al., 2007) and is known to be up-regulated by ligand bound PR (Ward et al., 2008, Labied et al., 2006). At present there is no available data on the level of FOXO1 in patients with endometrial hyperplasia. As FOXO1 is widely reported to be down-regulated in endometrial cancer (Goto et al., 2008, Myatt et al., 2010, Lam et al., 2012), it was of interest to see if the levels of this protein were affected in endometrial hyperplasia, particularly in cells with atypia as these are often cancer precursors. The expression of FOXO1 in the control and study groups was assessed at glandular and stromal levels in the nucleus and cytosol of endometrial cells (Figure 3.2).

Expression of FOXO1 in endometrial glands at nuclear and cytosol level was significantly different between study and control groups. A lower nuclear expression of FOXO1 was observed in the HA ($p>0.001$) and H ($p>0.001$) groups compared to controls. At cytosol level a similar pattern was observed, with lower expression of FOXO1 in the HA ($p=0.003$) and H ($p=0.02$) groups compared to controls (Figure 3.2).

In the stroma, the expression of FOXO1 was significantly lower in the HA group comparing to control at nuclear ($p<0.01$) and cytosol level ($p<0.05$). However no significant differences in FOXO1 expression were observed in the stroma between the H group and control or between the H and HA groups (Figure 3.2).

Although no previous research has been published on the levels of FOXO1 in endometrial hyperplasia, a loss of FOXO1 has been reported in endometrial cancer in several studies (Goto et al., 2008, Myatt et al., 2010, Lam et al., 2012). FOXO1 is involved in cell fate decisions and acts as a tumor suppressor (Greer and Brunet, 2005), so it is not surprising that FOXO1 is lost in HA patients where cells are abnormal and at a higher risk of developing into a cancerous tumor. There does appear to be an increasing reduction in FOXO1 protein expression in endometrial glands as disease progresses with a marked reduction in FOXO1 in both glands and

stroma from the hyperplasia with atypia group. These data therefore suggests that loss of glandular FOXO1 may be indicative of hyperplasia presence whereas loss of both gladular and stromal FOXO1 may be indicative of atypical hyperplasia suggesting FOXO1 could be a potential biological marker for both presence and progression of this disease.

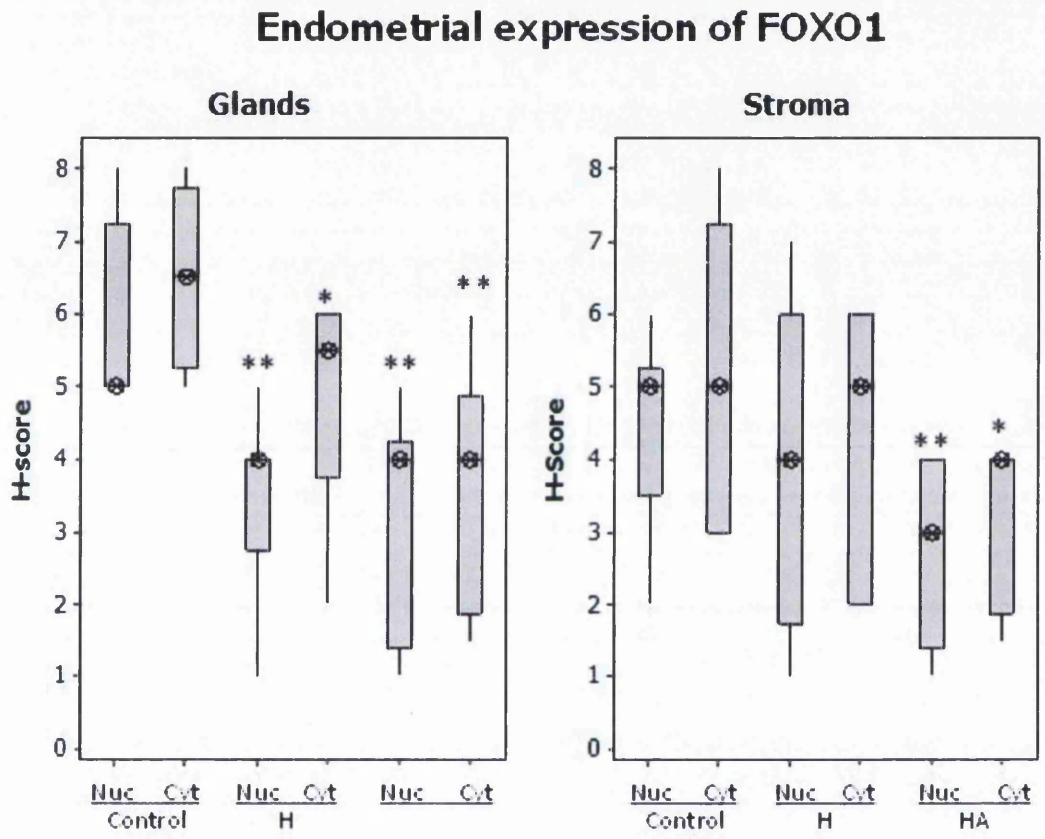


Figure 3.2: Expression of FOXO1 in endometrial samples from normal human endometrium (Control), hyperplasia without atypia (H) and hyperplasia with atypia (HA). Staining was assessed in the nuclear (Nuc) and cytosolic compartments (Cyt) using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix E. (n=20)

3.3.1.3 Differential Expression of AREG in Endometrium of Women with Hyperplasia Comparing to Controls

AREG is a gene regulated by ligand-bound PR and is expressed at varying levels in the endometrium throughout the menstrual cycle (Gui et al., 2008a). An assessment

of the endometrial expression of AREG in the control and hyperplasia study groups was performed to investigate the expression of this PR target as a potential marker for endometrial hyperplasia presence and stage (Figure 3.3). In both glands and stroma there was a significant decrease in nuclear expression of the AREG protein in both study groups compared to control (Figure 3.3). This decrease was not paralleled in cytosol with the exception of the HA stroma where cytosolic AREG was significantly reduced compared to control ($P<0.05$).

The AREG protein is known to interact with epithelial growth factor (EGF) and transforming growth factor alpha (TGFα) receptors to promote proliferation of normal epithelial cells (Plowman et al., 1990) and has been shown to be up-regulated in endometrial cancer cells (Ejskjær et al., 2007). It is thus surprising that such a significant reduction was observed. However, a high degree of functional redundancy compensation within the family of EGF receptor (EGFR) ligands has been reported (Schneider and Wolf, 2009) meaning it is possible that high levels of other EGFR ligands such as EGF and TGFα may be compensating for a loss of AREG. Indeed Nikura *et al* documented the presence of TGFa in hyperplasia patients, with highest levels in atypical hyperplasia (Niikura et al., 1996). Interestingly, in the same study, AREG was reported to be completely absent in all endometrial samples, including healthy, hyperplastic and cancerous endometrium (Niikura et al., 1996). Furthermore, endometrial hyperplasia is associated with reduced progesterone signaling (Herrinton and Weiss, 1993) and progesterone withdrawal is known to impact on many genes (Catalano et al., 2007, Critchley et al., 2003), therefore the loss of AREG may be a direct result of progesterone absence. Though the ability of estrogen to up-regulate this protein raises further questions into why a reduction was witnessed.

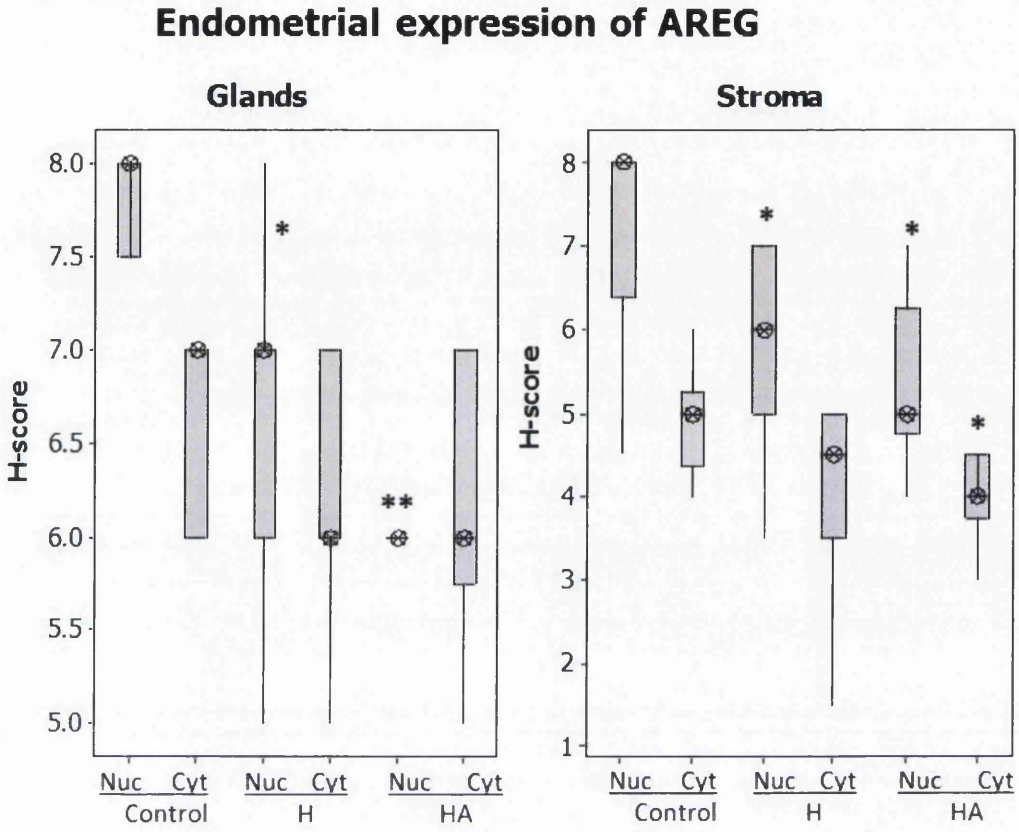


Figure 3.3: Expression of Amphiregulin in endometrial samples from normal human endometrium (Control), hyperplasia without atypia (H) and hyperplasia with atypia (HA). Staining was assessed in the nuclear (Nuc) and cytosolic compartments (Cyt) using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix F. (n=20)

3.3.1.4 Differential Expression of Lactoferrin in Endometrium of Women with Hyperplasia Comparing to Controls

LF is a major estrogen-inducible secretory protein with an elevation in its expression correlating directly with a peak in proliferation of endometrial epithelia (Kelver et al., 1996). The correlation between LF and endometrial proliferation prompted us to investigate its expression in hyperplasia given the proliferative nature of this condition. LF expression in the endometrial glands at nuclear and cytosol level was significantly different between study and control groups. At nuclear level, a

significantly lower expression of LF was observed in the endometrium of HA ($p<0.05$) and H ($p=0.01$) patients compared to controls. The down regulation was surprising as enhanced estrogen signaling is associated with the hyperplastic endometrium. Despite being significant, the down regulation was minimal and not seen in other locations. At cytosolic level the expression of LF in the HA and H groups was not statistically different in the glands when compared to controls (Figure 3.4). In the stroma, the LF expression was not statistically significantly different between the study groups and control in either the nuclear or the cytosolic compartments. There was also no statistical difference in LF expression whilst comparing study groups in both epithelia and stroma (Figure 3.8). Akin to this finding, Walmer *et al* (1995) reported no alteration in LF expression in women with endometrial hyperplasia although the authors highlighted one exception where a patient with atypical hyperplasia presented with elevated LF (Walmer et al., 1995).

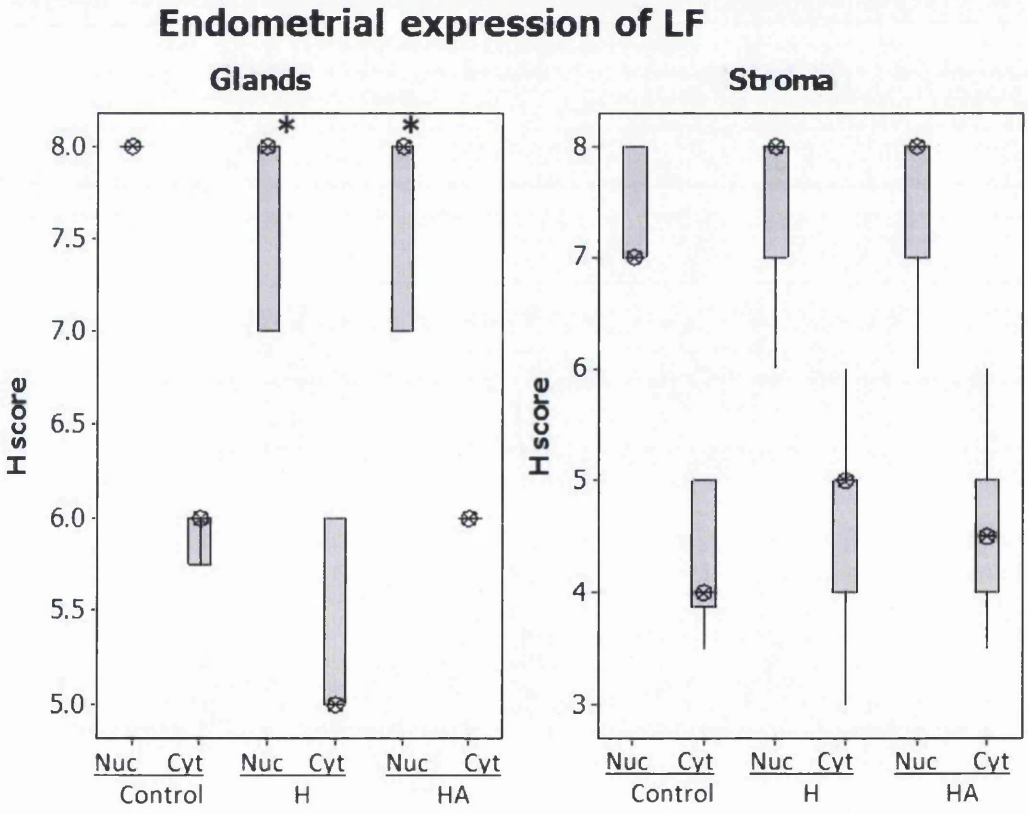


Figure 3.4. Expression of Lactoferrin in endometrial samples from normal human endometrium (Control), hyperplasia without atypia (H) and hyperplasia with atypia (HA). Staining was assessed in the nuclear (Nuc) and cytosolic

compartments (Cyt) using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix G. (n=20)

3.3.1.5 Summary of Section 3.3.1

The overall findings of this section can be summarised into three main points. 1. The data obtained confirmed that the target proteins are adversely expressed in endometrial hyperplasia suggesting they are clinically relevant proteins. 2. That PR is present at high levels in endometrial hyperplasia suggesting this condition is susceptible to progestin treatment. 3. PR, FOXO1, AREG and LF could be used as potential biomarkers of endometrial hyperplasia since their expression is altered compared to healthy patients. Therefore these targets are good candidates for investigation of progestins regulation through PR activity *in vivo*.

3.3.2 Expression of Progestogen-Regulated Proteins in Hyperplastic Endometrial Biopsies Pre and Post MPA treatment

Having determined the adverse expression of PR, FOXO1, AREG and LF in endometrial hyperplasia, it was next interesting to see if their expression was restored with progestin therapy. Therefore, the levels of these proteins were determined pre and post MPA treatment. The patients recruited for this part of the study were diagnosed with hyperplasia without nuclear atypia. Patients with atypical hyperplasia did not receive progestin therapy as they underwent hysterectomy for the management of their pathological condition.

3.3.2.1 The Expression of FOXO1 in Women with Hyperplasia Pre and Post Treatment with MPA *in Vivo*

As detailed in Chapter 1 (Section 1.7.2), *in vitro* treatment models have revealed that progestins directly induce FOXO1 via transcriptional activation to inhibit the growth of cancer and pre-cancerous endometrial epithelial cells (Ward et al., 2008, Labied et al., 2006), demonstrating FOXO1 as a target of MPA treatment. The pre and post MPA treatment samples from patients with hyperplasia without atypia were

investigated for the expression of this transcription factor and the results can be seen in Figure 3.5.

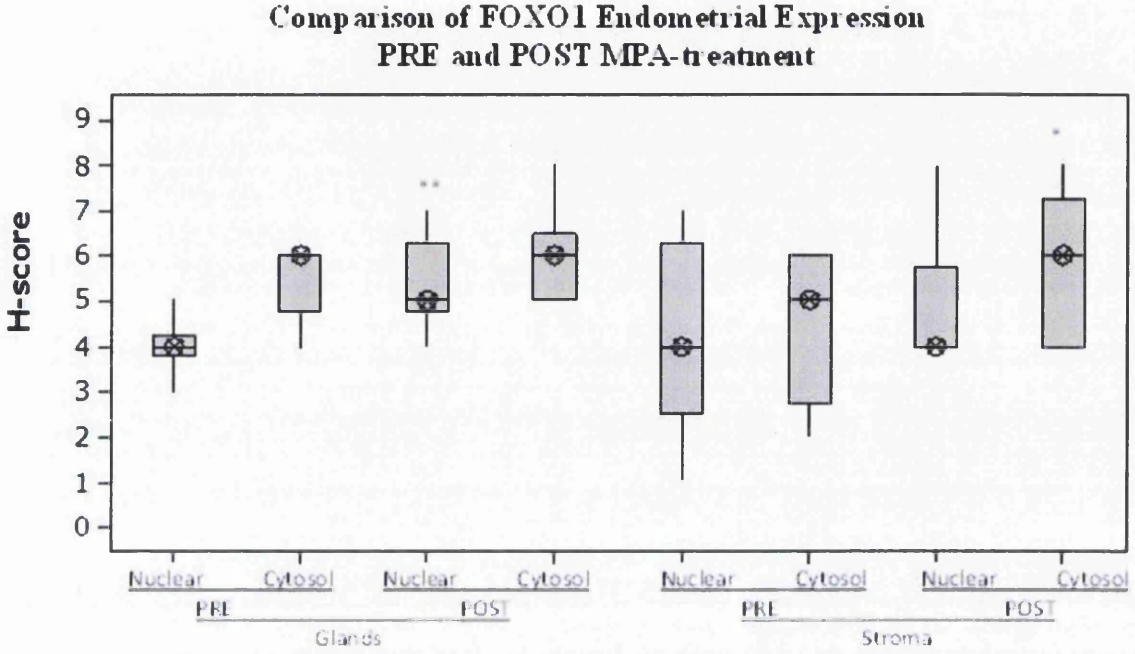


Figure 3.5. Expression of FOXO1 in endometrium of patients with hyperplasia without atypia before and after treatment with MPA (20mg/day) for 3 months. Staining was assessed in the nuclear and cytosolic compartments using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix H. (n=18)

An increased expression of FOXO1 was observed after progestin treatment in the nucleus of glandular cells (p=0.001) and in the cytoplasm of stromal cells (p>0.05). MPA treatment failed to significantly modulate FOXO1 expression in the cytosol of the glands or in the nucleus of stromal cells although the overall trend indicated a rise in FOXO1 protein post MPA treatment (Figure 3.5). This study has previously shown that FOXO1 is down-regulated in endometrial hyperplasia patients (Figure 3.2). However, MPA treatment appears to have restored the expression of this protein. This information is in line with previous findings that FOXO1 is a progestin target *in vivo* and suggests that MPA causes an induction of this protein; it also indicates that these patients have responded to progestin treatment. However, it

remains unknown whether the changes seen in FOXO1 would be the same had natural progesterone been administered instead of MPA.

3.3.2.2 The Expression of PR in Women with Hyperplasia Pre and Post Treatment with MPA *in Vivo*

Samples from 18 patients in the hyperplasia without atypia group were investigated for the expression of PR before and after treatment with MPA 20 mg/day for 3 months. PR has been previously reported to be down-regulated after progestin treatment due to a negative feedback loop (Graham and Clarke, 1997b). In line with the literature, progestin treatment induced a significant decrease in PR expression in the nucleus ($p>0.05$) and cytosol ($p>0.05$) of endometrial glandular and stromal cells post treatment (Figure 3.6). A study by Vereide *et al* (2006) revealed a significant down-regulation of PR in endometrial cells after progestin therapy. The group reported a reduction in PR after treatment with two progestins, LNG and MPA with a pronounced reduction in the LNG group and a more reserved reduction in the MPA group (Vereide et al., 2006). The extent of PR down-regulation post MPA treatment was similar between the previously published research and the data obtained within this study.

In contrast to epithelial cells, no significant changes were observed in stromal expression of PR in the nucleus or the cytosol between pre and post treatment groups (Figure 3.6). The maintenance of PR in endometrial stromal cells is not surprising as stroma retains PR in the progesterone-dominated phase of the menstural cycle suggesting this tissue is less sensitive to inhibitory effects of progesterone (Mote et al., 1999). Although there were no significant changes in stromal PR levels post-treatment, the mean PR H-score tended to be reduced in these samples, a similar trend was also reported by Vereide and co-workers whereby there was an overall decrease in stromal PR following MPA treatment (Vereide et al., 2006). The observed reduction in PR is likely to influence the long-term efficacy of the progestin treatment as a loss of receptor negates the actions of the progestins.

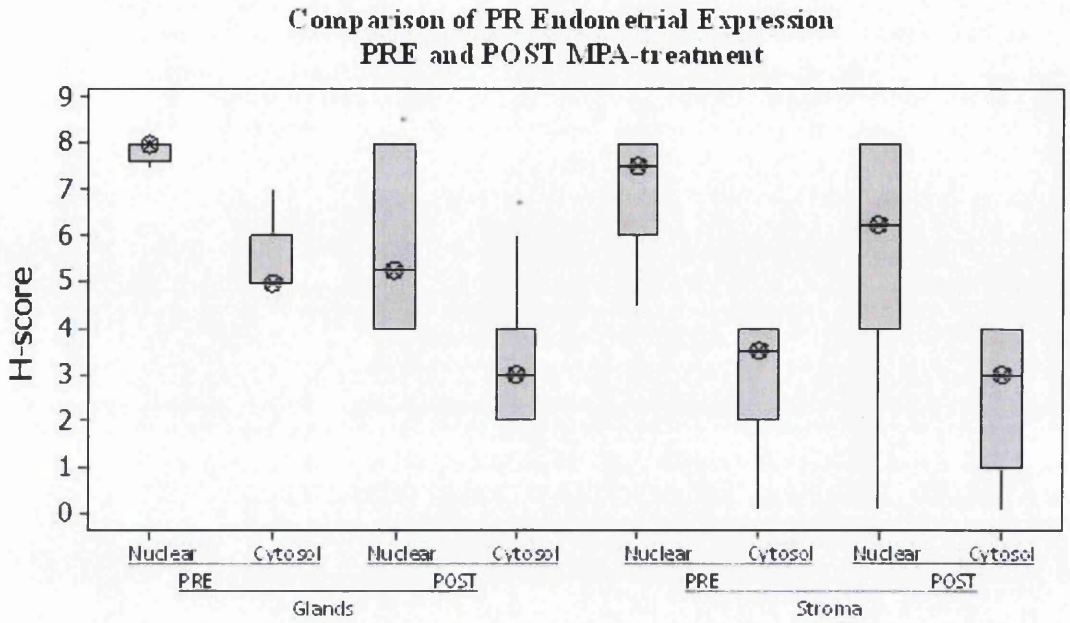


Figure 3.6: Expression of PR in endometrium of patients with hyperplasia without atypia before and after treatment with MPA (20mg/day) for 3 months. Staining was assessed in the nuclear and cytosolic compartments using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix I. (n=18)

Overall, *in vivo,* MPA serves to down-regulate the PR in endometrial epithelium but does not alter PR expression in Stroma.

3.3.2.3 The Expression of AREG in Women with Hyperplasia Pre and Post Treatment with MPA *in Vivo*

The pre and post MPA treatment samples from hyperplasia without atypia patients were investigated for the expression of AREG (Figure 3.7). Previous research has shown that MPA is able to upregulate AREG (Smid-Koopman et al., 2003), and that progestin-induced up-regulation occurs through ligand bound PR-A (Das et al., 1995, Mulac-Jericevic et al., 2000). Indeed a significantly higher expression of AREG (p=0.02) was observed in the stroma at nuclear level after treatment with this progestin. However, in the glandular cells at nuclear and cytosol levels as well as in the stromal cells at cytosol levels the expression of AREG was not significantly

different before and after MPA treatment (Figure 3.7). Whether natural progesterone would evoke an equivalent response remains to be determined.

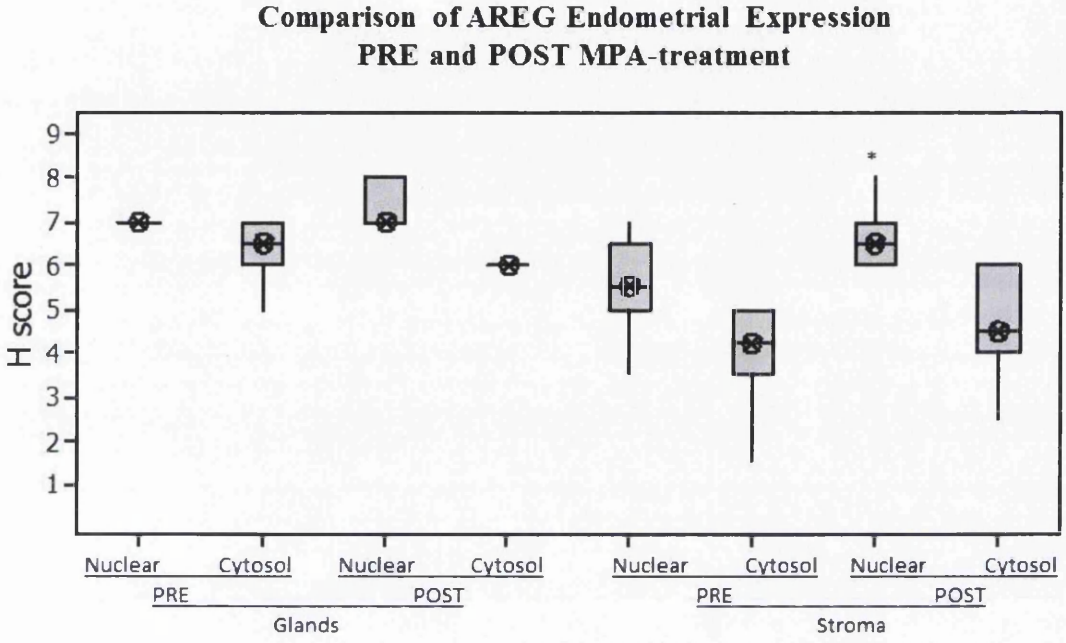


Figure 3.7. Expression of AREG in endometrium of patients with hyperplasia without atypia before and after treatment with MPA (20mg/day) for 3 months. Staining was assessed in the nuclear and cytosolic compartments using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix J. (n=18)

3.3.2.4 The Expression of LF in Women with Hyperplasia Pre and Post Treatment with MPA *in Vivo*

LF is known to be inhibited through ligand-bound PRB in epithelial and stromal cells. Pre and post MPA treatment samples from hyperplasia patients were investigated for the expression of LF (Figure 3.8). A statistically significantly higher expression of LF was observed in the glands at nuclear (p=0.04) and cytosol (p=0.04) level after treatment with progestin. The up-regulation of LF after MPA treatment was unexpected. Immunohistochemical analysis of LF in healthy primate endometrium suggests LF is down-regulated by progestin *in vivo* (Teng et al., 2002).

Yet in the case of hyperplastic endometrial glands; there was a significant increase which may suggest adverse gene regulation by MPA that differs from the natural drug. On the other hand, in the stromal cells at nuclear and cytosol level, the endometrial expression of LF did not change significantly after MPA treatment (Figure 3.8).

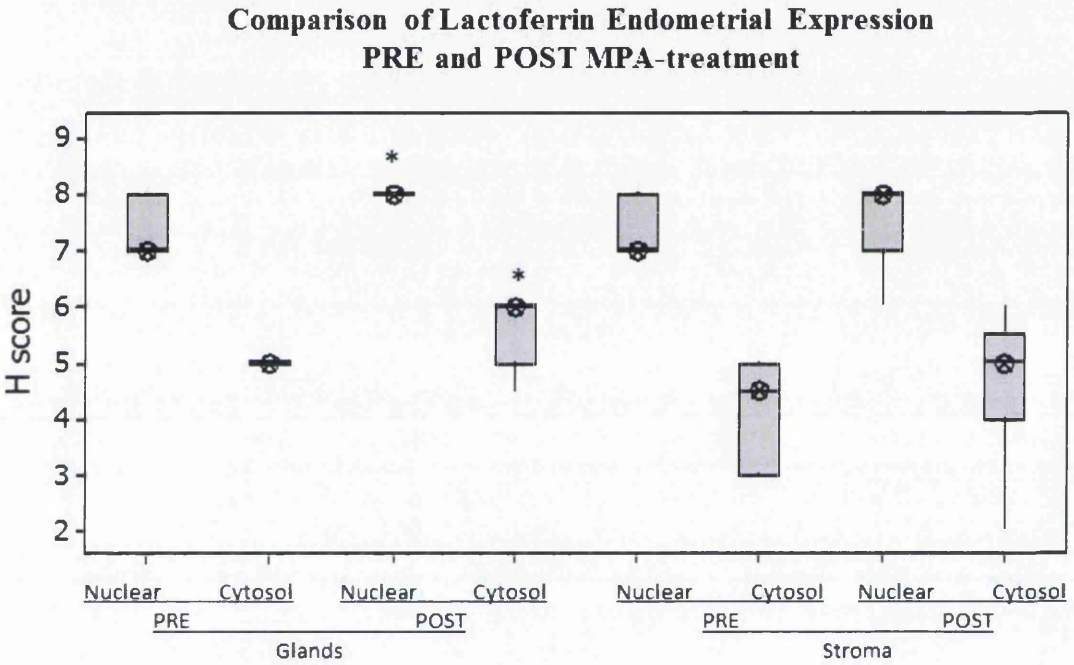


Figure 3.8: Expression of LF in endometrium of patients with hyperplasia without atypia before and after treatment with MPA (20mg/day) for 3 months. Staining was assessed in the nuclear and cytosolic compartments using the H-score system with 3 independent scorers. Statistical analysis was performed using a Mann Whitney test. *p≤0.05, **p≤0.01. Corresponding IHC images can be seen in Appendix K. (n=18)

3.3.2.5 Summary of Section 3.3.2

The data presented within this section shows the regulation of FOXO1, PR, AREG and LF by MPA *in vivo*. It was not possible to determine the regulation of these proteins by LNG as biopsies were not available from women receiving this treatment. It was also not possible to assess the regulation of these proteins by progesterone as this natural product is not prescribed to women for the treatment of

hyperplasia. To summarise the findings obtained within this section, a table was produced showing the regulation of the four proteins by MPA (Table 3.2).

Protein	Glands		Stroma	
	Nuc	Cyt	Nuc	Cyt
FOXO1	↑	-	-	↑
PR	↓	↓	-	-
AREG	-	-	↑	-
LF	↑	↑	-	-

Tables 3.2: Changes in the expression of target proteins after MPA treatment *in vivo*. Green indicates an increase in protein expression, blue indicates a decrease and pink indicates no change.

3.3.3 PR, ER and AR are expressed in human endometrial epithelial and stromal cell lines

As it was not possible to compare the regulation of targets by natural and synthetic progestins *in vivo,* work to assess differences in gene regulation by natural and synthetic progestins was assessed *in vitro* in two endometrial cell lines; Ishikawa cells and Human Endometrial Stromal Cells (HESCs). Ishikawa cells were used as a model cell line for human endometrial epithelium and HESCs were used as a model stromal cell line.

In order to assess the actions of P_4 MPA and LNG in these cell lines, it was first important to establish whether the cell lines expressed the main receptor through which these hormones signal; the progesterone receptor (PR). As estrogen was used in combination with the progestins to treat Ishikawa cells it was also important to assess the presence of the estrogen receptor (ER) in this cell line. Further to this, side-effects associated with synthetic progestin are often attributed to their ability to bind to and signal via the androgen receptor (AR) (Bentel et al., 1999, Kloosterboer et al., 1988, Burton et al., 2003, Phillips et al., 1990). Therefore, the presence of AR was also quantified in both cell lines.

The human endometrium naturally expresses PR, ER and AR and these levels have been shown to fluctuate throughout the menstrual cycle (Ingamells et al., 1996, Lessey et al., 1988, Mertens et al., 2001). Furthermore, different isoforms of these receptors are also expressed at precise ratios. The PR has two main isoforms, PR-A and PR-B. PR-B is the larger of the two isoforms with a molecular weight of 116kDa, PR-A contains the same amino acid structure as PR-B but is truncated at the N-termins resulting in a smaller protein with a molecular weight of 99kDa (Giangrande and McDonnell, 1998). Both isoforms are seen at different ratios throughout the menstrual cycle with both appearing at similar levels in proliferative phase and PR-A appearing to be the most dominant in the secretory phase (Mangal et al., 1997, Mote et al., 1999).

There are also two dominant ER isoforms, ERα and ERβ, which have again been shown to be regulated at an intricate balance throughout the monthly cycle (Mylonas et al., 2004, Lecce et al., 2001). The smaller ER isoform, ERβ, has a molecular weight of 52-54kDa and ERα has a molecular weight of 66kDa, splice variants of both isoforms also exist (Wang et al., 2005, Peng et al., 2003).

The molecular weight of the full length AR-B is 110kDa. The AR-A isoform has a truncated N-terminus leading to its lower molecular weight of 87kDa (Lubahn et al., 1988).

The basal expression of PR, ER and AR RNA was quantified within the Ishikawa and HESC cell lines through RT-PCR. The whole PR-A RNA sequence lies within the coding region of the longer PR-B isoform and the primers used for this analysis lay within the coding region of PR-A thus causing both isoforms to be amplified during the single PCR reaction. The same scenario occurs for AR, whereby the primers used amplify both isoforms of the protein. Thus, the normalised starting quantity of PR and AR seen in Figure 3.9 is the total RNA inclusive of all isoforms. Separate sets of primers were used to analyse ERα and β. The results indicate the presence of the hormone receptors at RNA level, though only very low levels of ER were detected in HESCs.

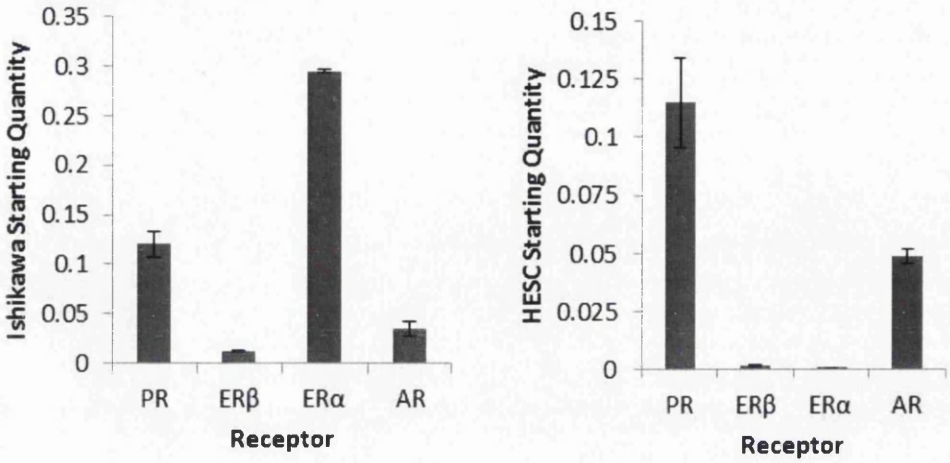


Figure 3.9: mRNA expression levels of nuclear receptors in the Ishikawa (A) and HESC (B) cell lines.

RNA was extracted was extracted from Ishikawa and HESC cells using the Quiagen RNeasy kit, the RNA was then converted to cDNA, and amplified using PCR. The average starting quantity of the target mRNA was calculated by obtaining the cycle number at which the gene was first amplified and plotting this number within a standard curve. The results were normalised against reference gene GAPDH. Error bars represent standard deviation from the mean of 3 repeats.

To assess whether the RNA detected for the hormone receptor is translated into protein, the levels of PR, ER and AR protein in the Ishikawa and HESC cell lines was determined by immunoblot analysis. Due to the different molecular weight of the individual isoforms found within PR, AR and ER, they could be visualised separately on the immunoblot membrane.

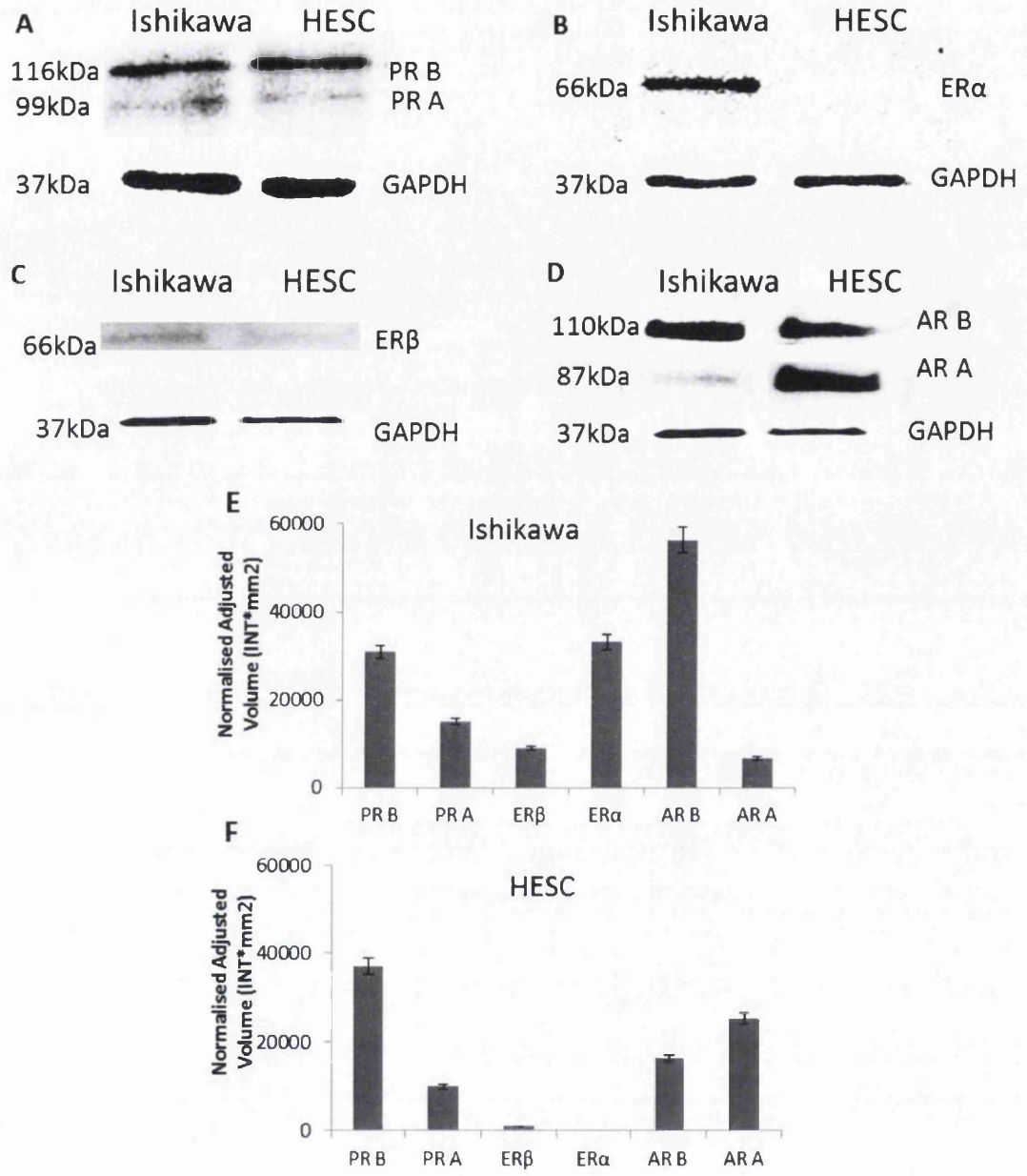


Figure 3.10: Protein immunoblot images showing PR-A and B (A), ERα (B), ERβ (C) and AR-A and B (D) protein expression in Ishikawa and HESC cell lines. Graphs show quantified band density of receptor levels in the Ishikawa (E) and HESC (F) cell lines.

Ishikawa and HESC cells were grown to confluencey in triplicate in a 6-well plate. Cells were scraped in extraction buffer (Section 2.1.3) for protein extraction. Immunoblots were separated by SDS-PAGE then immunoblotted

against GAPDH (sc-25778, Santa Cruz) PR (H10: sc-7208, Santa Cruz), ERα (HC-20 sc-542 Santa Cruz), ERβ (PA1-311 Pierce Ltd) and AR (N-20, Santa Cruz) antibodies.

Band density was determined using Quantity One software (Bio-Rad). The Band Density of GAPDH samples were also quantified and used to normalise the data. Band density of PR-A, PR-B, ERα, ERβ, AR-A and AR-B are shown.

Protein blot analysis of receptor expression in the Ishikawa cell line revealed that these cells express each of the protein receptors analysed at varying levels with AR-B being the most highly expressed (Figure 3.10 E). The expression of AR has been previously shown in the Ishikawa cell line (Lovely et al., 2000) and this was confirmed in this study with the expression of AR-B being far higher than AR-A.

The expression of PR in Ishikawa cells has also been confirmed in previous work (Hata and Kuramoto, 1992). This study revealed that the level of PR-B expression is greater than the levels of PR-A, although the levels of PR-A were lower, protein was still detectable (Figure 3.10 A and E). ERα and ERβ were also detected in the Ishikawa cell line which is in line with previous findings (Nishida et al., 1985).

In the HESC cell line the expression of AR-A was greater than AR-B but both were expressed at high levels (Figure 3.10 D and F). Both PR-A and B were also detectable in this cell line with the expression of PR-B being greater than PR-A (Figure 3.10 A and F). However, ER expression was very poor. The ERβ could be detected at low levels but no ERα could be detected at all (Figure 3.10 B, C and F).

The expression of the relevant hormone receptors in the Ishikawa cell line suggests their sensitivity to gene alterations after hormone exposure. The absence of ERα and low expression of ERβ in the HESC cell line should not influence the results as the combined estrogen treatment will not be applied to these cells. However, the high expression of PR and AR protein subtypes will allow differences in signalling patterns to be seen.

3.3.4 MPA, LNG and P_4 differentially regulate target gene expression in endometrial epithelial cells.

Given the expression of PR and ER has been determined in the Ishikawa cell line, these cells were used as a model for the human endometrium. Confluent cell monolayers were cultured in the presence of P_4, MPA or LNG either alone or in combination with E_2 in order to compare their target regulation. Thereafter, PR signalling pathways were assessed through the PR targets described in Table 3.1.

For this investigation, cells were treated with 1μM of progestogen for 48 hours. The treatment time of 48 hours was chosen as FOXO1, the positive control for this study, is known to be affected by progestin treatment at this time-point in both endometrial epithelial and stromal cells (Kyo et al., 2011, Labied et al., 2006, Zoumpoulidou et al., 2004). Likewise, 1μM of progestin has been shown to be a sufficient concentration to obtain a progestogenic response in the endometrium *in vitro* (Labied et al., 2006, Zoumpoulidou et al., 2004, Smid-Koopman et al., 2003)

3.3.4.1 FOXO1 is significantly up-regulated by MPA, P_4 and LNG in the Ishikawa cell line.

The first target to be analysed was FOXO1. FOXO1 was utilised as a positive control for the action of P_4, MPA and LNG in Ishikawa cells. Indeed progesterone-induced FOXO1 up-regulation has been well characterised, and is shown to occur specifically through the PRB (Kyo et al., 2011, Wang et al., 2009, Ward et al., 2008). Furthermore, this up-regulation has been demonstrated in the Ishikawa cell line after progesterone and progestin treatment (Kyo et al., 2011, Ward et al., 2008), thus making FOXO1 an ideal target for confirming the sensitivity of the current cell line to progestin treatment.

Moreover, a direct comparison between the regulation of FOXO1 by MPA, LNG and P_4 has yet to be made. Therefore, as well as using FOXO1 as a positive control, the differences in regulation of FOXO1 between the progestogens will also be assessed.

Figure 3.11 B shows that MPA, P_4 and LNG each significantly up-regulated FOXO1 gene expression in the Ishikawa cell line compared to untreated control. P_4

significantly up-regulated FOXO1 gene expression by 1.6 fold ($P<0.05$) while MPA caused a 1.4 ($P<0.05$) fold increase, although the induction was modest, the up-regulation was consistent between repeats and deemed significant by students T-test. LNG caused a 1.75 fold induction of the FOXO1 gene which was greater than that of MPA and P_4; however there was no significant difference in the regulation of FOXO1 gene between the progestogens.

The modest but significant increase in FOXO1 RNA after progestin treatment was sufficiently translated to protein (Figure 3.11 C). Figure 3.11 A (Control) shows basal FOXO1 protein expression in Ishikawa cells and the intensity of the red stain can be seen to increase after treatment with MPA, P_4 and LNG (Figure 3.11 A) suggesting the amount of protein present has increased. This observation was confirmed by the quantitative analysis of the stain intensity which showed that MPA, P_4 and LNG significantly up-regulated the FOXO1 protein (Figure 3.11 C). These results are in concurrence with the findings observed *in vivo* in patients diagnosed with hyperplasia and treated with MPA (Fig 3.5).

Furthermore, FOXO1 is a transcription factor that shuttles in and out of the nucleus and control samples from this study show that FOXO1 is predominantly expressed in the nucleus in Ishikawa cells (Figure 3.11A, Control). In line with previous findings (Ward et al., 2008), treatment with progestins up-regulated both cytoplasmic and nuclear FOXO1 protein at the same level with the difference between nuclear and cellular intensity remaining the same pre and post hormone treatment (Figure 3.11D).

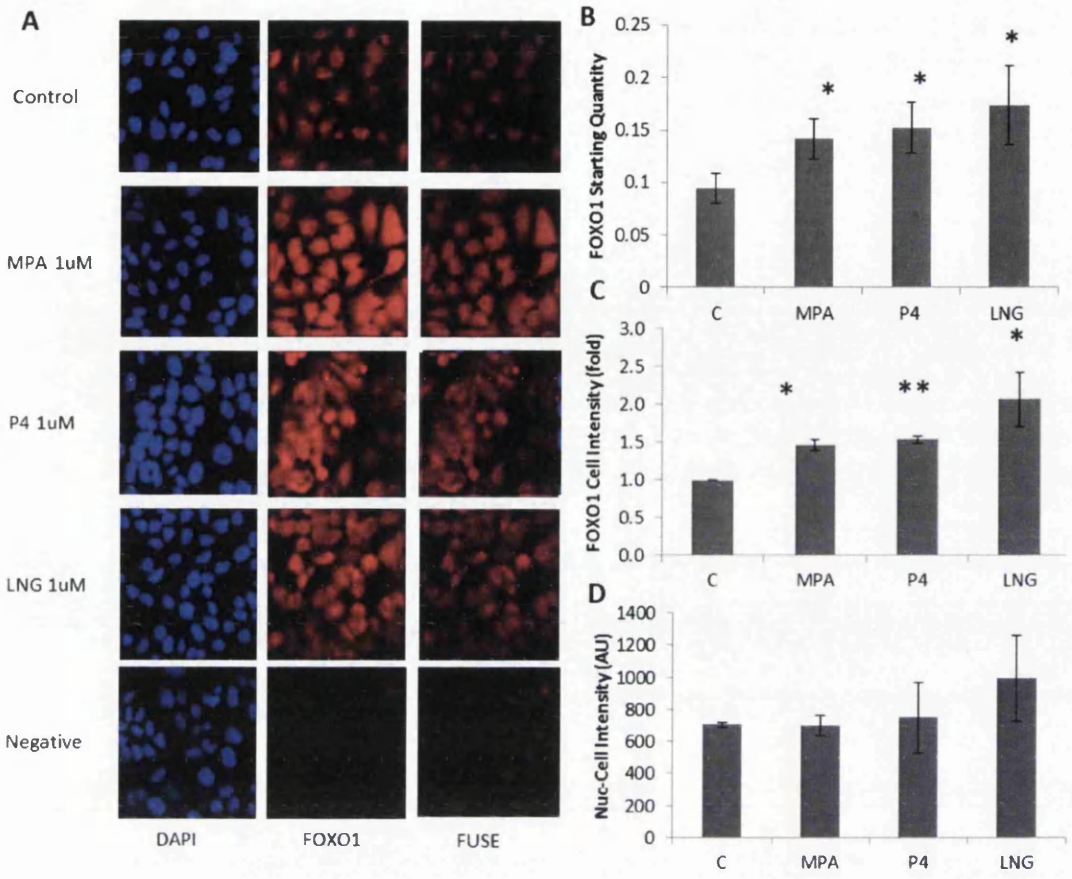


Figure 3.11: FOXO1 gene and protein expression in the Ishikawa cell line following hormone treatment for 48h with 1µM of hormone. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Nuclear minus Cell Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. The difference between these concentrations is plotted. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

Like progesterone, E_2 is capable of up-regulating FOXO1 through binding its receptor (Lengyel et al., 2007, Schuur et al., 2001). When Ishikawa cells were treated with E_2 alone there was an average 2-fold increase of FOXO1 RNA, however, this was not significant due to noticeable deviations from the mean (Figure 3.12B). Yet, when E_2 was delivered in combination with P_4 or MPA, FOXO1 gene expression was significantly up-regulated (1.8 and 1.4 fold respectively). Despite a 1.5 fold increase in FOXO1 RNA after E_2 plus LNG treatment, this was not significant.

After incubation with MPA combined with E_2 there was no change seen in the expression of FOXO1 protein in the Ishikawa cells compared with untreated control (Figure 3.12C). This was contrary to the 1.5 fold increase seen in treatment with MPA alone (Figure 3.11C). On the other hand, when cells were treated with E_2 in combination with LNG there was a significant 1.5 fold increase in FOXO1 protein (Figure 3.12C). P_4 + E_2 treatment also caused a significant up-regulation in FOXO1 protein at the same level as when cells were treated with P_4 alone (both 1.5 fold) (Figure 3.11C and 3.12C). Overall, progestogen treatment alone caused a significant increase in FOXO1 RNA and protein which was reduced with the addition E_2, particularly when E_2 was added to MPA, where protein induction was diminished (Figure 3.12C). This result implies that patients treated with MPA may need to be monitored for estrogen levels since estrogen may affect the action of this progestin. In fact a group of patients recruited for this study were treated with MPA and did not respond to treatment but due to their management and care it was not feasible to obtain endometrial biopsies.

Moreover, previous work has shown that ligand bound ER is able to interact directly with members of the FOXO family of proteins (Schuur et al., 2001) and agonist-bound ER can lead to the inactivation of FOXO1 (Lengyel et al., 2007) which may have led to the observed decrease in FOXO1 after E_2 treatment.

After E_2 treatment, the red protein stain can be seen over a broad range compared to control where it appears compact and nuclear localised (Figure 3.12A). To assess whether this was indeed the case the intensity of protein stain (Texas Red) was calculated in the region above the DAPI-stained nucleus only as well as over the

whole cell as described in Section 2.1.5. The cellular intensity was then subtracted from the nuclear intensity. Figure 3.12D shows that there is little difference between nuclear and cytosolic FOXO1 protein with E_2 treatment confirming FOXO1 displacement. This data correlates with previous findings that show E_2 causes downstream phosphorylation of FOXO1 leading to its movement out of the nucleus (Lengyel et al., 2007). As expected, after the combined treatment of Ishikawa cells with the progestogens and E_2 there was an increase in nuclear positioning compared to E_2 alone (Figure 3.12D). However, it can be seen that, after $P_4 + E_2$ treatment, the location of the FOXO1 protein was not significantly different to the control cells (Figure 3.12D). After treatment with E_2 and progestins there was a significant difference in nuclear location. Existing research shows that androgens negatively regulate FOXO1 (Huang et al., 2004) and that ligand-bound AR can inactivate the FOXO1 protein (Li et al., 2003), thus, the ability of MPA and LNG to bind and activate the AR may have influenced the current data.

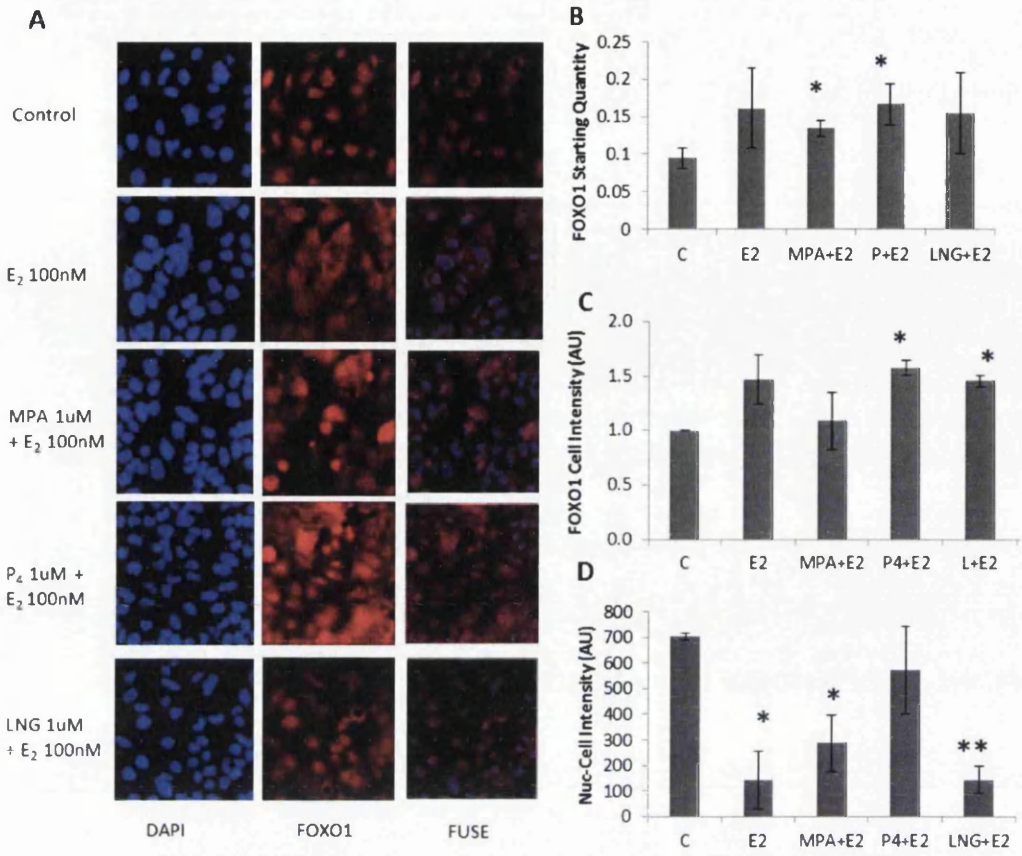


Figure 3.12: FOXO1 gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. MPA, P_4 and LNG treatment concentration was 1µM whereas E_2 treatment concentration was 100nM A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Nuclear Intensity minus Cell Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. The difference between these concentrations is plotted. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-

Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

The overall findings relating to FOXO1 as a positive control are indicative that genes in the Ishikawa cell line can be influenced by progestogens, therefore suggesting the cell line is sufficient for the analysis of the remaining target genes. Moreover no differences were observed between the progestogens in the regulation of the FOXO1 gene in the presence or absence of E_2.

3.3.4.2 Progesterone Receptor is differentially regulated by P_4, MPA and LNG in the Ishikawa cell line.

The second target to be analysed is the Progesterone Receptor (PR). The PR itself is regulated by its own ligand, and potential differential regulation of this receptor by synthetic or natural ligands has not previously been investigated. As the presence of PR in the Ishikawa cell line has already been confirmed, this section will focus on the regulation of PR by the progestogens. The PR contains estrogen and progesterone response elements within its promoter region and can therefore be regulated through ligand-activated PR and ER (Petz and Nardulli, 2000, Vegeto et al., 1993). Progesterone has been widely reported to down-regulate the PR (Graham and Clarke, 1997b) whereas estrogen has been reported to up-regulate the PR (Hata and Kuramoto, 1992, Jamil et al., 1991). MPA and LNG have also been shown to down-regulate PR in several studies (Hanifi-Moghaddam et al., 2006, Critchley et al., 1998, Vereide et al., 2006) with one exception that suggested LNG does not alter PR levels (Meng et al., 2010).

Furthermore, work carried out on the Ishikawa cell line has shown that PR is down-regulated by progesterone after being primed with estradiol (Lessey et al., 1996). The down-regulation of PR by MPA has also been shown in another epithelial cell line (Hanifi-Moghaddam et al., 2006) though with a vastly reduced treatment concentration (1nM) compared to this study (1μM). Although there is currently no data available on the effect of LNG on the PR in endometrial cell lines, data from patient samples suggest that LNG also down-regulates PR in glandular epithelia (Critchley et al., 1998, Vereide et al., 2006) though there is conflicting information in

luminal epithelia whereby some have reported an increase in PR and others have reported no change (Critchley et al., 1998, Vereide et al., 2006, Meng et al., 2010). The effects of the progestogens on PR in the Ishikawa cell line were assessed to determine whether they differentially regulate this receptor.

Figure 3.13B shows that, contrary to previous publications, P_4 and LNG significantly up-regulated PR gene expression in the Ishikawa cell line. MPA alone, however, did not change the level of PR RNA compared to the control. The increase in PR gene expression after treatment with P_4 was further confirmed at protein level whereby an obvious increase in protein intensity can be seen after P_4 ttreatment in Figure 3.13A. Upon quantifying this up-regulation, it was found that PR expression was more than 8 fold of that seen in control cells (Figure 3.13C). On the other hand, MPA caused no change in PR protein expression compared to control (Figure 3.13C), this is different from *in vivo* data where a significant decrease in PR was observed after MPA treatment (Figure 3.6).

Although LNG caused a marginal increase in PR protein (1.3 fold), this was by no means significant (Figure 3.13C). Moreover the up-regulation of the PR protein by P_4 was significantly different to both LNG and MPA ($P>0.05$). The evident up-regulation of PR by P_4 is contradictory to previous findings, however, previous work utilised different hormone concentrations and incubation times as well as priming cells with E_2 treatment which must have had an influence on the downstream target regulation (Lessey et al., 1996, Hanifi-Moghaddam et al., 2006).

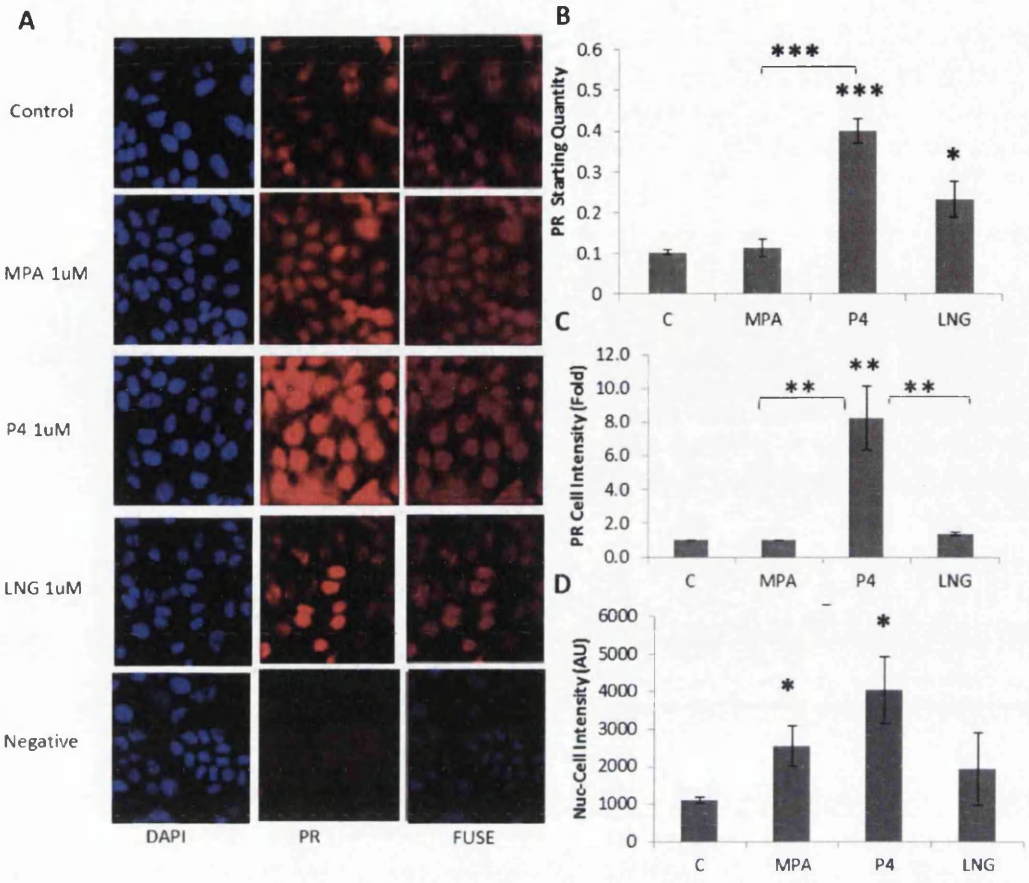


Figure 3.13: PR gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. Each treatment was at a final concentration of 1µM. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Nuclear minus Cell Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. The difference between these concentrations is plotted. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

It is well documented that estrogen causes an increase in PR (Hata and Kuramoto, 1992, Jamil et al., 1991) and this was confirmed at RNA level in the Ishikawa cell line (Figure 3.14B). To establish whether this effect was translated to protein, the InCELL analyser was again used to quantify PR protein level before and after treatment with E_2 alone and E_2 combined with the progestogens (Figure 3.14).

E_2 alone caused a significant up-regulation of PR RNA and further increased the levels of PR when applied to the cells in combination with MPA or P_4. In particular, there was a noticeable increase in the average starting quantity of PR RNA after treatment with MPA combined with E_2 (6.5 fold). Despite the large average increase, the induction was not statistically significant (P=0.061). However, in the presence of E_2, up-regulation of PR by LNG was very similar as in its absence (2.3 fold in both) (Figure 3.14B).

At protein level, treatment with E_2 also caused a marked increase in PR both alone and in combination with the progestogens (Figure 3.14C). The increase caused by each of the combined treatments was confirmed to be significant by student t-test but, although the overall fold increase was greater (2.5 fold), the increase was not significant with E_2 treatment alone (P=0.054). Furthermore, in the combined treatments, no significant difference was observed between treatment groups.

Interestingly, the combination of P_4 and E_2 caused a lesser increase in PR expression than treatment with P_4 alone, although both were significant (P>0.05).

Similarly to the FOXO1 protein, PR protein appears to be localised to the nucleus, particularly after progestogen treatment both alone (Figure 3.14D) and in combination with E_2 (Figures 3.14D). When PR binds to its ligand it is translocated to the nucleus where it regulates target genes and proteins (Leonhardt et al., 2003a), therefore the increase in nuclear PR after progestogen treatment falls in line with expectation.

LNG and MPA altered PR level in a significantly different manner to P_4 in Ishikawa cells. The differential regulation of PR by the progestogens may be detrimental in an *in vivo* situation.

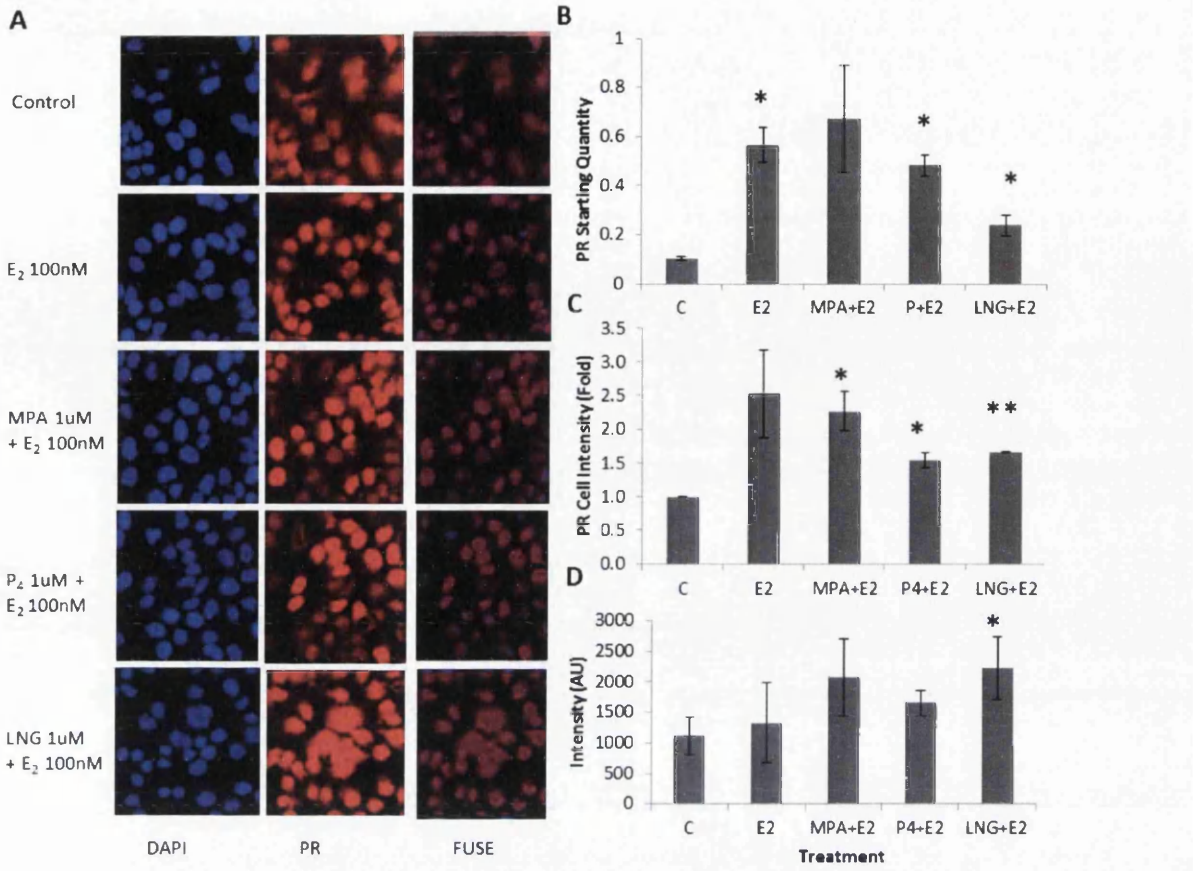


Figure 3.14: PR gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. MPA, P_4 and LNG treatment concentration was 1µM whereas E_2 treatment concentration was 100nM A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Nuclear minus Cell Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. The difference between these concentrations is plotted. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.4.3 Amphiregulin gene and protein expression is not altered after progestogen treatment in the Ishikawa cell line.

The third target to be assessed is amphiregulin (AREG). The regulation of AREG by P_4 was first described in mouse uterine epithelium whereby a rise in AREG correlates with rising P_4 and is blocked by a PR antagonist (Das et al., 1995). A rise in AREG in the human endometrium has also been shown to increase with rising P_4 (Gui et al., 2008a). This up-regulation has been reported to be mediated specifically through the PR-A isoform (Conneely and Jericevic, 2002, Mulac-Jericevic et al., 2000), though this finding is controversial (Smid (Smid-Koopman et al., 2003). If AREG is indeed regulated through the PR-A isoform its regulation may be affected by the low level of PR-A expressed in the Ishikawa cell line (Figure 3.10A) which is likely to influence P_4 regulation of AREG expression. Furthermore, AREG is stimulated by androgen in prostate (Sehgal et al., 1994) suggesting potential for MPA and LNG to further influence AREG expression through binding the AR.

Despite the reported up-regulation of AREG by P_4, after 48 hours of incubation with the progestogens there was no significant change in the expression of AREG RNA in the Ishikawa cell line. However, LNG up-regulated AREG 1.5 fold but with noticeable deviation from the mean rendering the result insignificant (Figure 3.15B). A similar result was seen at protein level, whereby there was some up-regulation after MPA and LNG treatment (1.4 fold) but this was not significant (Figure 3.15A and C). The absence of a significant increase of MPA has been reported once previously; study by Geilen and colleges (2006) found that AREG was not significantly up-regulated by MPA in the endometrium though there appeared to be an average overall increase in the protein (Gielen et al., 2006). Although there was no statistically significant change in AREG expression, an interesting trend was observed whereby MPA and LNG treatment up-regulated AREG at both RNA and protein level (Figure 3.15B and D). A non-significant increase in nuclear AREG was also observed in the glandular epithelium post MPA treatment in endometrial hyperplasia samples (Figure 3.7), suggesting that the synthetic progestins may weakly regulate this protein.

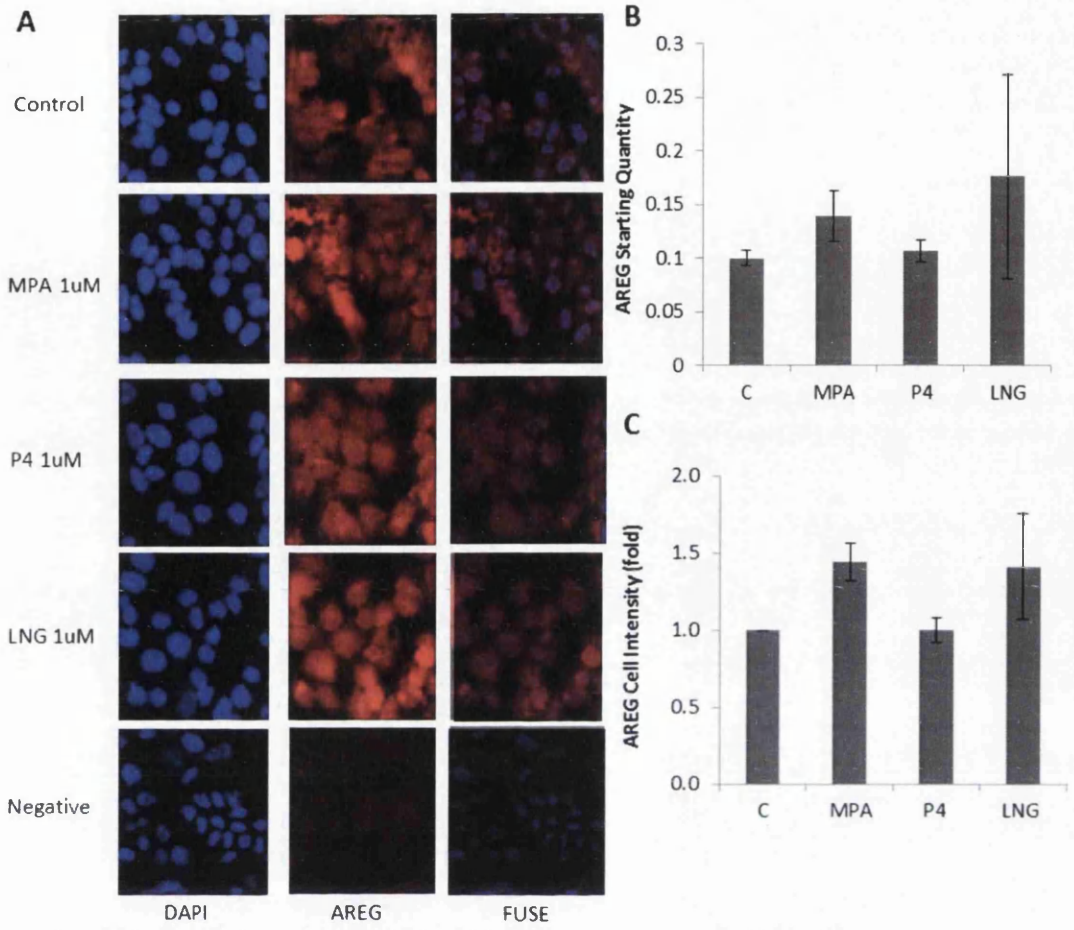


Figure 3.15: AREG gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. All treatment concentrations were 1µM. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained AREG protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of AREG normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

Estrogen has also been previously shown to up-regulate AREG gene expression, this has been shown in breast tissue (Martinez-Lacaci et al., 1995, LaMarca and Rosen, 2007) and in endometrium (Gielen 2005, Gielen 2006). Within this study, the expression of AREG was up-regulated by E_2 by 1.5 fold at both RNA protein level but was only deemed statistically significant at RNA level ($P<0.05$) (Figure 3.16). A similar observation was also made after treatment with E_2 in combination with each of the progestogens though the apparent up-regulation was not statistically significant (Figure 3.16). In sum, LNG, MPA and P_4 +/- E2 similarly regulate AREG at both RNA and protein level in the Ishikawa cell line after 48 hours of treatment.

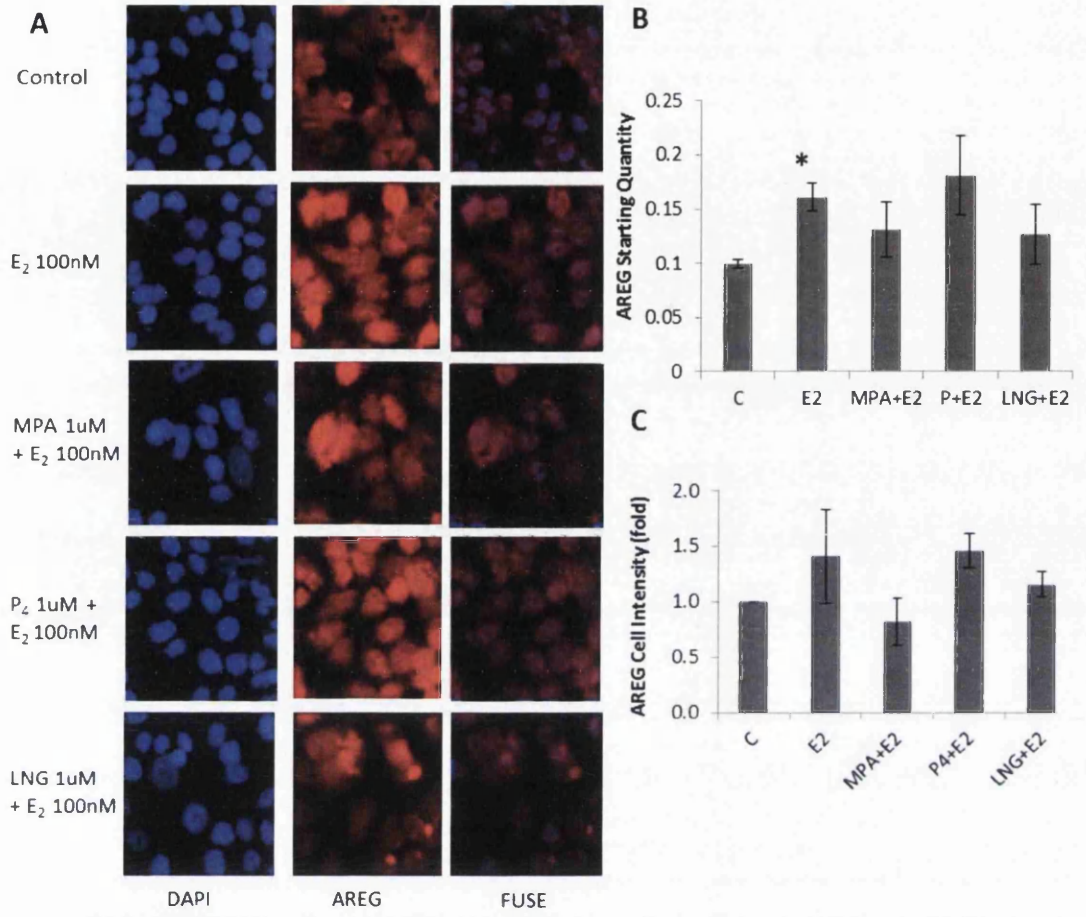


Figure 3.16: AREG gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. MPA, P_4 and LNG treatment concentrations were 1µM whereas E_2 treatment concentration was 100nM A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained AREG protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of AREG normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.4.4 Lactoferrin is significantly down-regulated in the Ishikawa cell line after treatment with P_4, MPA and LNG.

LF is expressed in the human and mouse endometrium at varying levels throughout the menstrual cycle (Masson et al., 1968, Newbold et al., 1992). During the mouse estrous cycle levels of LF increase during the estrogen dominated phase and decrease during the progesterone dominated phase suggesting its down-regulation by progesterone in this species (Newbold et al., 1992, McMaster et al., 1992). Moreover, in both human epithelial and human stromal cells LF is suppressed by progesterone and this occurs specifically through the PRB (Tibbetts et al., 1998, Kurita et al., 2000, Conneely and Jericevic, 2002). On the other hand, LF is up-regulated by estrogen (Zhang and Teng, 2000, Kelver et al., 1996) and is not altered through androgen signalling (Yu and Chen, 1993) therefore the synthetic progestins cannot adversely regulate LF through binding the AR.

In line with the literature, data from Ishikawa cells showed that LF was significantly down-regulated after treatment with each of the progestogens at the RNA level (Figure 3.17 B). However, the extent of LF inhibition differed with the different progestogen treatments. After treatment with P_4, LF was reduced to less than 0.2 fold of that found in the untreated control, however, after treatment with LNG or MPA, LF was only reduced to approximately 0.6 fold of the control. The difference in LF inhibition between MPA and P_4 treatment was statistically significant. Moreover, the regulation of LF by MPA in the Ishikawa cell line was the opposite of that observed *in vivo*, whereby LF was upregulated in glandular epithelium *in vivo* and down-regulated in this cell line.

The significant LF down-regulation by the progestogens appears to have been translated to protein. However, the extent of LF down-regulation was similar between all the treatments with each treatment similarly causing a decrease of between 0.7 and 0.8 fold, this can be seen in Figure 3.17 C and can be visualised in Figures 3.17 A.

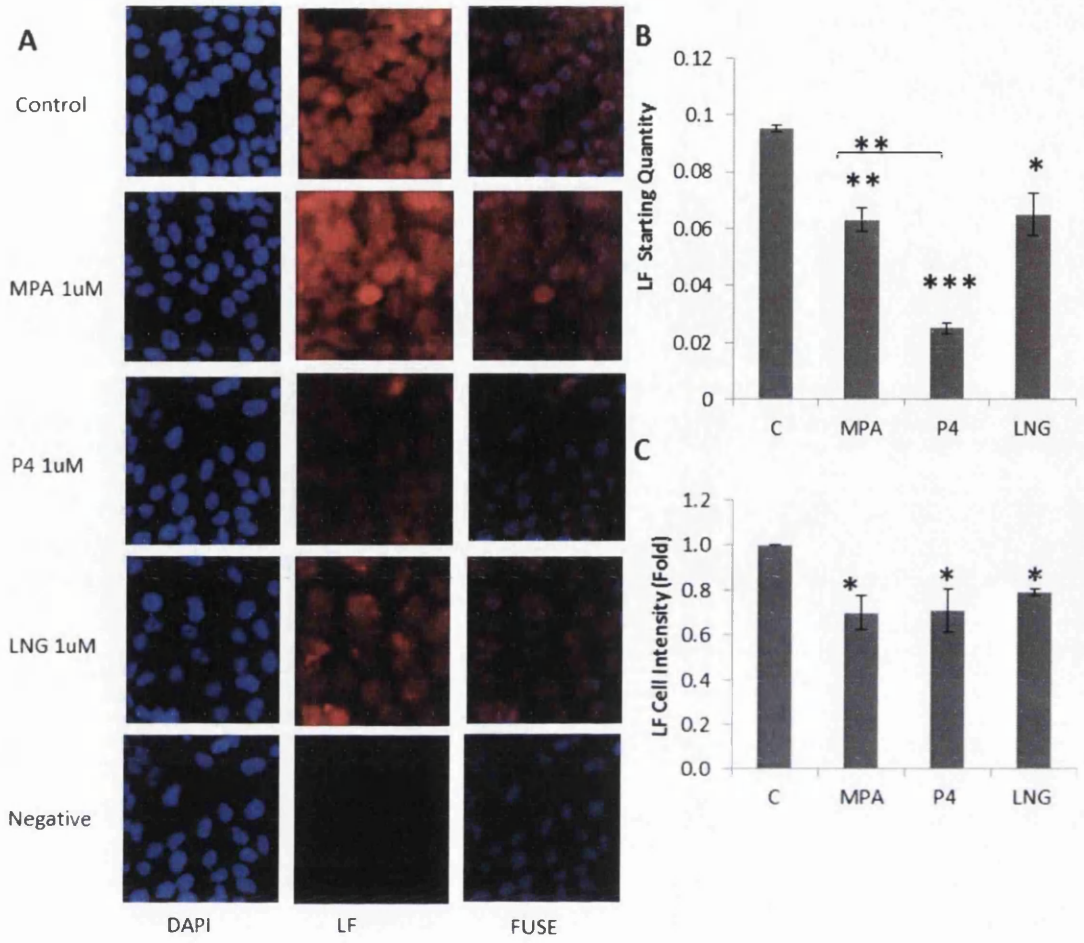


Figure 3.17: LF gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. All treatment concentrations were 1µM. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

The combined treatment of E_2 and P_4 hampered the down-regulation of LF, causing an overall reduction of 0.75 fold (Figure 3.18) compared to the striking 0.2 fold decrease seen with P_4 treatment alone. On the other hand, the addition of E_2 to LNG and MPA treatment caused little change. The combination of E_2 and LNG or MPA caused 0.6 and 0.5 fold decrease in LF RNA respectively; however, the decrease was not significant after LNG treatment. Conversely, MPA plus E_2 treatment caused a significant down-regulation in LF RNA (Figure 3.18B).

When treated with E_2 alone, the expression of LF protein in Ishikawa cells did not change (Figure 3.18C). However the addition of E_2 to LNG and P_4 abolished the significance of the down-regulation although it did not have an influence on the overall fold decrease (Figure 3.18C). On the other hand, LF protein expression remained significantly less than control in Ishikawa cells after treatment with E_2 plus MPA (Figure 3.18C).

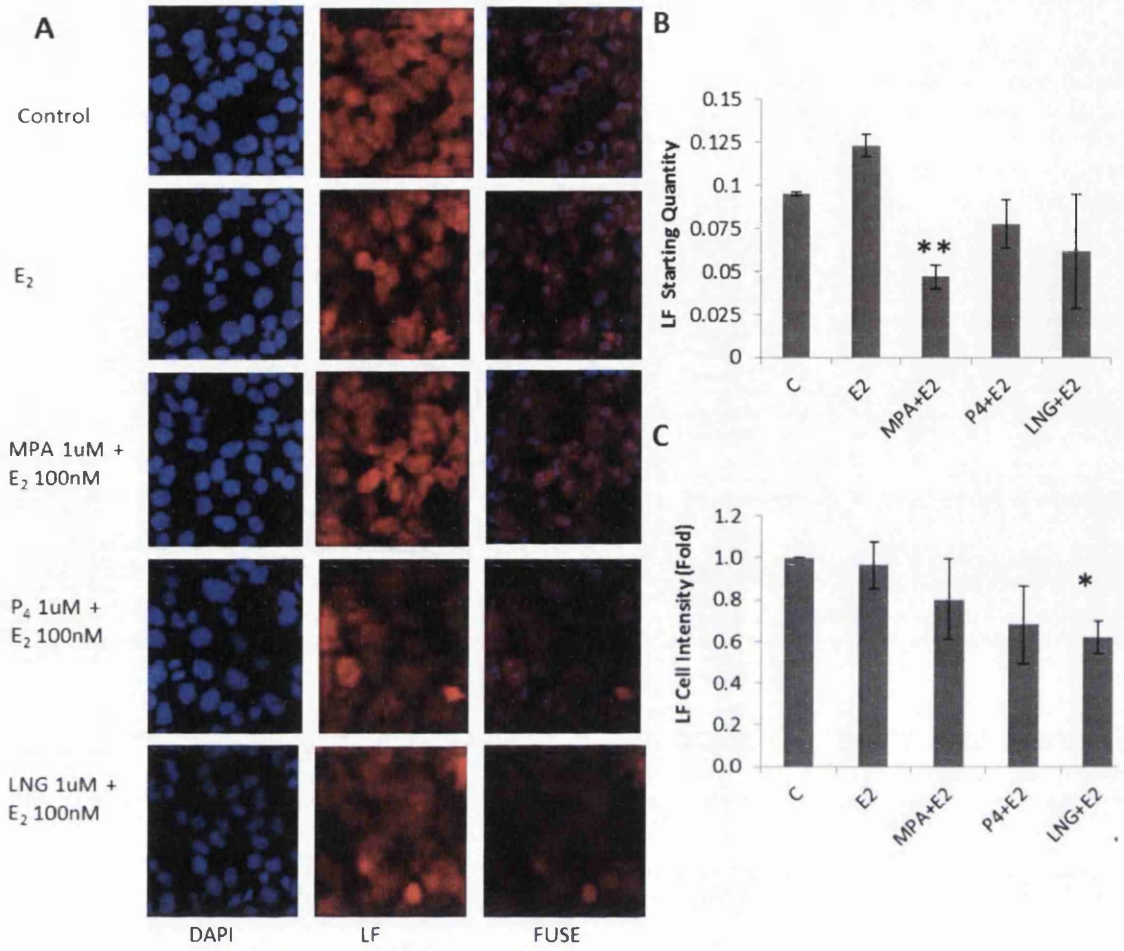


Figure 3.18: LF gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. MPA, P_4 and LNG treatment concentrations were 1μM whereas E_2 treatment concentration was 100nM A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.4.5 GREB1 is induced by progestogenic as well as estrogenic signalling in the Ishikawa cell line.

The next target to be assessed was GREB1. As described in Section 1.7.5, the GREB1 gene is an early response gene in the estrogen receptor-regulated pathway (Pellegrini et al., 2012) and is a direct target of estrogen (Ghosh et al., 2000) GREB1 is also an androgen target and contains a progesterone response element in its promoter (Rae et al., 2006).

Although the presence of a progesterone response element suggests that GREB1 may be influenced by PR binding, the regulation of GREB1 by progesterone has not been reported until now. The present study shows that P_4, LNG and MPA significantly up-regulate GREB1 gene expression in the Ishikawa cell line (Figure 3.19B). Moreover, GREB1 was up-regulated significantly more by P_4 than MPA ($P<0.01$) and the average increase in GREB1 RNA was far greater after treatment with LNG (2.8 fold) compared to P_4 (2.2 fold) and MPA (1.5 fold).

The significant changes seen in GREB1 RNA were not seen at protein level using the InCell analyser (Figure 3.19B). Despite the lack of statistical significance, up-regulation at protein level was still apparent which could potentially be confirmed with further repeats or the use of a more sensitive antibody.

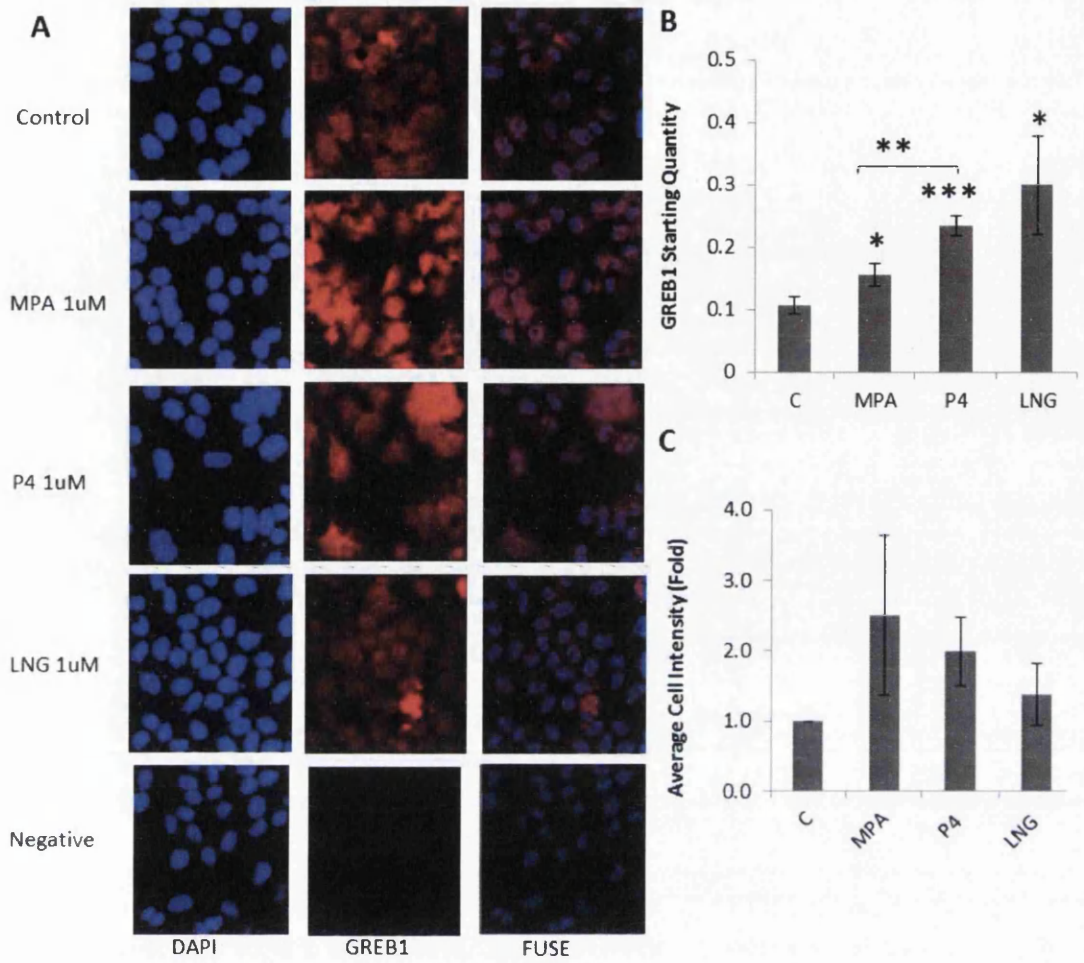


Figure 3.19: GREB1 gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. All treatment concentrations were 1µM. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained GREB1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of GREB1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

It is worth noting that one study by Pellegrini *et al* (2012) investigated GREB1 expression in women suffering with endometriosis and looked at patients taking or not taking contraception in their control group. Although there is limited information available, Pellegrini reported that there is no difference in GREB1 levels in the human endometrium within the control group suggesting that the intake of progestins does not influence GREB1 expression *in vivo* (Pellegrini et al., 2012). This data however, does not elaborate on the type of progestin taken or the duration of use.

GREB1 expression is known to be induced by estrogen in the Ishikawa cell line (Naciff et al., 2009, Gori et al., 2011) and was therefore used as a positive control for the estrogen responsiveness of the Ishikawa cell line. After E_2 treatment, GREB1 RNA was indeed significantly up-regulated. A 3.2-fold induction was observed at RNA level ($P>0.05$) (Figure 3.20B) and 2.8 fold induction observed at protein level ($P>0.01$) (Figure 3.20C). GREB1 protein expression also increased after combined E_2 and progestogen treatment but not significantly (Figure 3.20C).

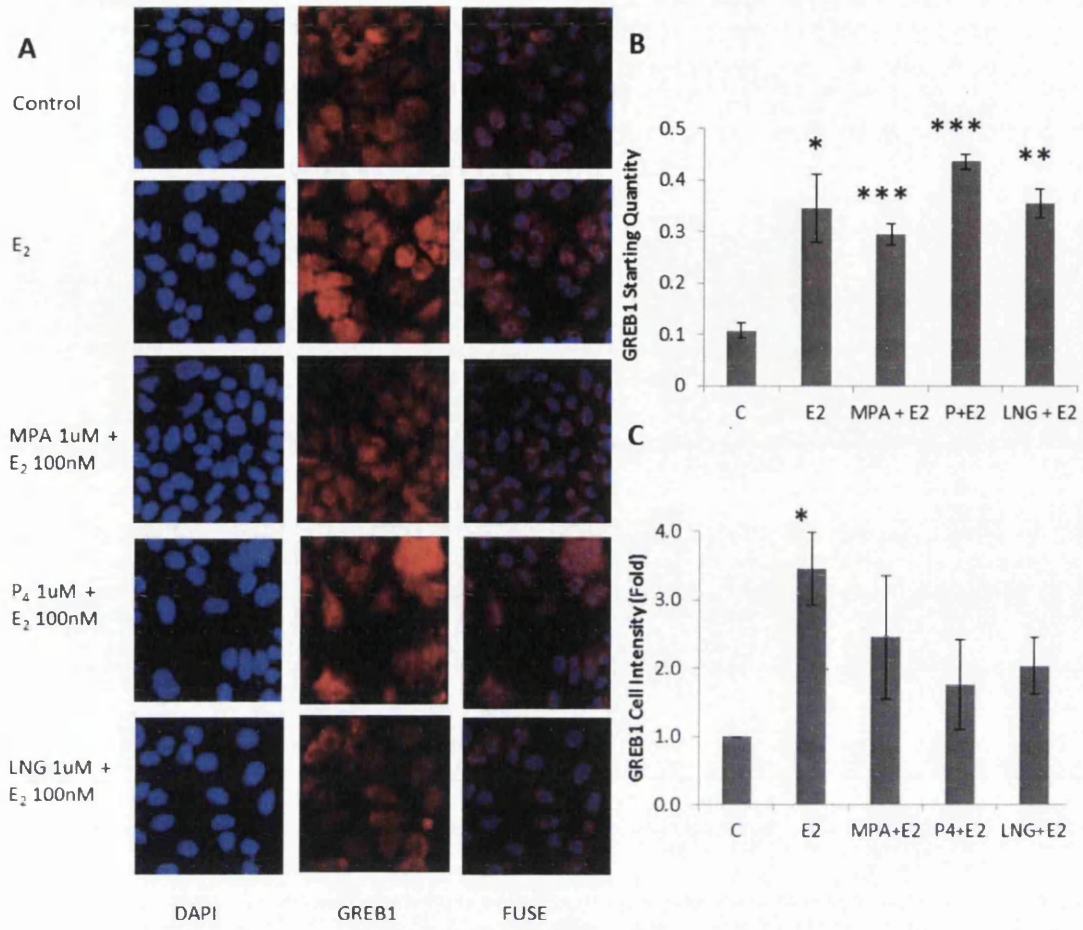


Figure 3.20: GREB1 gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. MPA, P_4 and LNG treatment concentrations were 1µM whereas E_2 treatment concentration was 100nM A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained GREB1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of GREB1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.4.6 IGF1 gene expression is enhanced in the presence of MPA and LNG but not in the presence of P_4.

The final gene to be assessed was insulin-like growth factor 1 (IGF1). IGF1 is not regulated by estrogen or progesterone (Weihua et al., 2002, Ace and Okulicz, 1995) but is enhanced by ligand-activated androgen receptor (Zhu and Kyprianou, 2008). It is thus possible that MPA and LNG are able to regulate this gene through binding the AR. Indeed the results showed that IGF1 was significantly up-regulated by LNG and MPA but not affected by P_4 (Figure 3.21). MPA binds the AR with more than twice the affinity of progesterone and LNG binds with more than 3 times the affinity (Africander et al., 2011) which has inevitably affected the differences seen in the regulation IGF by these hormones. Furthermore there was a significant different between P_4 and both MPA and LNG ($P<0.05$).

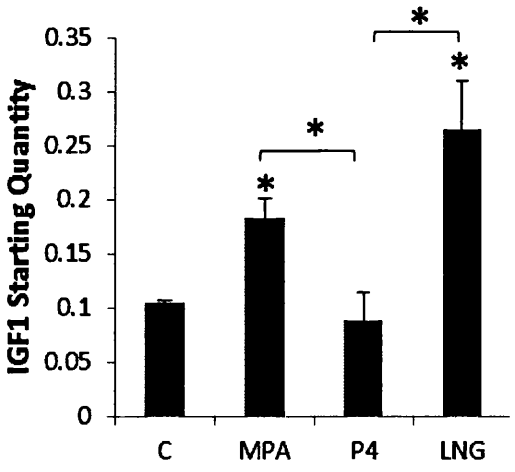


Figure 3.21: Normalised starting quantity of IGF1 RNA in Ishikawa cells after 48h of treatment with 1μm of P4, MPA or LNG. RNA was extracted was extracted from Ishikawa cells using the Quiagen RNeasy kit, the RNA was then converted to cDNA, and amplified using PCR. The average starting quantity of the target mRNA was calculated by obtaining the cycle number at which the gene was first amplified and plotting this number within a standard curve. The results were normalised against reference gene GAPDH. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed statistically

using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All PCR data presented in this section was subjected to the same analysis.

3.3.4.7 Summary of Section 3.3.4

A

Gene	Regulation by Progestogens in Ishikawa		
	MPA	P4	LNG
FOXO1	1.5*	1.5*	1.7*
PR	1	4***	2.3*
AREG	1.4	1	1.7
LF	0.06**	0.02***	0.06*
GREB1	1.5*	2.2***	3*
IGF1	1.8*	0.9	2.5*

B

Protein	Regulation by Progestogens in Ishikawa		
	MPA	P4	LNG
FOXO1	1.5*	1.5**	2*
PR	1	8**	1
AREG	1.4	1	1.4
LF	0.7*	0.7*	0.8*
GREB1	2.5	2	1.5

C

Gene	Regulation by Progestogens in the presence of E2 in Ishikawa cells			
	E2	MPA	P4	LNG
FOXO1	1.70	1.50*	1.60*	1.75
PR	5.50*	6.52	4.68*	2.32*
AREG	1.61*	1.31	1.81	1.27
LF	1.30	0.49**	0.82	0.65
GREB1	5.53*	3.06***	4.52***	3.61**

D

Protein	Regulation by Progestogens in the presence of E2 in Ishikawa Cells			
	E2	MPA	P4	LNG
FOXO1	1.23	1.31*	1.57*	1.45
PR	2.53	2.27*	1.54*	3.47*
AREG	1.41	0.82	1.46	1.16
LF	0.72	0.63	0.68	0.62*
GREB1	1.91**	1.26	1.59	1.77

Tables 3.3 A-D: Tables summarise the regulation of target genes (A) and proteins (B) by progestogen treatment alone and in combination with estradiol (C and D) in Ishikawa cells. Green indicates an increase in protein expression, blue indicates a decrease and pink indicates no change.

The data presented within this study highlighted some differences in gene and protein regulation in Ishikawa cells by P_4, MPA and LNG. To help visualise these differences, tables summarising the effect of each progestogen at gene (Table 3.3A and B) and protein level (Table 3.3C and D) were produced.

The regulation of PR, FOXO1, AREG and LF by MPA was not always in line with *in vivo* data. This may be owed to the fact that Ishikawa cells are cancerous in origin and the molecular pathology of endometrial cancer is different to endometrial hyperplasia, particularly without atypia (Matias-Guiu et al., 2001). This suggests that Ishikawa cells may be a poor model for determining differential gene regulation by natural and synthetic progestins *in vivo* in hyperplasia patients. Nonetheless, the data still shows that synthetic progestins are capable of adversely regulating a number of genes and proteins.

3.3.5 MPA, LNG and P4 differentially regulate target gene and protein expression in human endometrial stromal cells (HESCs).

As with the Ishikawa cell line, the regulation of a number of genes and proteins by P_4, MPA and LNG was assessed in the HESC cell line. As described in Section 2.1.2, the HESC cell line is shown to display similar outcomes to primary, unpassaged, stromal cells after treatment with ovarian hormones. Furthermore, a high expression of PR and AR was determined in the HESC cell line (Figure 3.10). Although the levels of ER were low, this should not limit outcomes as estradiol treatments were not used with the HESC cell line. Therefore, during this section HESCs were used to assess genetic and morphological changes induced by P_4, compared to MPA and LNG.

3.3.5.1 FOXO1 is up-regulated by progestogen combined with cyclic AMP in the Stroma but is not significantly up-regulated by progestogen alone.

Several studies have been carried out to identify the role of FOXO1 in endometrial stroma. Collectively, these studies have shown that FOXO1 is involved in regulating the expression of decidual marker genes and is markedly induced upon decidualisation both *in vivo* and *in vitro* (Christian et al., 2002, Gellersen and

Brosens, 2003, Labied et al., 2006, Gellersen et al., 2007). *FOXO1 binds to target genes as part of a complex of transcription factors in response to cyclic AMP and progesterone dependant signalling* (Gellersen et al., 2007, Christian et al., 2002). *Prolactin is a* prominent marker of decidualisation and is an example of a FOXO1 target gene regulated in this way (Christian et al., 2002, Kim et al., 2003). The addition of cyclic AMP to cultured human endometrial stromal cells has been shown to cause an up-regulation of FOXO1 RNA and protein which is enhanced by the addition of a progestogen. The added progestogen not only enhances the expression of FOXO1 in differentiating stromal cells but simultaneously induces cytoplasmic retention and inactivation of the FOXO1 protein and withdrawal of progestin causes rapid nuclear re-accumulation (Labied et al., 2006). Interestingly treatment with progestin alone does not appear to enhance FOXO1 expression in stromal cells (Labied et al., 2006). In addition to this, androgens have been shown down-regulate FOXO1 in prostate (Huang et al., 2004) which may suggest that MPA and LNG can influence FOXO1 to some extent through binding to the androgen receptor.

As previously shown by Labied *et al* (2006), treatment with progestin or progesterone alone did not induce FOXO1 RNA expression in HESC cells (Figure 3.22B). This was also the case at protein level, though interestingly the level of FOXO1 protein did increase after treatment with MPA and although this was not significant, a similar pattern can be seen at RNA level (Figure 3.22C) which may suggest MPA is having some effect on the FOXO1 protein unlike the other progestogens. In line with this observation, a significant increase in cytosolic FOXO1 in the endometrial stroma of hyperplasia patients was observed after MPA treatment (Figure 3.5).

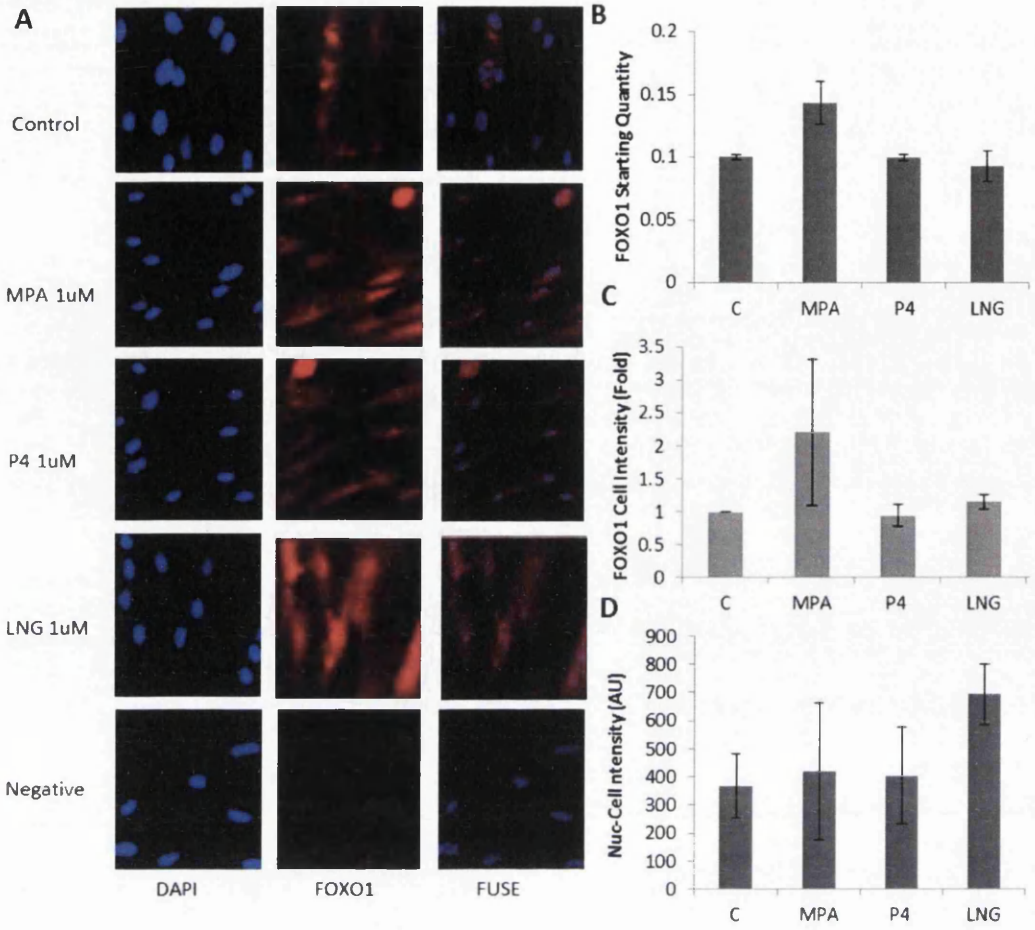


Figure 3.22: FOXO1 gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of hormone. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

The addition of cyclic AMP to MPA, P_4 or LNG caused a marked induction of the FOXO1 gene (Figure 3.23B). Cyclic AMP alone caused a 6 fold increase in FOXO1 RNA which was enhanced to 11.4 fold when combined with MPA. The combined treatment of cyclic AMP with P_4 or LNG also caused a significant up-regulation of the FOXO1 gene (6 and 4.9 fold respectively) however the increase was at a similar level to that seen with cyclic AMP alone making it is difficult to certify the influence of these hormones on FOXO1 induction. Again in line with RNA data, treatment of HESC cells with cyclic AMP caused a marked increase in the level of FOXO1 protein. Treatment with cyclic AMP alone caused a significant induction of FOXO1 protein ($P<0.01$), and this was again enhanced by the addition of MPA and LNG. However, the combination of cyclic AMP and P_4 did not cause a greater increase than with cyclic AMP alone. Although the fold increase did not change with the addition of P_4, its addition clearly had a similar effect on the protein to the other progestins (Figure 3.22B and C). Figure 3.23A shows FOXO1 protein (red) in Ishikawa cells, after treatment with cyclic AMP alone it appears that the level of protein is greater in the nucleus compared to the rest of the cell. Figures 3.23A also shows Ishikawa cells after treatment with cyclic AMP plus P_4, MPA and LNG. The level of protein in these images not only appears to increase but also appears homogenous throughout the cell rather than localised to the nucleus. To confirm this observation the whole cell intensity was subtracted from the nuclear intensity and difference was plotted (Figure 3.23D). There was indeed a significantly larger difference between nuclear and cytosolic FOXO1 in cells treated with cyclic AMP alone compared to control and this difference was decreased by the presence of the progestogens which is in line with findings by Labied (2006).

Based on the images shown in Figure 3.23A, it is tempting to speculate that HESC cells become rounder after cyclic AMP plus progestogen treatment due to the process of decidualisation. However, it was not possible to quantify cell roundness in the absence of a membrane stain using this technique, nonetheless, this observation was confirmed under light microscope in Section 3.3.6.

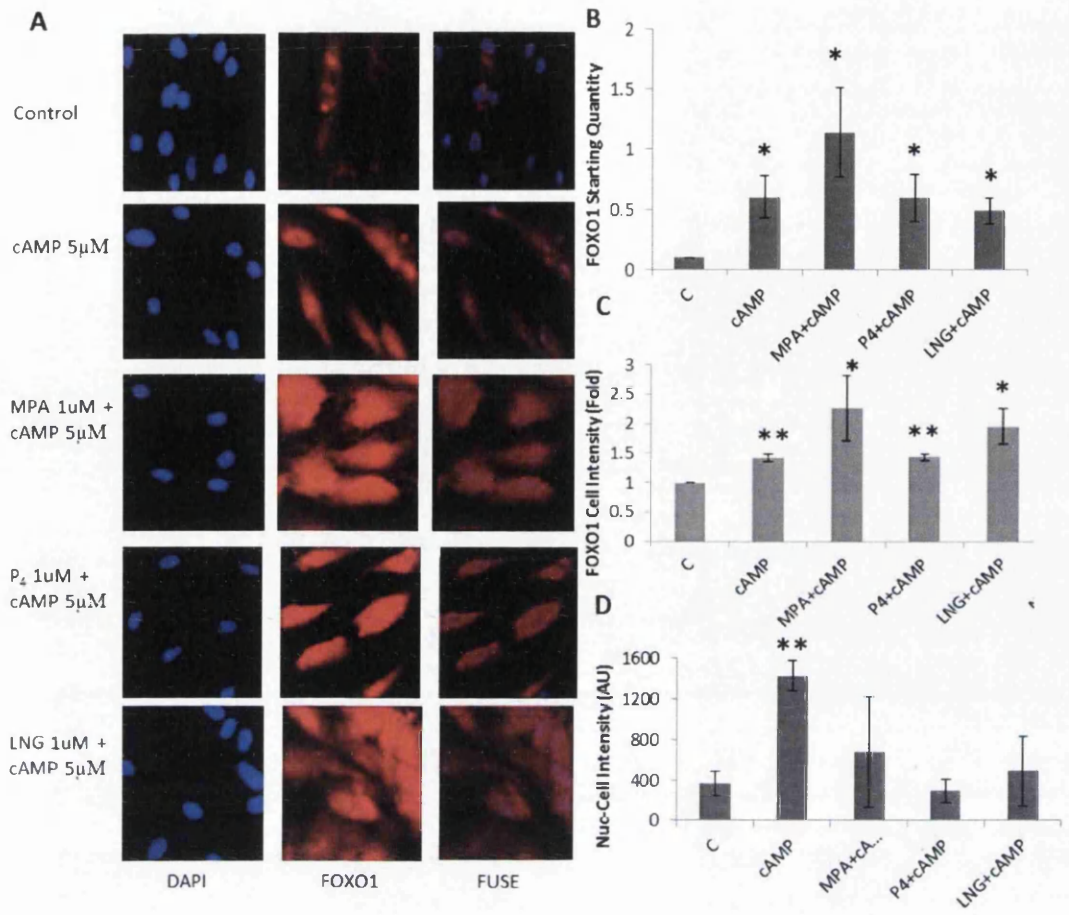


Figure 3.23: FOXO1 gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of progestin and/or 5μM cyclic AMP. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

In summary there was no statistically significant difference in the regulation of FOXO1 by the progestogens in HESCs whereby FOXO1 was not altered by progestogen alone and was significantly induced by progestin combined with cyclic AMP.

3.3.5.2 PR is upregulated after progestogen treatment in Human endometrial stromal cells.

In the literature progestogens have been shown to down-regulate the PR in endometrial eptihelium (Hanifi-Moghaddam et al., 2006, Critchley et al., 1998, Vereide et al., 2006). However, an increase in PR expression between 2 and 10 fold has been reported in MPA and P_4-treated stromal cells (Tseng and Zhu, 1997). Data obtained within this study confirmed that PR RNA is significantly induced by P_4 by more than 1.5 fold ($P>0.05$) after 48hours of incubation (Figure 3.24B), however this was not seen with the protein analysis (Figure 3.24C). MPA treatment also had no effect on PR RNA or protein levels in HESC cells (Figure 3.24B and C). Furthermore, data obtained within this study from patients taking MPA *in vivo* is in concurrence with these observations, showing no change in stromal PR expression after MPA treatment (Figure 3.6). The PR induction by MPA found in the 1998 study by Critchley took at least 2-3 days therefore a longer incubation time may be necessary to replicate this result (Critchley et al., 1998).

Unlike MPA, LNG caused a small but significant increase in PR RNA in HESC cells after 48 hours (Figure 3.24B) but again this was not significant at protein level (Figure 3.24C). No study has been previously carried out to investigate the effects of LNG on PR in cultured stromal cells, although the use of an LNG inter-uterine device has been shown to cause no change in stromal PR levels (Critchley et al., 1998). Changes seen during *in vivo* work may be affected by paracrine signalling between epithelial and stromal compartments of the endometrium (Kurita et al., 1998) as well as the presence of other hormones.

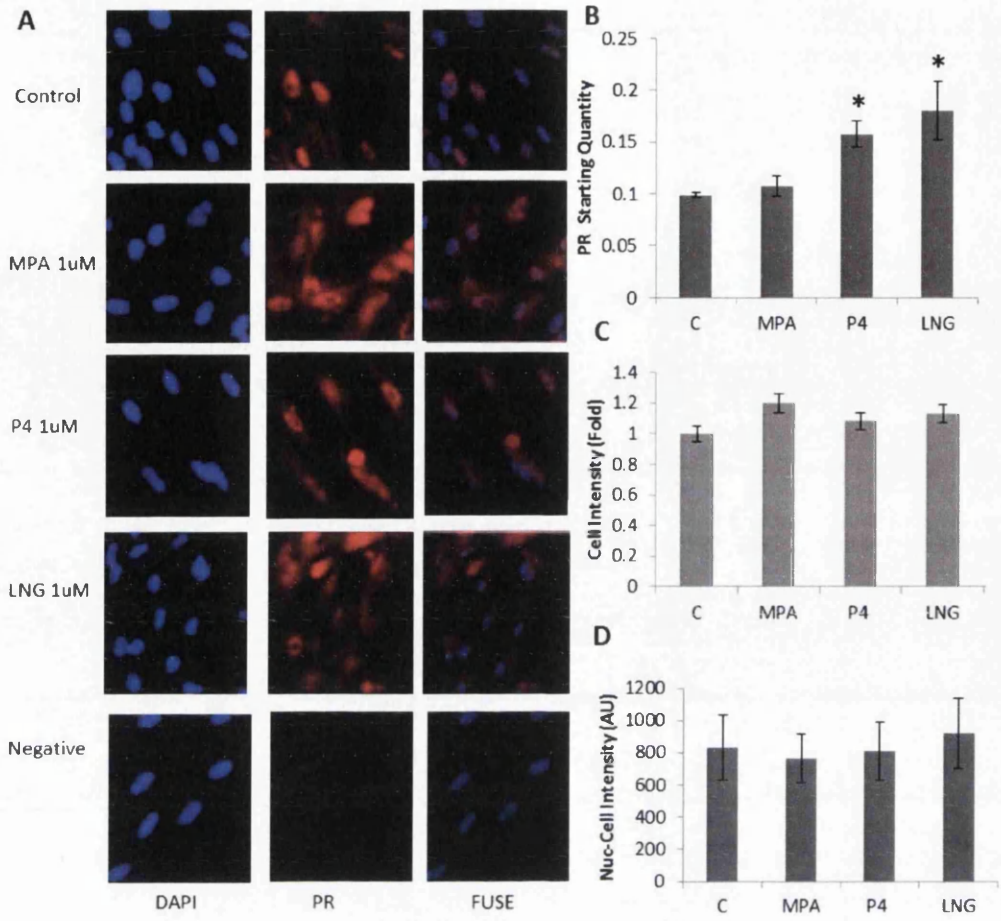


Figure 3.24: PR gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of progestin. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

As well as progesterone, work has been carried out to determine the regulation of PR by cyclic AMP. Cyclic AMP is able to stimulate PR expression without the requirement of a ligand (Rowan et al., 2000, Aronica and Katzenellenbogen, 1991). Although a marginal induction of PR was seen after cyclic AMP treatment alone, the addition of progesterone was required to cause a significant increase of 1.9 fold (Figure 3.25B) at the RNA level. Although the addition of MPA or LNG to cyclic AMP treatment did not lead to significant changes in PR RNA levels, a slight up-regulation was observed which was sufficient to cause changes at protein level whereby all cyclic AMP incubations, both alone and in combination with a progestogen, caused a small but significant increase in PR protein (Figure 3.25C). Synergistic action of ligand-bound PR and cyclic AMP is vital for decidualisation and pregnancy establishment as well as to drive decidualisation in IVF patients with luteal defects. Thus any differential gene regulation by the progestins through this pathway could have major consequences *in vivo* in particular for IVF patients who are sometimes administered with synthetic progestins (Chakravarty et al., 2005, Kuang et al., 2015). However, no differences between progestogen treatments with cyclic AMP were observed in the regulation of PR.

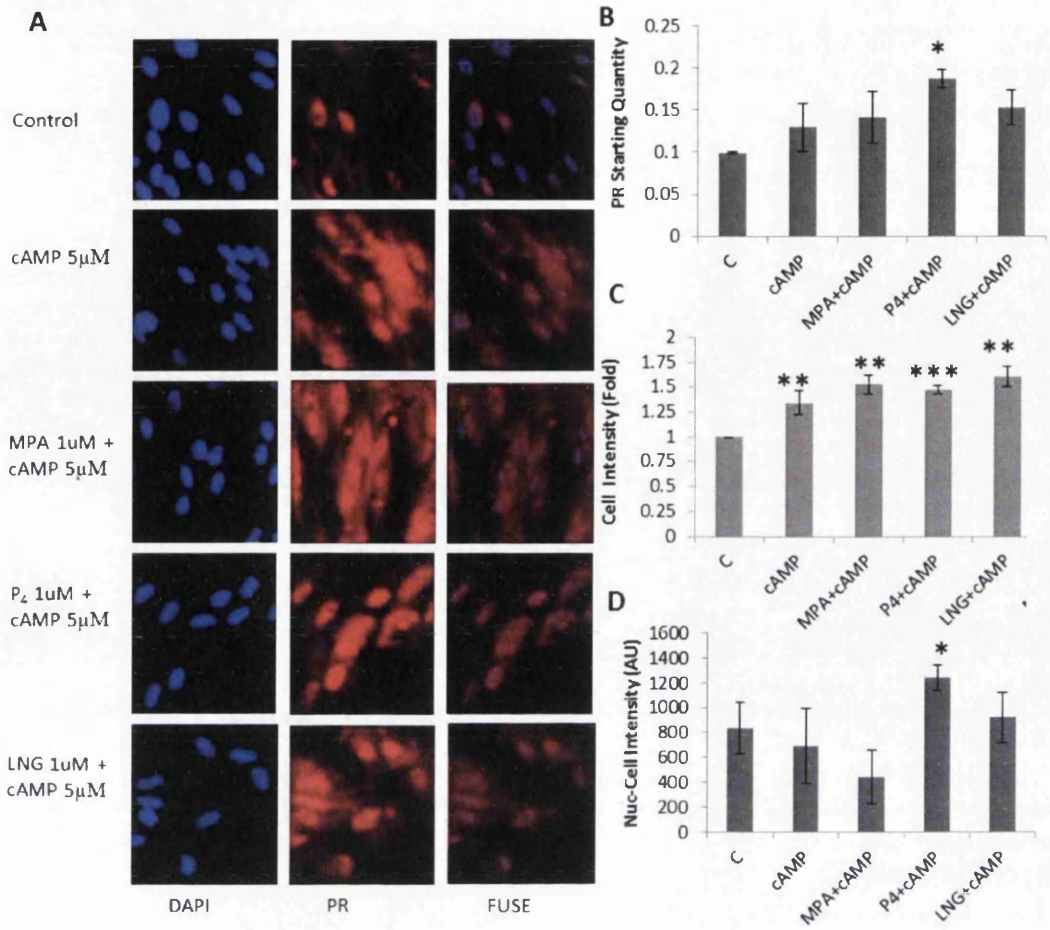


Figure 3.25: PR gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of progestin and/or 5μM cyclic AMP. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.5.3 Lactoferrin is down-regulated by progestogens in HESC cells and this is further amplified by the addition of cyclic AMP.

The amount of data available on the regulation of LF in human endometrial stroma is limited. However, the current literature suggests that LF is regulated in a similar manner as in the endometrial epithelium whereby it is inhibited by progesterone through the PRB in both tissue types (Kurita et al., 2000). The regulation of LF by cyclic AMP has yet to be reported. The data obtained within this study suggests that 1μM of P_4 or LNG sufficiently inhibits LF gene expression (Figure 3.26B) whereas MPA does not alter the expression of LF RNA in HESC cells. Data obtained from the InCell Analyser also showed that the LF is significantly down-regulated by progesterone at the protein level (Figure 3.26C, $P>0.01$). MPA and LNG also caused a slight reduction in LF protein but this was not significant. These results are very similar to those observed in stromal PR in hyperplasia patients (Figure 3.6).

The addition of cyclic AMP to progestogen treatment enhanced the progestin-induced down-regulation of LF RNA and protein causing LF RNA to decrease by more than half with each progestin/cyclic AMP combination (Figure 3.27B). Furthermore, combined MPA/Cyclic AMP treatment caused a significant down-regulation of the LF gene ($P<0.01$) and protein ($P<0.05$) (3.26B and C) whereas MPA treatment alone had no effect (3.26B and C). Cyclic AMP alone did not alter LF gene expression implicating synergistic action between cyclic AMP and the progestogens (Figure 3.27).

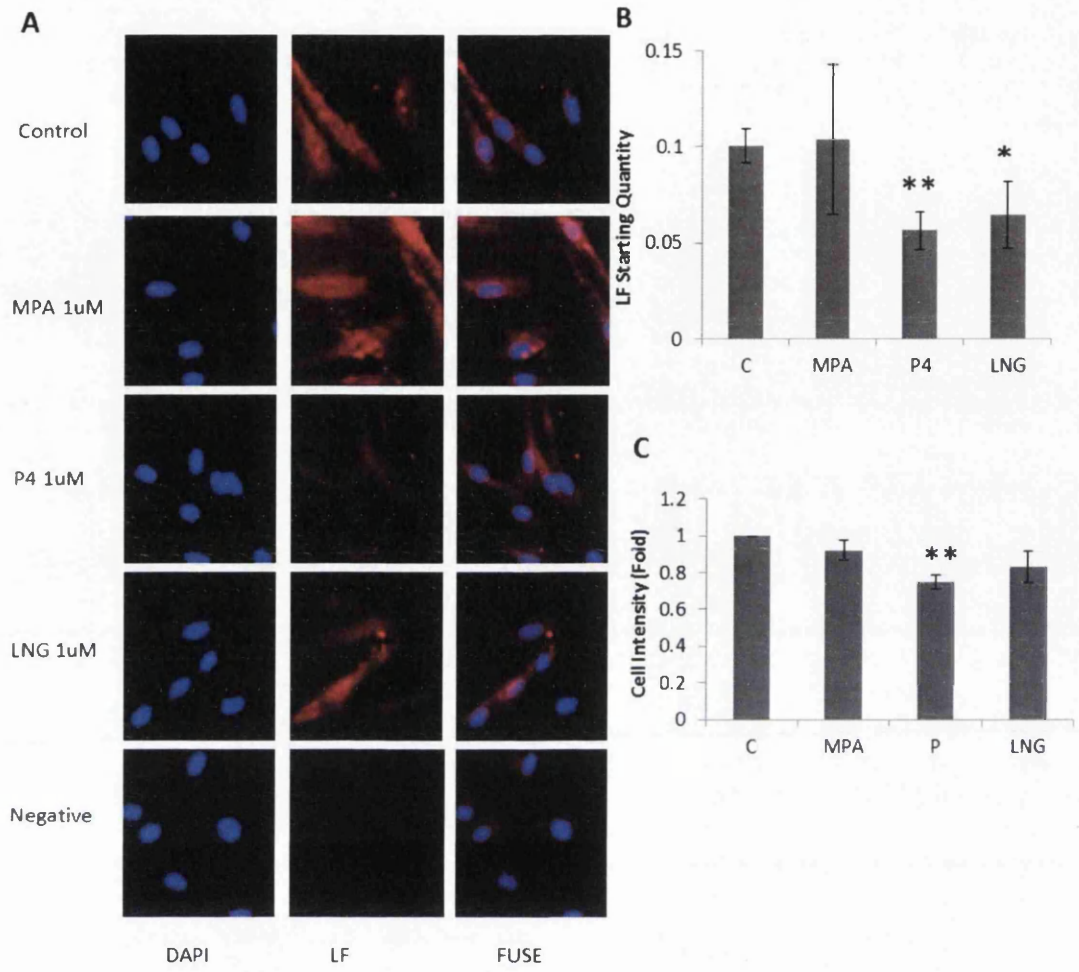


Figure 3.26: LF gene and protein expression in the HESC cell line following hormone treatment for 48h with 1µM of progestin. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

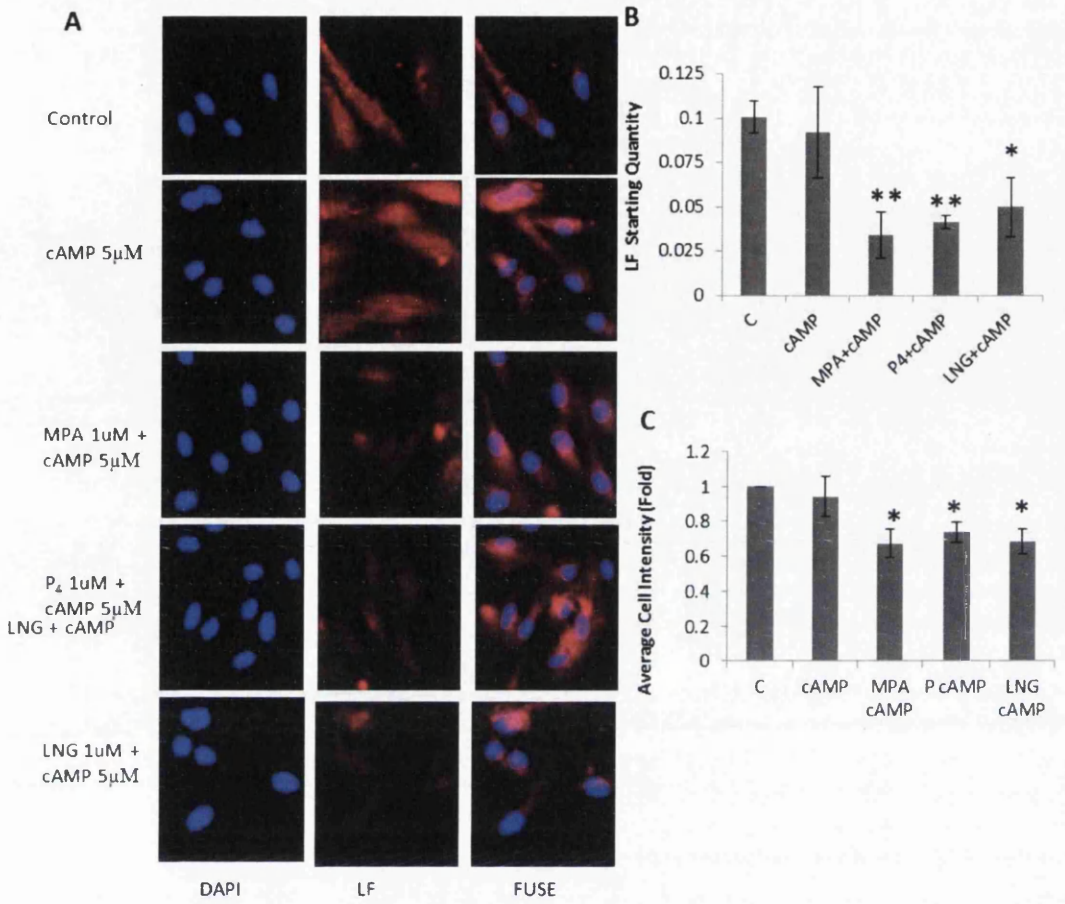


Figure 3.27: LF gene and protein expression in the HESC cell line following hormone treatment for 48h with 1µM of progestin and/or 5µM cyclic AMP. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.5.4 GREB1 is not regulated by P_4, MPA or LNG alone but is significantly up-regulated by cyclic AMP both alone and in combination with the progestogens.

GREB1, a gene of unknown function, has been shown to be up-regulated by estrogen in breast and endometrium (Pellegrini et al., 2012, Ghosh et al., 2000) and was thus used as a positive control for estrogen regulation earlier in this study. During this investigation it was determined that, as well as estrogen, progesterone is also able to significantly up-regulate GREB1 gene expression in endometrial epithelium. There is currently no data on the effects of progesterone on the GREB1 gene in endometrial stromal cells, however due to the substantial changes seen in the Ishikawa cell line after progestin treatment it was decided to assess changes in GREB1 RNA and protein after progestogen treatment in endometrial stromal cells.

However, after 48 hours of treatment with P_4, MPA or LNG no changes were observed in GREB1 RNA or protein between control and treated samples in HESC cells (See Figure 3.28 for graphical data. For images see Appendix L).

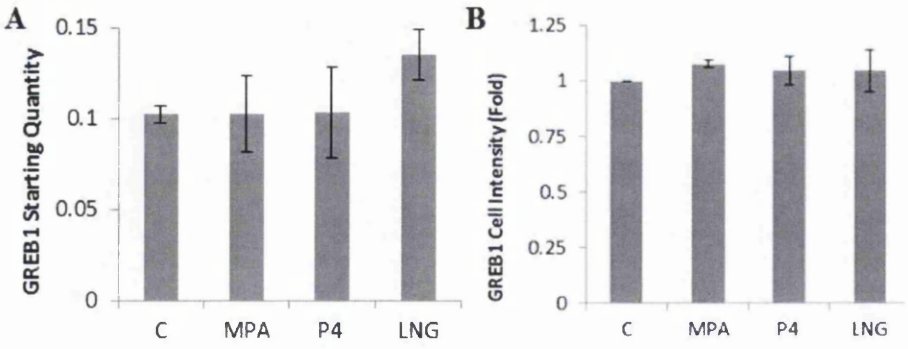


Figure 3.28: GREB1 gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of progestin. A) RT-PCR data showing basal transcript level of GREB1 normalised to GAPDH. B) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. InCell Analyser Data is normalised to background based on negative control. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05,

****P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.**

When treatments were combined with cyclic AMP, however, there was a substantial increase in GREB1 gene expression. Cyclic AMP alone induced a more than 5 fold increase in GREB1 RNA. The observed increase was also visible after treatment with cyclic AMP combined with MPA, P_4 and LNG but only significant with P_4. (Figure 3.29B). Interestingly, the fold induction of GREB1 RNA was higher in HESC cells after treatment with cyclic AMP alone compared to the combined treatment of cyclic AMP and the progestogens suggesting progestogens may be hampering cyclic AMP in terms of this target. After cyclic AMP treatment, GREB1 protein was significantly up-regulated by 1.5 fold (P>0.01), a similar up-regulation could be observed after treatment with cyclic AMP plus each of the progestogens whereby they all caused a significant increase of between 1.4 and 1.5 fold (P>0.05) (Figure 3.25C). The changes seen in GREB1 RNA and protein after cyclic AMP treatment in endometrial cells suggest a potential role for GREB1 in processes such as stromal decidualisation. However, as there was no significant differences between cyclic AMP treatment alone and in combination with the progestins, it is possible that the observed changes were as a result of cyclic AMP alone. Based on the data obtained within this study, the role of GREB1 in the endometrium certainly warrants more investigation.

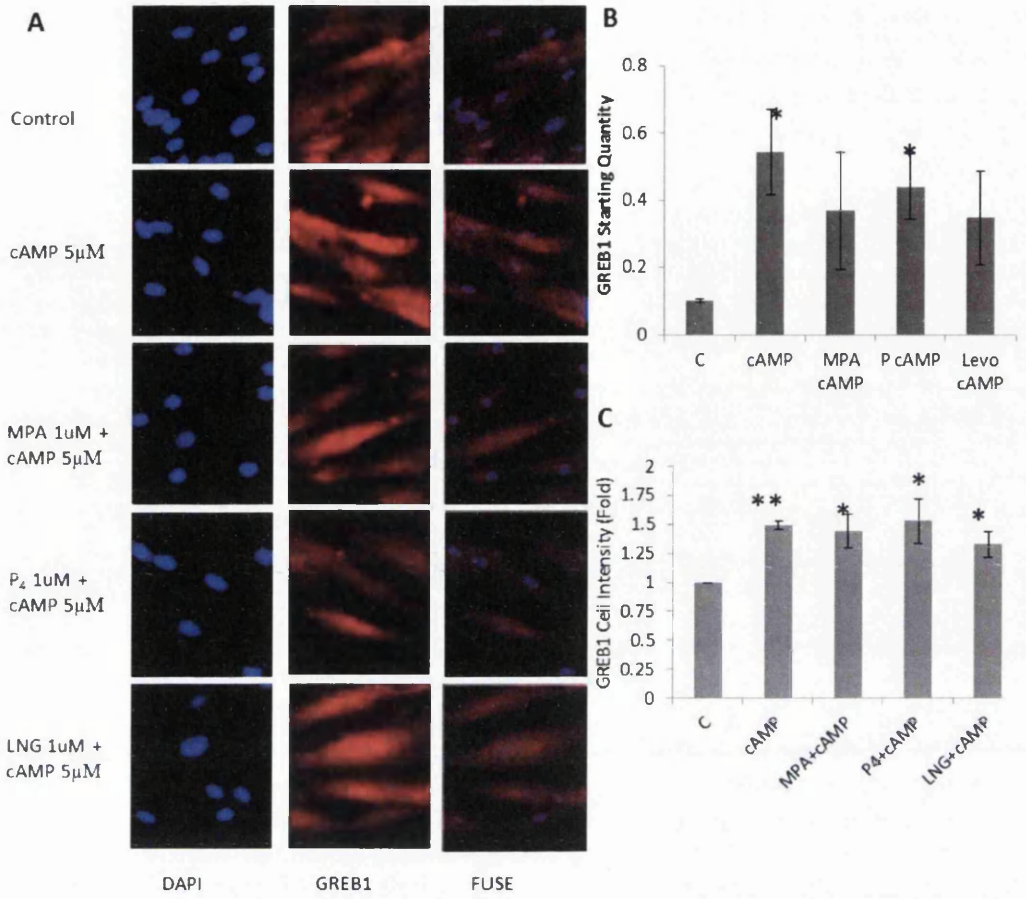


Figure 3.29: GREB1 gene and protein expression in the HESC cell line following hormone treatment for 48h with 1μM of progestin and/or 5μM cyclic AMP. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained GREB1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of GREB1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

3.3.5.6 Prolactin is induced by LNG, MPA and P_4 when in combination with cyclic AMP and is also induced by LNG alone.

Prolactin (PRL) is a multifaceted gene regulated through the PR in differentiating human endometrial stromal cells. Progestogens alone are very weak inducers of the PRL gene, yet, the role of progestogens in PRL regulation is important as they synergistically enhance cAMP-induced PRL expression (Gellersen et al., 1994, Telgmann et al., 1997, Brosens et al., 1996, Brar et al., 1997, Tang and Gurpide, 1993). In line with the literature, after incubating HESC cells with P_4 or MPA for 48 hours, no significant change in PRL gene expression was observed (Figure 3.26). After incubation with LNG on the other hand, there was a significant, 3.8 fold, up-regulation of the PRL gene which was also significantly different to its regulation by P_4 ($P=0.011$). The regulation of PRL by LNG in endometrial stromal cells has not been reported previously.

After incubating HESCs with P_4, MPA or LNG combined with cyclic AMP, there was an obvious induction in PRL gene expression. Cyclic AMP alone induced an caused PRL starting quantity to rise from 0.01 to >200,000, this rose further to >1350,000 with the addition of MPA. Treatment with cyclic AMP and LNG or P_4 both increased PRL starting quantity to more than 100,000, which was less than the increase seen with cyclic AMP alone (Figure 3.30). The difference in PRL starting quantity after treatment with MPA plus cyclic AMP and P_4 plus cyclic AMP was also significant, highlighting some potential differences in the effects of this synthetic progestin on genes involved in stromal differentiation.

It is worth noting that the likely reason for such substantial increases is due to the low levels of PRL found at basal levels in HESCs, thus a 10 cycle increase can lead to vast numbers when calculating starting quantity.

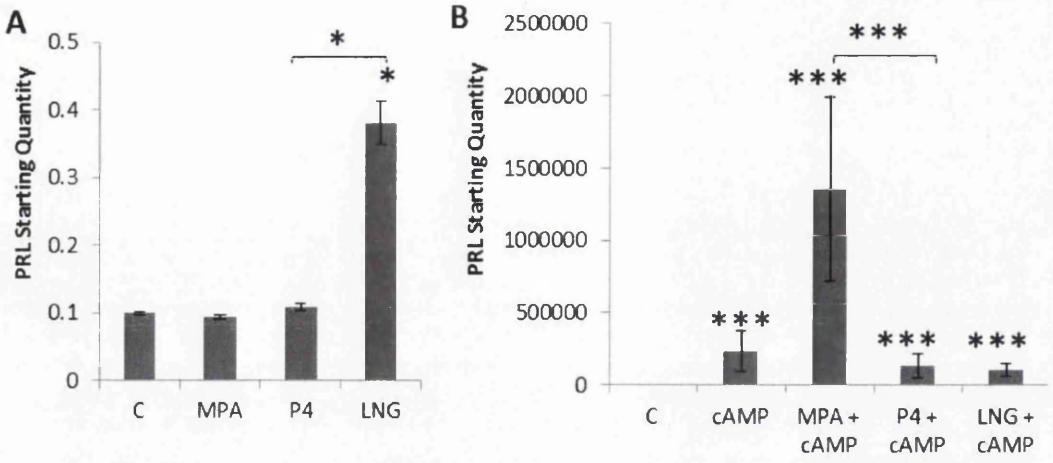


Figure 3.30: The effects of P4, MPA and LNG on PRL gene expression in HESC cells in the absence (A) and presence (B) of cyclic AMP

The transcription of the PRL gene leads to the synthesis of the PRL protein which is secreted into surrounding fluids (Rong Huang et al., 1987). The amount of secreted prolactin in the culture media surrounding HESC cells during treatment was therefore assessed. After 48 hours of incubation with P_4 the levels of secreted PRL remained unchanged from HESC cells. However, in the presence of MPA and LNG, PRL secretion was up-regulated. Despite no change in PRL gene expression in HESCs after MPA treatment, MPA significantly increased the secreted PRL protein by 4.2 fold ($P>0.05$). In line with the RNA data, LNG increased PRL protein by more than 20 fold, though this was not significant (Figure 3.31).

After incubating HESCs with P_4, MPA or LNG combined with cyclic AMP, there was an obvious induction in secreted PRL protein which correlated well with data obtained at RNA level. Cyclic AMP alone induced PRL protein 37 fold, this rose to 73 fold with the addition of MPA. As with RNA, treatment with cyclic AMP combined with P_4 or LNG increased PRL gene expression but by the same or less than the increase seen with cyclic AMP alone (Figure 3.31).

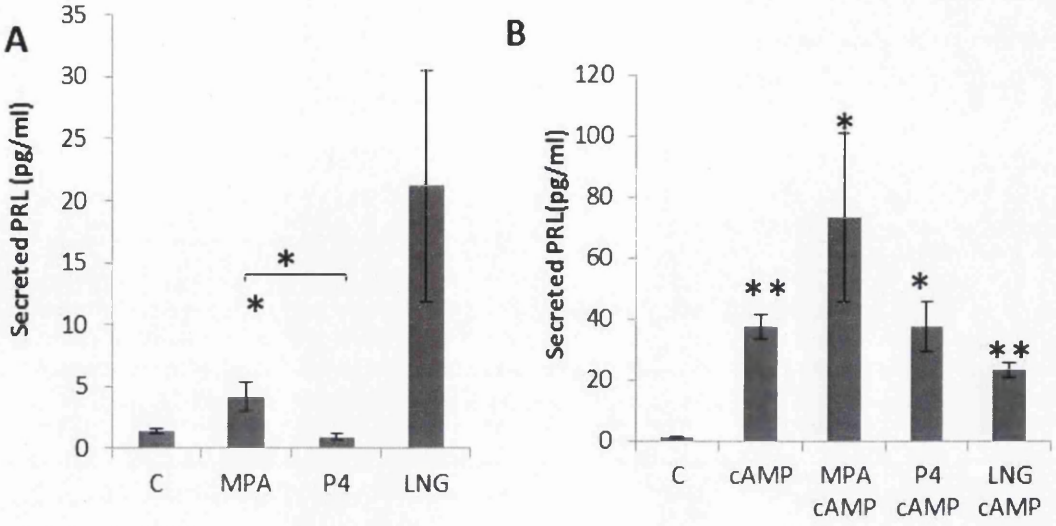


Figure 3.31: Secreted PRL levels in response to MPA, P4 and LNG in the presence (A) or absence (B) of cyclic AMP.

The ELISA technique was conducted to measure hormone-stimulated levels of secreted PRL in HESCs. Values are given as average and standard deviation. Student's t-test was used to test significance. Significant changes are represented by * (*, P≤0.05 **, P≤0.01).

The differences observed in the regulation of the PRL gene and protein by P_4 and MPA may be due to indirect signalling by MPA or potentially due to the difference in affinity for the PR by these hormones. Such differences have not been researched previously but may have profound effects on stromal cell phenotype *in vivo* as well as within this study.

3.3.5.7 The HESC cell line decidualises after treatment with cyclic AMP and combined treatment of cyclic AMP and each of the progestogens but not with the progestogens alone.

The increase in PRL expression is indicative of stromal decidualisation, therefore the extent of decidualisation was visualised using light microscopy and further confirmed by calculating the level of cellular roundness using Image J, where a value of 1 represents a perfect circle and this value decreases with a reduction in circularity.

HESCs were imaged after 48 hours of incubation with P_4, LNG and MPA in the presence or absence of cyclic AMP or with cyclic AMP alone. Figure 3.32A shows untreated HESCs as a control, in this image cells appear to be elongated and flowing. ImageJ analysis gave a value of 0.61 for cell roundness in the control cells (Figure 3.32B). The addition of P_4, MPA and LNG to the cells resulted in a similar appearance of cells to untreated control (Figure 3.32A). Analysis of circularity in these samples further confirmed no change in roundness (Figure 3.32B). The addition of cyclic AMP to cells, however, caused a marked change in cellular appearance which was further enhanced by the addition of a progestogen (Figure 3.32A). The changes observed visually were further confirmed by ImageJ analysis for roundness. Cellular roundness after cyclic AMP treatment increased from 0.61 (control) to 0.69($P>0.05$) and was further increased to 0.71($P>0.01$), 0.72($P>0.05$) and 0.73($P>0.05$) when cyclic AMP was combined with MPA, P_4 and LNG respectively (3.32D).

Despite the vast differences seen in the levels of PRL gene and protein after treatment with different progestogens, the changes observed in cell shape were similar between all 3. Although no obvious difference in cell shape was observed the marked changes in PRL expression is likely to cause changes in the expression of other genes.

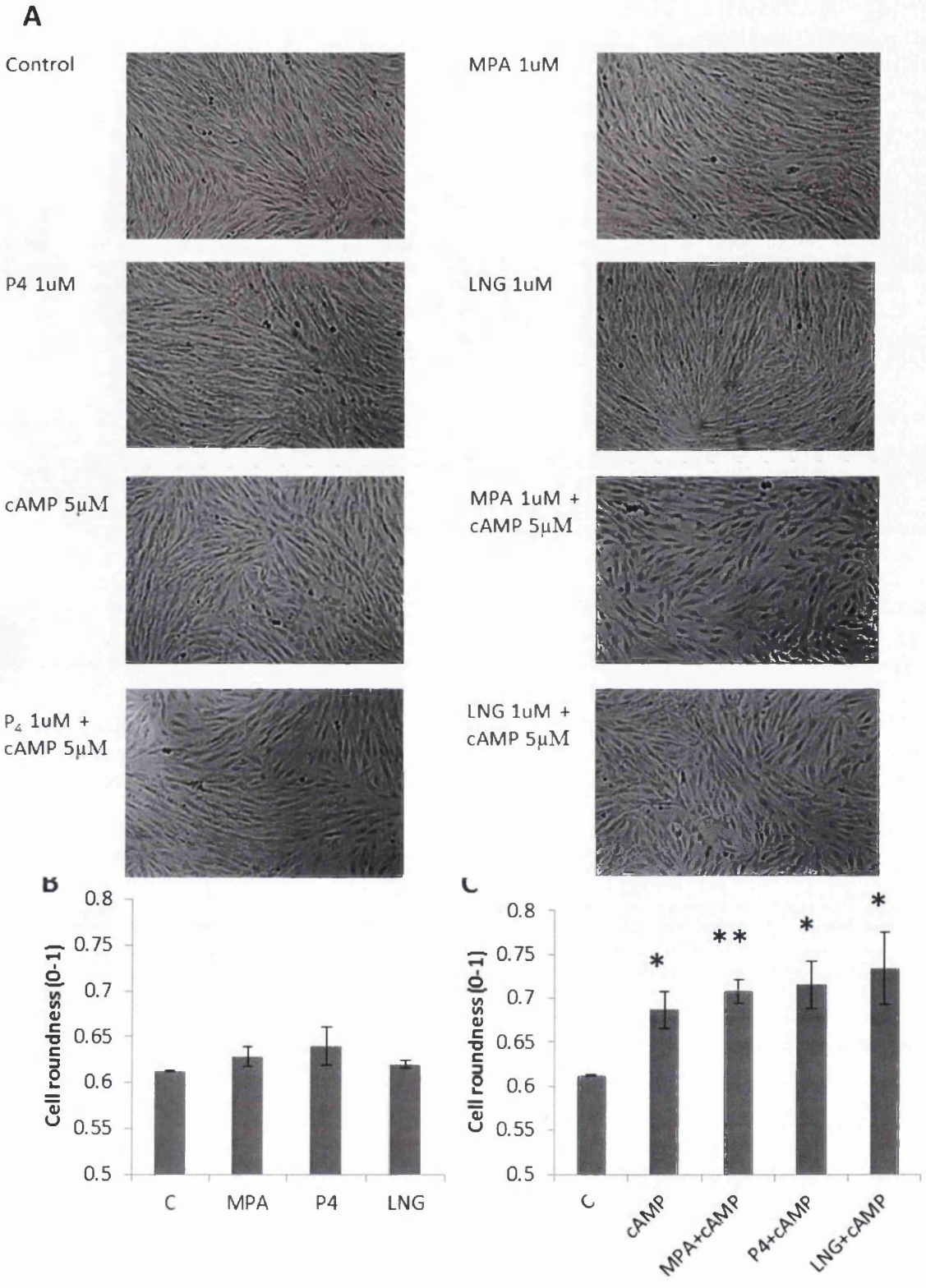


Figure 3.32: The effects of P_4, MPA and LNG on HESC cell roundness. Cell morphology can be visualised in images shown in panel A. Roundness was quantified using imageJ in the absence (C) and presence (D) of cAMP.

3.3.5.8 Summary of Section 3.3.5

Within this section the regulation of a subset of target genes and proteins by natural progesterone and the two synthetic progestins was analysed within the HESC cell line. In order to more clearly view this regulation four tables were made summarising the data (Tables 3.4A-D).

Furthermore, the data obtained in the HESC cell line was in line with findings *in vivo* in Section 3.3.1. Endometrial hyperplasia without atypia is largely self-limited with little apparent relationship to carcinoma (Horn et al., 2007), suggesting its regulation is more like that found in the normal endometrium. The HESC cell line is representative of primary cells of the healthy endometrium (Krikun et al., 2004) so it is not surprising that similar patterns in protein regulation were obtained between HESC cells and patient biopsies. What is of particular interest is that several of these proteins were differentially regulated by P_4 and LNG in the HESC cell line compared to MPA, this suggests that differential protein regulation may also occur *in vivo*.

A

Gene	Regulation by Progestogensin HESC		
	MPA	P4	LNG
FOXO1	1.4	1.08	1.08
PR	0.81	1.5*	1.7*
LF	1.03	0.56**	0.64*
GREB1	1.00	1.01	1.3
PRL	0.94	1.09	3.83

B

Protein	Regulation by Progestogens in HESC		
	MPA	P4	LNG
FOXO1	2.20	0.94	1.15
PR	1.19	1.08	1.13
LF	0.92	0.75**	0.83
GREB1	1.08	1.05	1.04
PRL	4.18*	0.95	21.18

C

Gene	Regulation by Progestogens in the presence of cAMP			
	cAMP	MPA	P4	LNG
FOXO1	6.02*	11.36*	5.96*	4.89*
PR	1.31	1.44	1.89*	1.55
LF	0.92	0.34**	0.42**	0.5*
GREB1	5.3*	3.60	4.3*	3.39
PRL	2.4e6***	1.4e7***	1.4e6***	1.1e6***

D

Protein	Regulation by Progestogens in the presence of cAMP			
	cAMP	MPA	P4	LNG
FOXO1	1.41**	2.26*	1.44**	1.95*
PR	1.35**	1.53**	1.47***	1.61**
LF	0.94	0.67*	0.74*	0.69*
GREB1	1.49**	1.44*	1.53*	1.33*
PRL	37.71**	73.42*	37.72*	23.36**

Tables 3.4 A-D: Tables summarise the regulation of target genes (A) and proteins (B) by progestogen treatment alone and in combination with estradiol (C and D) in HESC cells. Green indicates an increase in protein expression, blue indicates a decrease and pink indicates no change.

3.4 Comparison of the regulation of target proteins by MPA in vivo and in vitro

As several differences were visually observed between *in vivo* and *in vitro* data, a formal analysis was used to test for significant differences between these data sets. A student's t-test was carried out between patient's pre and post MPA treatment and control and MPA treated cells. In order to clearly visualise differences/similarities between the two data sets, all data was converted to fold and a direct comparison was made. A side-by-side comparison of fold data can be seen in Table 3.5.

Protein	**Fold increase compared to untreated patient (*In* Vivo) or control sample (*In Vitro*)**					
	In Vivo	Ishikawa	P Value	*In Vitro*	HESC	P Value
FOXO1	1.34	1.23	0.3	1.26	2.20	0.28
PR	0.83	1.01	0.58	0.65	1.14	**0.03***
LF	1.13	0.06	**0.01****	0.97	0.93	0.65
AREG	1.03	1.47	**0.04***	-	-	-

Table 3.5: The regulation of target proteins by MPA *in vivo* and *in vitro*. Green indicates an increase in protein expression, blue indicates a decrease and pink indicates no change. Differences between *in vitro/in* vivo data were tested by Student's T-test and P values are given (*, P≤0.05 **, P≤0.01).

In some instances the regulation of target proteins by MPA is similar *in vitro* as *in vivo*. However, there were significant differences between 3 targets. Most notably is the LF protein was up-regulated *in vivo* and down-regulated in the Ishikawa cell line (Table 3.5). The data suggests that the cell lines may be poor representatives of the *in vivo* situation suggesting hyperplasia cell lines would be preferable when making such a comparison. Even though the cell lines may not be representative of endometrial hyperplasia, they clearly show adverse regulation between the progestins and natural progesterone that requires addressing. Moreover, in instances where data

is similar (e.g LF in HESC) the *in vitro* data shows a significant difference between MPA and P_4 which may indeed be occurring in the patient receiving therapy.

3.5 Discussion

The first line of treatment for non-atypical endometrial hyperplasia is synthetic progestin therapy with either MPA or LNG (Armstrong et al., 2012). However, a number of side-effects have been attributed to synthetic progestin use due to adverse gene and protein regulation by these compounds (Bafaloukos et al., 1999, Thigpen et al., 1999). Thus, the main aim of this chapter was to determine the regulation of a subset of target genes and proteins by MPA and LNG differed from that of P_4. To determine this we identified a number of PR target genes and evaluated their expression *in vivo* in samples from endometrial hyperplasia patients to assess whether they were adversely expressed in this condition. Following this, endometrial biopsy samples were taken from patients pre and post progestin treatment. It was not possible to obtain samples from patients treated with LNG or P_4, especially as P_4 is not currently prescribed for this condition. Thus, samples were taken from patients receiving MPA treatment only. In order to compare gene and protein regulation by P_4, MPA and LNG, several targets were also assessed in endometrial cell lines and this data was compared with *in vivo* MPA data.

IHC was used to assess four protein targets in the patient samples; these were FOXO1, PR, AREG and LF. The level of each of these proteins was found to be significantly altered in hyperplasia patients compared to control. Moreover, the expression of these targets significantly changed in at least one compartment of the endometrium when patients were subject to MPA regime.

Although Ishikawa cells are a good model of human endometrial epithelium they may not be fully representative of what happens *in vivo* in hyperplasia patients. Previous research has suggested that PR is a major mediator of stromal and epithelial crosstalk and that progesterone effects on uterine epithelium may also be mediated through stromal PR (Kurita et al., 1998). Since a mono-cell culture model was not used, cell-cell communications or interactions that can mediate progesterone action in endometrium would not have been detected. Nonetheless, the detailed analysis

carried out within this study was able to reveal subtle yet important differences in target gene and protein regulation by the progestogens.

The regulation of LF was particularly interesting whereby MPA up-regulated LF protein expression in patient samples and caused no change in its expression in Ishikawa cells, whereas LNG and P_4 both caused a significant down-regulation of the LF protein. This data is not the first to suggest that MPA has opposite effects to natural progesterone. When investigating the effect of progestins in the pathogenesis of endometriosis, MPA was shown to represses the expression of the chemokine named RANTES in endometrial stroma (Zhao et al., 2002), whereas natural progesterone has been reported to elevate the expression of the RANTES gene (Ramhorst et al., 2006). Another example of this is with intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 where both MPA and LNG have been shown to increase these molecules but natural progesterone is not known to stimulate them (Tatsumi et al., 2002).

As well as LF, the regulation of the PR was also differentially altered by P_4 and the synthetic progestins. P_4 was seen to markedly increase PR protein compared to MPA and LNG (both $P<0.01$), whereas MPA was seen to down-regulate the PR *in vivo.* The PR is a transcription factor for a number of genes and can act in a ligand dependent and independent manner (Leonhardt et al., 2003a, Weigel and Zhang, 1998) therefore even slight alterations in its regulation could have major consequences *in vivo.* Furthermore, for the treatment of diseases such as endometrial hyperplasia, synthetic progestins serve to antagonise estrogen-driven growth but these benefits are short lived as the progestins serve to down-regulate their own receptor, counteracting the initial effect (Satyaswaroop et al., 1992). Thus, the data obtained from this cell line suggests natural progesterone may cause an increase in PR in certain cell types. *In vivo* this would serve to prolong treatment effectiveness and ultimately improve patient outcome. Although these observations need validation they certainly warrant further research.

Another gene differentially regulated by the progestogens in the Ishikawa cell line was IGF1. IGF1, which is an androgen-regulated gene but not a target of

progesterone (Weihua et al., 2002, Ace and Okulicz, 1995), was upregulated by LNG and MPA but not by P_4. This differential regulation is likely due to the ability of MPA and LNG to bind the AR and activate androgen target genes. P_4, which is unable to bind the AR, had no effect on this gene. As PR and LF are not androgen targets it is possible that adverse signalling through other receptors or differences in binding affinity were the main cause for the dissimilarities in their regulation.

Even though the Ishikawa cell line is not wholly representative of what happens *in vivo*, the differential regulation of target protein by the progestogens certainly highlights the importance of adverse gene regulation by the synthetic progestins.

As endometrial hyperplasia is often associated with over-exposure to E_2, target gene regulation was also determined in the presence of this hormone. Indeed, the addition of estradiol to progestogen treatment was shown to influence target regulation in Ishikawa cells; for example the down-regulation of LF was reduced when estradiol was included in the treatments. Despite this, there were no significant differences recorded between progesterone and the progestins in this particular set of target genes after combined treatment with E_2. However, studies have shown that estrogen can have a strong influence on a number of factors when added to progestogens *in vivo* such as decreasing glucose tolerance (Wynn et al., 1979) and elevating high-density lipoprotein (Bradley et al., 1978, Wynn and Niththyananthan, 1982). Therefore, even though no significant differences were seen in this study, the potential role of E_2 in affecting progestogen gene regulation should not be overlooked.

Overall, the results obtained in the Ishikawa cell line showed that P_4, MPA and LNG regulate selected targets in a similar manner; however, in a few instances the data highlighted differential target regulation by the three progestogens which could potentially have profound consequences in an *in vivo* situation.

Unlike the Ishikawa cell line, the regulation of proteins by MPA the HESC cell line was found to be similar to *in vivo* patient samples in the stromal compartment. This suggests HESCs may be a good model for predicting *in vivo* protein regulation. However, all synthetic progestins have been shown to bind PR with different binding

affinities, binding affinity also differs depending on tissue type or cell-line highlighting the need for caution when interpreting these results (Africander et al., 2011). Nonetheless, target regulation by MPA was determined *in vivo* in endometrial stroma and *in vitro* in HESC cells and the regulation in HESCs was also compared to LNG and P_4. This was also carried out in the presence of cyclic AMP.

In both patient biopsies and HESC cells, cytosolic FOXO1 protein was found to be up-regulated by MPA alone, however, in HESC cells, LNG or P_4 alone did not alter FOXO1 expression. Previous research, as well as data from this study, shows that cyclic AMP plus progestin causes cytoplasmic retention and inactivation of FOXO1 and progestin withdrawal causes nuclear re-accumulation and activation of FOXO1 (Labied et al., 2006). However, progestogen alone is not known to alter FOXO1 expression (Labied et al., 2006). FOXO1 is a key transcription factor with pro-apoptotic activity. Therefore, if MPA alone causes FOXO1 up-regulation in cytoplasm, even in the absence of cyclic AMP, it may influence the apoptotic state of stromal cells. Apoptosis in a proportion of decidualised stromal cells, is a cardinal feature of impending menstruation (Dahmoun et al., 1999, Kokawa et al., 1996) and evasion of apoptosis is linked with cancer (Hanahan and Weinberg, 2011) thus showing the importance of FOXO1 regulation in these cells. Despite some clear trends being observed in the regulation of FOXO1 *in vitro* and *in vivo*, the differences seen in FOXO1 regulation were not significant between the progestins.

Akin to FOXO1, P_4, MPA and LNG did not differentially regulate PR, LF or GREB1 RNA or protein in HESC cells. The extent of cellular decidualisation was also similar with the three treatments both alone and in combination with cyclic AMP. Conversely, PRL was differentially regulated by the progestogens whereby the PRL protein was up-regulated by MPA and LNG but not altered by P_4 (Figure 3.26, 3.27). The addition of cyclic AMP to the HESC cell line also caused significant changes in PRL gene expression. When combined with MPA, cyclic AMP caused a significantly higher PRL gene-induction than when combined with P_4 (Figure 3.26, 3.27). Cross-talk between different progestogens and second messengers has not been investigated but previous research has reported that PRs found in the plasma membrane (mPRs) can modulate cyclic AMP levels in response to progesterone (Zhu et al., 2008, Sen

and Hammes, 2010). Although research into synthetic progestin interaction with mPR has not been undertaken, such differences could certainly attribute to adverse gene and protein regulation such as that seen here.

There are also several other reasons that progesterone targets in the endometrium may be differentially regulated by the progestins. Firstly, MPA and LNG have a higher affinity for binding the PR than P_4 which could lead to changes in rate and extent of up or down-regulation of target genes causing differences in gene concentration to be seen at 48 hours of treatment. Secondly, differences in binding affinities for the different PRs may have influenced gene and protein regulation. Humans express two ligand-regulated PR isoforms; these are PR-A and PR-B. The two PR isoforms have different physiological functions owing to the extra activation factor on the N-terminus of the PR-B. Indeed, Schoonen *et al* showed that the sytntetic progestins, LNG and gestodene, displayed different binding affinities for PR-A and PR-B (Schoonen et al., 1998) Considering this; it may be possible that the different PR isoforms mediate different physiological responses to natural progesterone compared to other PR agonists, such as MPA and LNG. In the Ishikawa cell line the level of PR-A was shown to be much lower than PR-B which may explain why the expression levels of PR-B regulated proteins (LF, FOXO1) were more inducible than the PR-A regulated protein (AREG). However, an up-regulation in AREG was seen after MPA treatment both *in vitro* (non-significant) and *in vivo* (significant) suggesting that MPA may be a stronger transactivator of the PRA. This is especially important as PR-A is a known suppressor of PR-B (Vegeto et al., 1993).

Alternatively, the obvious regulation of FOXO1 compared to AREG may be as a result of the PR mechanism of action. Ligand-bound PR directly activates promoters containing PREs, such as those found in FOXO1 (Kyo et al., 2011). FOXO1 was similarly up-regulated *in vitro* with each of the progestogens and *in vivo* with MPA. This may indicate that progestins similarly control targets that are regulated through direct signalling mechanisms. Indeed in the breast cancer cell line, T47D, a number of progestins have been shown to regulate direct PR targets with a highly similar mechanism of action (Bray et al., 2005). However, PR ligands can also activate genes by tethering to other transcription factors to regulate promoters lacking PREs, such as

that of AREG, whereby the PR binds DNA via proteins such as SP1(Cheng et al., 2006) and tethers SRC3 in order regulate transcription (Han et al., 2006). Moreover, different PR isoforms are able to recruit different transcription factors (Giangrande et al., 2000) and if the progestins have different affinities for these isoforms it is certainly possible gene regulation could be altered in this way.

Furthermore, the regulation of progesterone target genes may occur in a time and dose dependent manner (Yie et al., 2006), therefore these factors combined with progestin binding affinities may have further influenced the differences seen in target regulation.

Overall, data from this study and others show the adverse effects of progestin treatments (Wynn et al., 1979, Wynn and Niththyananthan, 1982, Ottoson et al., 1984, Gruber and Huber, 2003, Lockwood et al., 2000, Murphy et al., 1986, Ellmann et al., 2009). Adverse signalling and gene regulation by progestins is of great importance. Not only can it cause minor side effects (Whitelaw et al., 1966, Hickey and d'Arcangues, 2002), but it may also affect levels of genes and proteins that can cause more serious problems such as the adverse regulation of oncogenes. Indeed progestin treatment has been linked to increased incidence of breast and endometrial cancer (Fournier et al., 2005, Stanford et al., 1995, Seeger et al., 2003, Kelley et al., 1976, Kaaks et al., 2002).

Despite the reasons behind the differential regulation of these targets not being fully understood, it is clear that some differences are present. Although, in general, the clinical profile of synthetic progestins is accepted by the user, the known and unknown risks involved in their administration are cause for concern. Thus, natural progesterone is considered to be a better option in hormone therapies, particularly in the case of endometrial hyperplasia where serious side-effects caused by synthetic progestins have been described (Bafaloukos et al., 1999, Thigpen et al., 1999).

Moreover, the use of a vaginally delivered natural progesterone (micronized) cream to treat endometrial hyperplasia has been previously explored with complete regression in over 90% of patients and minimal side effects (Affinito et al., 1994, Baker et al., 2012). Furthermore, there has been a flurry of interest in the use of

orally delivered natural, micronised, progesterone for endometrial hyperplasia treatment in recent years. Micronised progesterone has been shown to be effective given in 14 or 21 day regimens at 400mg per day causing decreased proliferative activity and activation of apoptosis in the hyperplastic endometrium (Chernukha et al., 2013). Micronised progesterone has also been shown to increase the regression rate of both simple and complex hyperplasia (Marra et al., 2014, Stanosz et al., 2014). However, research suggests that synthetic progestins have better endometrial control (Tasci et al., 2014) which may be due to wide inter and intra patient variability associated with micronized progesterone (Levine and Watson, 2000b).

Effectively overcoming the poor bioavailability of progesterone has hindered pharmaceutical companies in producing a main-stream drug containing the natural product for the treatment of endometrial hyperplasia and other diseases. The poor bioavailability of progesterone can be largely attributed to its first pass metabolism. Thus, the following chapter will investigate the reasons behind the poor bioavailability of orally administered progesterone in order to probe the pharmacokinetics leading to the poor oral bioavailability of this compound.

3.6 Summary

Altogether, the data obtained within this study has highlighted key differences in the regulation of target genes and proteins by MPA, LNG and P_4 in endometrial cell lines. The regulation of proteins by MPA in the HESC cell line also matched the regulation of proteins *in vivo* suggesting that the adverse regulation of these targets is also occurring in endometrial hyperplasia patients. This highlights the need for patients to receive a natural progesterone formulation.

The data obtained in this chapter also highlighted a number of progesterone targets as potential marker for the presence and progression of endometrial hyperplasia.

Chapter 4

Site Dependent Metabolism of Progesterone within the Gastro-Intestinal Tract

4. Site Dependent Metabolism of Progesterone within the Gastro-Intestinal Tract

4.1 Introduction

The use of a natural progesterone for the treatment of endometrial hyperplasia has long been desired but has never been achieved, this is largely due to its low aqueous solubility (8.81µg/ml) (Yalkowsky et al., 2010) and its susceptibility to first pass metabolism followed by rapid clearance (Yamazaki and Shimada, 1997, Sitruk-Ware et al., 1987). Thus, synthetic progestins have become the choice hormones for the treatment of hyperplasia, yet they often lead to adverse side effects including headache, dizziness and nausea (Berenson and Wiemann, 1993). Furthermore research shows that the use of natural progesterone has fewer side effects than the synthetic progestins, it does not cause changes in the levels of important lipids, such as cholesterol, and it also prevents osteoporosis (Sitruk-Ware et al., 1987, Murray, 1998, Rosano et al., 2000).

A large body of evidence has shown that the liver is responsible for a substantial proportion of progesterone first pass metabolism (Penning et al., 2000, Williams et al., 2004, Yamazaki and Shimada, 1997, Szklarz and Halpert, 1997a, Swart et al., 1993a, Nebert and Russell, 2002), however, there is very little information on progesterone metabolism in the intestine. The only data that appears to be available is in early studies carried out over 30 years ago by Nienstedt *et al (Harri et al., 1969, Nienstedt et al., 1980).* The intestine is likely to be the most significant site of drug biotransformation outside of the liver, which is due to the presence of metabolising enzymes such as Cytochrome P450s (CYPs) and aldo-keto reductases (AKRs) (Thelen and Dressman, 2009, Barski et al., 2008). Suggesting the intestine may play a substantial role in progesterone metabolism after oral delivery.

Furthermore, different regions of the intestine have different metabolising capabilities due to the presence of enzymes within the intestinal mucosa, pancreatic enzymes and enzymes secreted from bacterial microbes. The presence of microbes increases substantially along the GI tract; in the stomach and small intestine the microbial count is 10^2-10^4 cfu/ml, this increases significantly in the lower part of the

small intestine to 10^5-10^7 cfu/ml and increases further in the colon to approximately 10^{11}-10^{12} cfu/ml (Jung and Kim, 2010).

Thus the aim of this study is to obtain an understanding of the first pass metabolism of progesterone following its oral administration; this will be done in order to identify an optimal site for delivery of progesterone where degradation is less significant. There are a number of objectives required in order to achieve this aim.

The first objective is to compare progesterone metabolism between liver and intestine in order to understand how relevant the metabolism by the latter is in decreasing the oral bioavailability of progesterone. This will be assessed in the presence and absence of inhibitors of all AKR isoforms independently and together. Enzyme inhibitors of potential non-NADPH dependent enzymes will also be incubated with liver cytosol to identify the potential enzymes involved in NADPH independent degradation of progesterone.

The second objective is to investigate the metabolic degradation of progesterone by intestinal mucosal enzymes by incubating with whole intestine homogenates and with homogenates of different sections of the intestine of rats. In order to obtain this goal the stability of progesterone in these homogenates will be determined in the presence and absence of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is a cofactor that acts as a source of electrons for redox reactions of several enzymes implicated in progesterone metabolism including CYPs and AKRs (Lemley and Wilson, 2010). Therefore, by incubating progesterone with and without NADPH, it can be determined to what extent progesterone metabolism can be accounted to NADPH dependent enzymes whose concentration throughout the GI tract is well documented (Thelen and Dressman, 2009, Mitschke et al., 2008, Chhabra, 1979).

The final objective is to investigate the stability of progesterone to enzymes present in the lumen of the gastro-intestinal tract. A simulated colonic fluid produced from faeces of healthy adult males will be used to evaluate stability to metabolism by faecal bacteria. As human fluids of the small intestine are difficult to obtain, simulated fluids containing pancreatin will be used as an to assess the stability of progesterone in this region. The currently available information on progesterone

stability to intestinal bacterial metabolism is insufficient and this work will help clarify the true role of gut metabolism in progesterone bioavailability.

Investigation into the metabolism of progesterone throughout the GI tract will not only give novel insight into the complex degradation of this hormone, but it will also highlight the region of the GI tract where progesterone is most stable and least susceptible to enzymatic degradation indicating an optimum site for oral delivery.

4.2 Materials and Methods

Materials and methods used within this chapter are described in detail in Chapter 2.2. In brief, the stability of progesterone was determined in rat and human liver and intestinal homogenates using a multiwall plate assay with manual sampling at pre-determined time-points. Progesterone concentration was then determined using LC-MS/MS. This methodology will be used as well to determine the stability of progesterone to liver and intestinal cytosol in the presence and absence of specific enzyme inhibitors detailed in Table 2.9. The stability of progesterone to simulated human intestinal and faecal fluid was determined as described above but UV-HPLC was used instead of LC-MS/MS for progesterone concentration quantitation.

4.3 Results and Discussion

4.3.1 Progesterone metabolism by human and rat liver homogenates.

Rat liver homogenates, prepared as described in Methods (Section 2.2.1), were incubated with progesterone for two hours in the presence and absence of NAPDH to determine the in *vitro* stability of progesterone in these conditions (Figure 4.1). Progesterone was shown to be completely degraded below the limit of detection within 10 minutes of incubation in the presence of NADPH with only 8% of parent remaining at 5 minutes. An early study by Wiswel and Samuels (1953) showed that the α,β-unsaturated ketone structure in ring A of progesterone disappears on incubation with rat liver tissue *in vitro.* Rapid A-ring reduction has also been reported as the main route of progesterone metabolism in other studies in the rat liver (Yates et al., 1958, Shirley and Cooke, 1968). These suggest that A-ring reduction

may be the route of rapid parent depletion observed in rat liver homogenates in the presence NADPH observed in this study (Figure 4.1). In the absence of NADPH, the depletion of progesterone is significantly slower than in the presence of NADPH ($P<0.001$) with over 40% of drug still remaining after two hours of incubation (Figure 4.1). Together these data suggest that enzymes of the NADPH pathway play a major role in the degradation of progesterone in the rat liver. Another investigation into the stability of progesterone in common lab animal livers has been carried out previously. Rao and Taylor (1964) described the rate of degradation of radiolabelled progesterone in homogenised liver tissue of the rat, guinea-pig, rabbit and hamster. They observed that progesterone was rapidly degraded in rat, rabbit and hamster livers but at a slower rate in the guinea-pig. The authors also showed that gender can influence the rate of progesterone metabolism in these species. For example, their data showed that that progesterone is diminished to approximately 10% within 10 minutes in the male rat but it is less rapidly diminished in the female (15-20% in 10minutes). The present study only included male rat livers since female rat livers were unavailable, however, the data obtained is in agreement with the previous finding reported by Rao and Taylor.

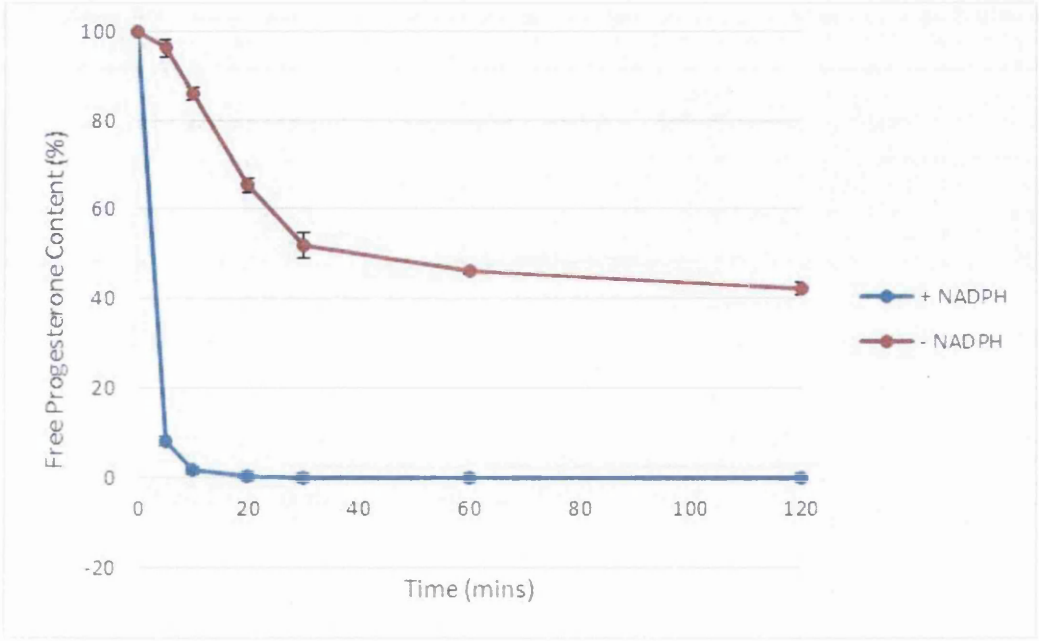


Figure 4.1: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with homogenates of the rat liver in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

Akin to the degradation found in rat liver homogenates, natural progesterone was also completely degraded *in vitro* in the human liver with only 7% of parent compound detectable after 5 minutes of incubation in the presence of NADPH, this reaction was again slowed significantly in the absence of the cofactor ($P>0.001$) (Figure 4.2). In fact, the rate of metabolism was found to be significantly reduced in the absence of NADPH at each timepoint ($P<0.001$). In the absence of NADPH the initial rate of depletion was slow with >87% of parent compound detectable at 20minutes, the rate of metabolism then increased rapidly with over 30% of the drug being metabolised between 20 and 30minutes, but as described for the rat liver, 40% of progesterone remained after the 2h incubation period.

It has been previously reported that a major route of NADPH dependent metabolism is through CYP pathways. CYP mediated oxidative metabolism of progesterone predominantly occurs through hydroxylation, counting for 75% of metabolites found after progesterone incubation with liver microsomes (Waxman, 1988). However, it has been previously reported that the reduction of progesterone to a 3-hydroxy-5-pregnane derivative occurs before hydroxylation takes place. Following this reaction the progesterone molecule becomes sensitive to hydroxylation at various positions giving rise to a large quantity of metabolites (Kremers et al., 1981) which would explain the vast amount of hydroxylated metabolites seen in the study by Waxman *et al.*

The susceptibility of progesterone to CYP enzymes is well documented. Known CYP enzymes that catalyse the metabolism of progesterone include CYP2C3, CYP2C5, CYP2C9, CYP2C19, CYP3A4, CYP17A1 and CYP21A2 (Williams et al., 2004, Yamazaki and Shimada, 1997, Szklarz and Halpert, 1997a, Swart et al., 1993a, Nebert and Russell, 2002). In particular several studies have shown that progesterone is a major substrate of CYP3A4 (Yamazaki and Shimada, 1997, Szklarz and Halpert, 1997a, Williams et al., 2004). CYP3A4 oxidizes progesterone to form 16α-, 6β-, and 2β-hydroxyprogesterone as major products and 21-hydroxyprogesterone as a minor product (Yamazaki and Shimada, 1997). A noteworthy difference between rat and human livers is the presence of different CYP enzymes, surprisingly the CYP3A4 enzyme is absent in the rat liver (reviewed by (Martignoni et al., 2006), yet other

CYP3A enzymes are present in the rat liver and intestine such as CYP3A9, CYP3A62 and CYP3A1 may play a similar role to CYP3A4 (Matsubara et al., 2004, Chiba et al., 1997). More CYP enzymes known to catalyse progesterone are CYP 2C9 and 2C19 which are also present in the human liver yet absent in the rat liver (Martignoni et al., 2006). Nonetheless, no significant differences were observed in the rate of degradation (k) between human and rat liver in the presence or absence of NADPH within this study (Figures 4.1 and 4.2). However, it is worth noting that, when only looking at the 20minute time-point there is a significant difference between the rat and human samples ($P<0.001$).

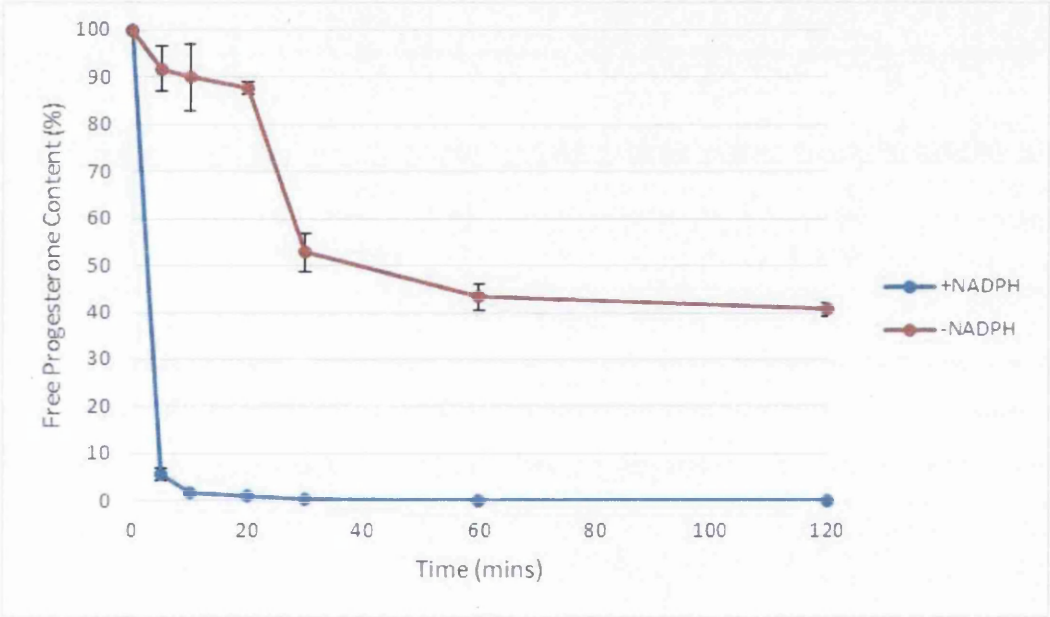


Figure 4.2: Concentration (expressed as percent of initial value) of progesterone as function of time of incubation with human liver homogenates in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

As well as members of the CYP family of enzymes, AKRs have also been reported to be involved in NADPH-dependent progesterone metabolism in the liver (Penning et al., 2000) and although their role in progesterone metabolism is less well documented than the role of CYPs, it is still possible that they are responsible for part of the degradation seen in liver homogenates within this study.

Differences between rat and human liver metabolism of some steroids has been analysed previously. One example of a difference in the metabolising capabilities of the human and rat livers is in the metabolism of the estrogen receptor antagonist, tamoxifen, whereby the amount of its hydroxylated metabolites are much lower in humans (Lim et al., 1994). Reduced steroidal glucoronidation has also been reported in the human liver compared to rats in the metabolism of the xenoestrogen, bisphenol A (Elsby et al., 2001).

Despite the differences in the apparent metabolising capabilities of rat and human livers, this study shows that the rate in which they are both capable of metabolising progesterone is similar. A rate constant of $0.014min^{-1}$ was obtained for progesterone depletion in human liver homogenate and a similar rate of $0.013min^{-1}$ was obtained in rat liver homogenate in the absence of NADPH (Table 4.2) based on 60 minutes of incubation. The half-life of progesterone was also similar in rat and human liver homogenates in both the absence and presence of NADPH. In the presence of NADPH the half-life of progesterone was 3.6 minutes in human liver and 3.7 minutes in rat liver and in the absence of NADPH the half-life of progesterone was 81minutes in human and 77minutes in rat. Both the rate constant and half-life of progesterone were similar in both human and rat suggesting, unlike with other steroids, the rat is a good model for assessing the rate of progesterone metabolism in liver tissue.

4.3.2 Progesterone Stability to homogenates of the Intestinal Mucosa

4.3.2.1 Progesterone metabolism by human and rat pooled small intestinal homogenates.

To further investigate differences in rat and human metabolising capabilities and to compare the rate of progesterone depletion in liver and intestine, progesterone was also incubated with pooled intestinal homogenates of human and rat.

There is no previous published data reporting the rate constant or half-life of progesterone in intestinal homogenates of any animal species. Indeed one study has looked at the metabolism of progesterone placed in the intestine of anaesthetised

dogs but their report shed light on metabolite formation as opposed to the stability of the whole drug in these conditions (Nienstedt and Hartiala, 1969). Thus the data presented herein provides novel insight into the stability of progesterone in the whole small intestine (pooled) as well as its stability in the proximal and distal small intestine and the large intestine of a rat model.

In the pooled intestine of the rat, including a combination of duodenum, jejunum and ileum, progesterone was degraded to 74% in 2 hours in the presence of NADPH (Figure 4.3). Interestingly progesterone concentrations remained high (95%) in the same time frame in the absence of NADPH suggesting that the majority of progesterone metabolism in the intestine is carried out by NADPH dependent enzymes (Figure 4.3).

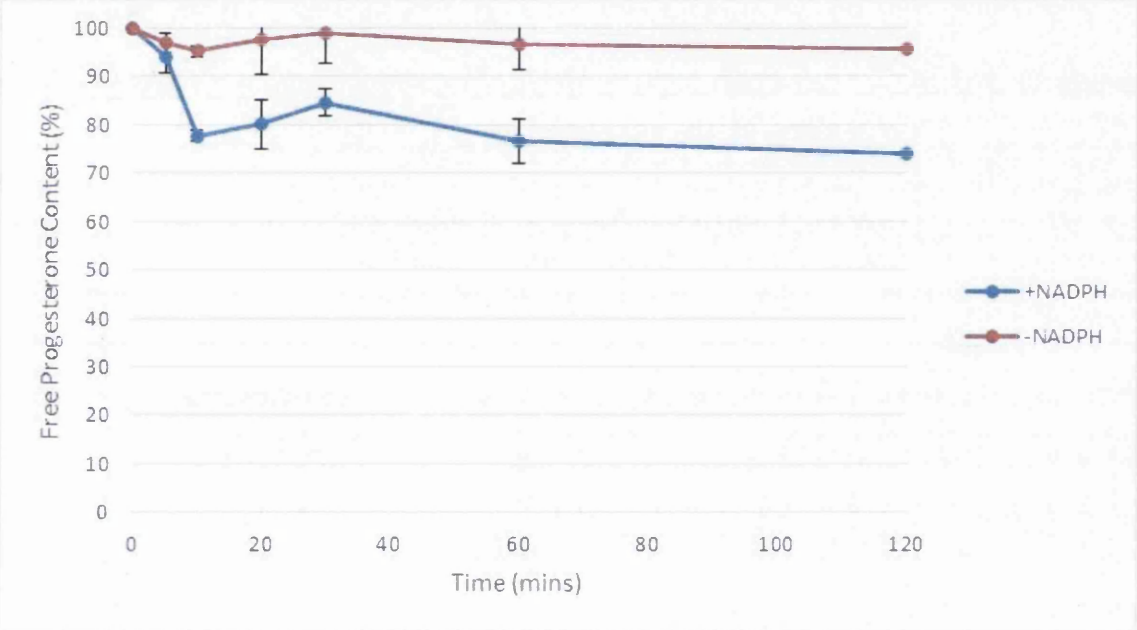


Figure 4.3: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with pooled rat intestinal homogenates in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

Unlike the rat, a different effect can be seen in the human intestinal homogenates. The stability of progesterone to homogenates of the pooled human intestine can be seen in Figure 4.4. After 120 minutes of incubation only 62% of parent drug remained detectable in samples containing NADPH. However in the absence of a co-factor progesterone was still degraded to 68%, suggesting that non-CYP enzymes

play a significant role in degradation of progesterone in the intestine. In both the presence and absence of NADPH the degradation of progesterone was gradual and unlike the rat did not plateau (Figure 4.4).

The stability of progesterone in the human intestine has only been reported in a single study by Nienstedt *et al. (1980). They* described progesterone metabolism in intestinal tissues of two adult male subjects and 13 human foetuses. The group obtained two ileum tissue samples and one colonic sample from the adult males and obtained intestinal homogenates from the foetuses. Although detailed information is available on metabolite formation, degradation rate and half-life are not disclosed, but from the data it can be deduced that after 2hours of incubation with radioactive progesterone between 38 and 48% of the drug remains in the adult male ileum and in the one colon sample progesterone was degraded to 36.9% in this time-frame (Nienstedt et al., 1980). No data on progesterone metabolism in the human duodenum and jejunum is available. It is thus difficult to determine to what extent different regions of the intestine have an effect on progesterone stability.

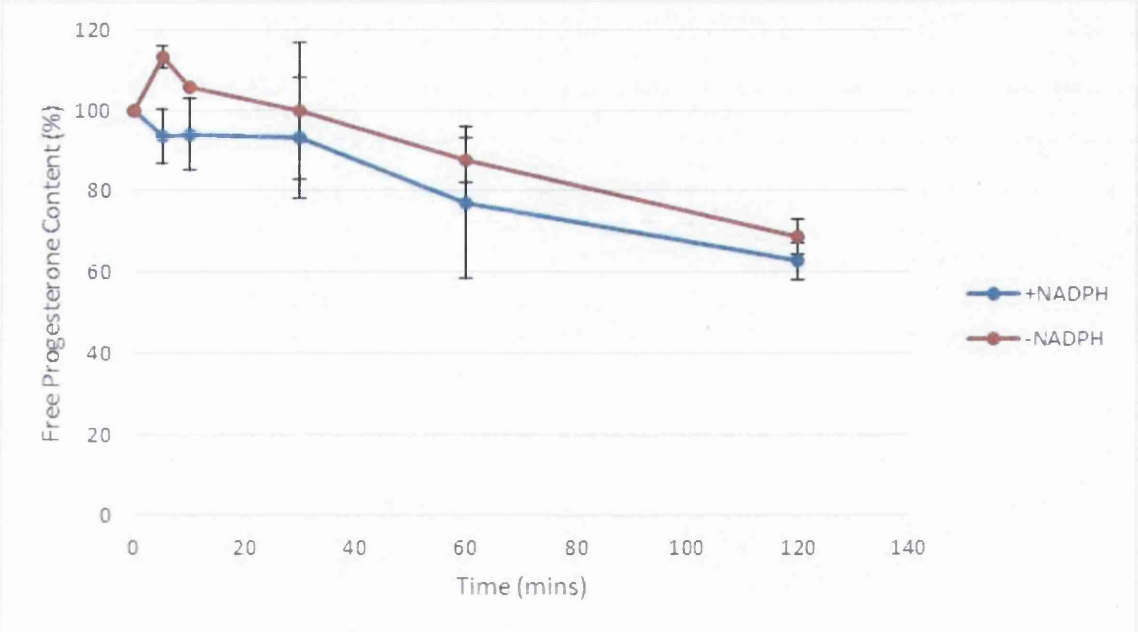


Figure 4.4. Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with pooled human intestinal homogenates in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

The half-life of progesterone in the rat pooled small intestinal homogenate was 248 minutes compared to 171minutes in the human intestine in the presence of NADPH (Table 4.1). Although the half-life appears much less in humans this was not statistically significant (P=0.24). Instead, a significant difference was observed in the half-life of progesterone between human and rat small intestine in the absence of NADPH (Table 4.2). Progesterone half-life was 1491 minutes in the rat and 166 in human which was deemed significant by t-test ($P<0.05$) (Table 4.2).

The human intestinal homogenate was obtained from a single individual, therefore these results could be different if intestinal homogenates obtained from various individuals were to be used. Moreover, this particular patient suffered from peritonitis, a condition that causes inflammation of the inner wall of abdominal organs, including the intestines (Verger et al., 1983). Peritonitis has been shown to alter enzyme expression in the intestinal mucosa (Ramachandran et al., 2002) so it is conceivable that the attained results are not representative of human healthy male individuals. Further to this, differences may have been found if samples had been obtained from females subjects.

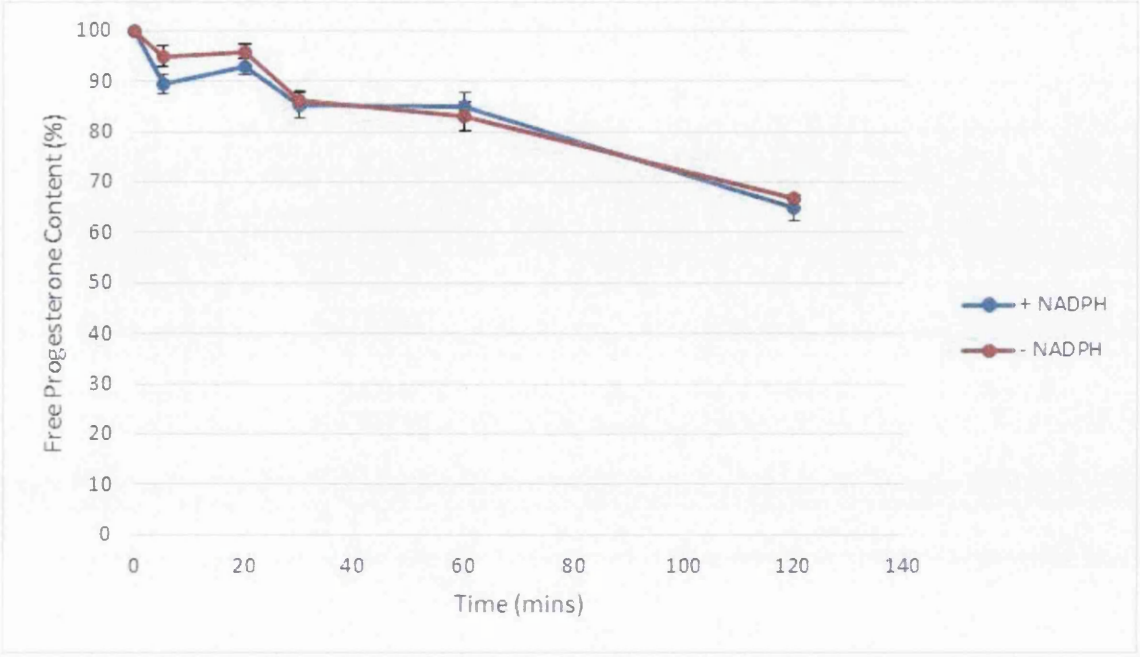
Although there was a significant difference between the rat and human pooled intestine in the absence of NADPH this may have been due to the reasons discussed and the remainder of the results proved similar between species. Therefore, the intestinal mucosa studies were subsequently carried out in rat intestine as a representative model for humans. This approach has been used previously when analysing progesterone metabolism in liver (Rao and Taylor, 1965, Waxman et al., 1988).

4.3.2.2 Progesterone metabolism by homogenates of the proximal small intestine of the male rat

There is no published data establishing the stability of progesterone in different regions of the small intestine in rat. When incubated with rat proximal intestine, approximating the duodenum and jejunum, progesterone degradation seemed independent of a co-factor; in fact in both samples, the percent depletion was similar at all time-points which also resulted in a similar half-life in presence and absence of

NADPH (described in Table 4.1 and 4.2). The overall degradation of progesterone was slightly greater in the rat jejunal mucosa compared to the duodenum with 64% of parent drug detectable in rat duodenum after 2hours compared to 58% detectable in jejunum in the presence of NADPH (Figure 4.5 Panel A vs Panel B). However in the pooled rat sample the cofactor seemed to have a greater impact on the degradation of progesterone which could potentially be due to the presence of distal small intestinal mucosa in the pooled intestine (Figure 4.3 compared to Figure 4.5). Unfortunately, the ileum was not available for analysis from individual male rat segments.

A



B

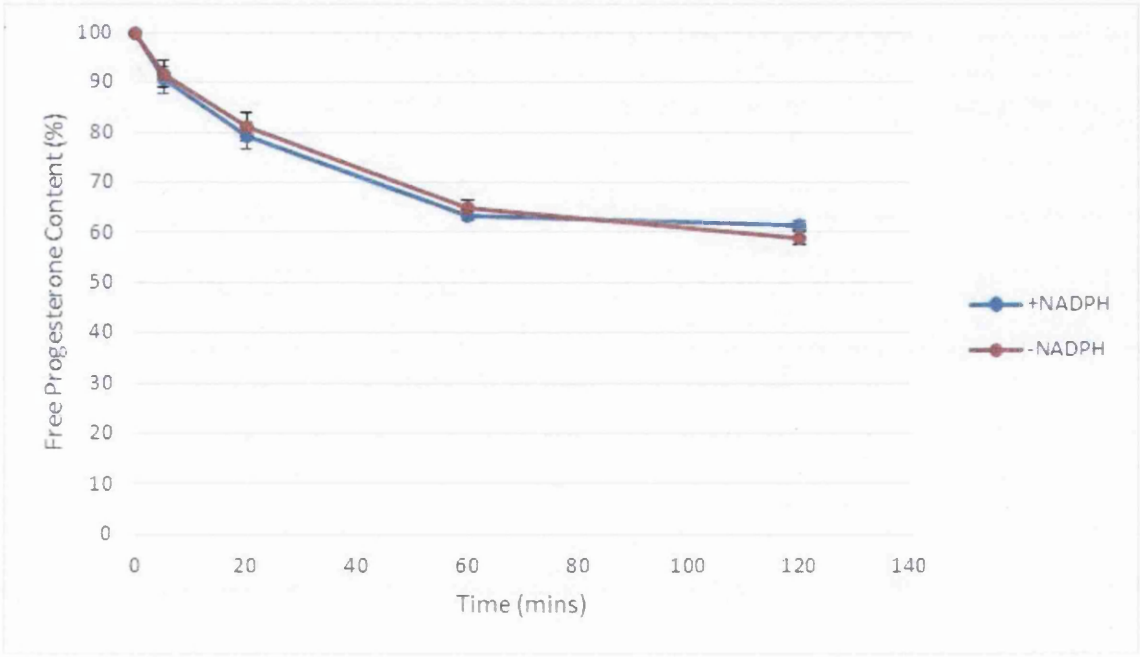


Figure 4.5: A and B. Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with male rat proximal small intestinal homogenate sections approximating the duodenum (A) jejunum (B) in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

4.3.2.3 Progesterone metabolism by homogenates of the proximal and distal small intestine of the female rat

It has been previously reported that compound degradation can differ between males and females (Bass et al., 1985, Martignoni et al., 2006), and this has also been demonstrated in the case of progesterone in the liver of rats (Rao and Taylor, 1965). To determine whether the proximal intestinal mucosa (approximating the duodenum) of the female rat differed from that of the male, the stability of progesterone to female proximal small intestine was determined (Figure 4.6). Due to sample unavailability the jejunal segment of the female rat was not analysed. After 2 hours progesterone was degraded to 70% in the absence of NADPH and to 63% in the presence of NADPH (Figure 4.6).

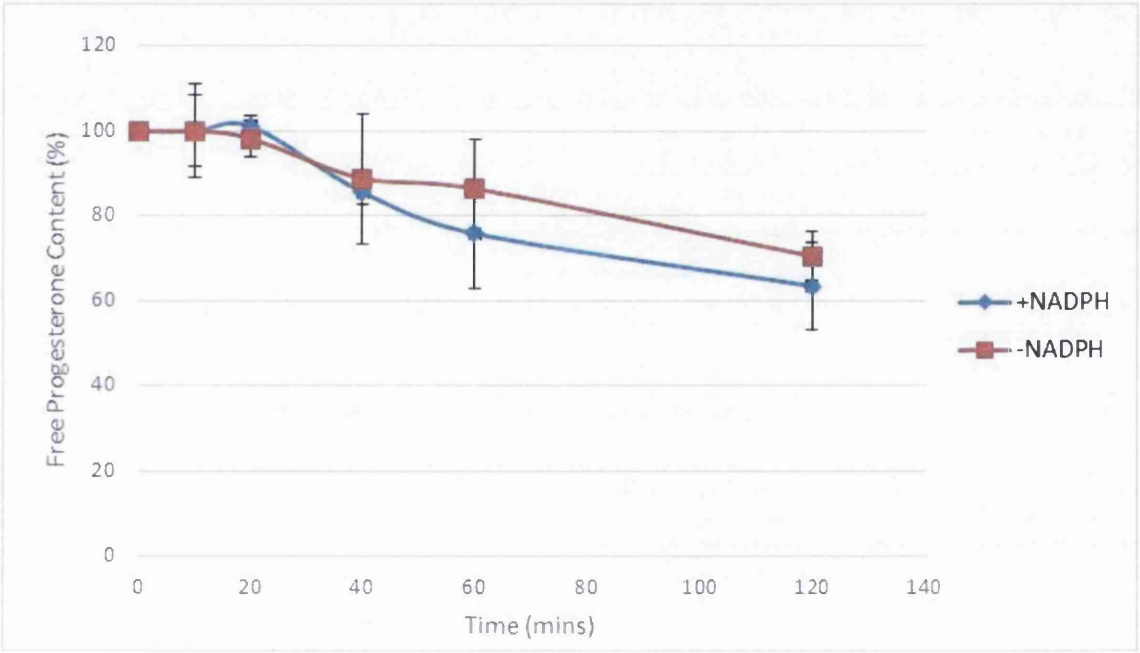


Figure 4.6: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with female rat proximal small intestinal homogenate approximating the duodenum in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

Similarly, in the male rat, there was a drop in free progesterone to 66% in the absence of NADPH and 65% in the presence of NADPH (Figure 4.5 panel A). In the female rat, NADPH appeared to cause some acceleration in progesterone depletion

though there was no significant difference in rate constant between samples incubated with NADPH and those incubated without (Table 4.1 and 4.2). The difference between male and female rats was not found to be significant. However, due to the availability of sample and the fact that this drug is intended for female consumption further work into the stability of progesterone in GI mucosa was carried out in female rats.

Akin to the proximal small intestine, the addition of NADPH to homogenates of the distal small intestine of the female rat had no significant effect on the degradation of progesterone. After 2 hours of incubation 70% of free progesterone was detected in the presence of NADPH and 57% was detected in the absence (Figure 4.7). The fact that amount of depletion was greater in the presence of NADPH suggests that the co-factor had absolutely no influence on progesterone stability in the distal small intestinal mucosa of the female rat.

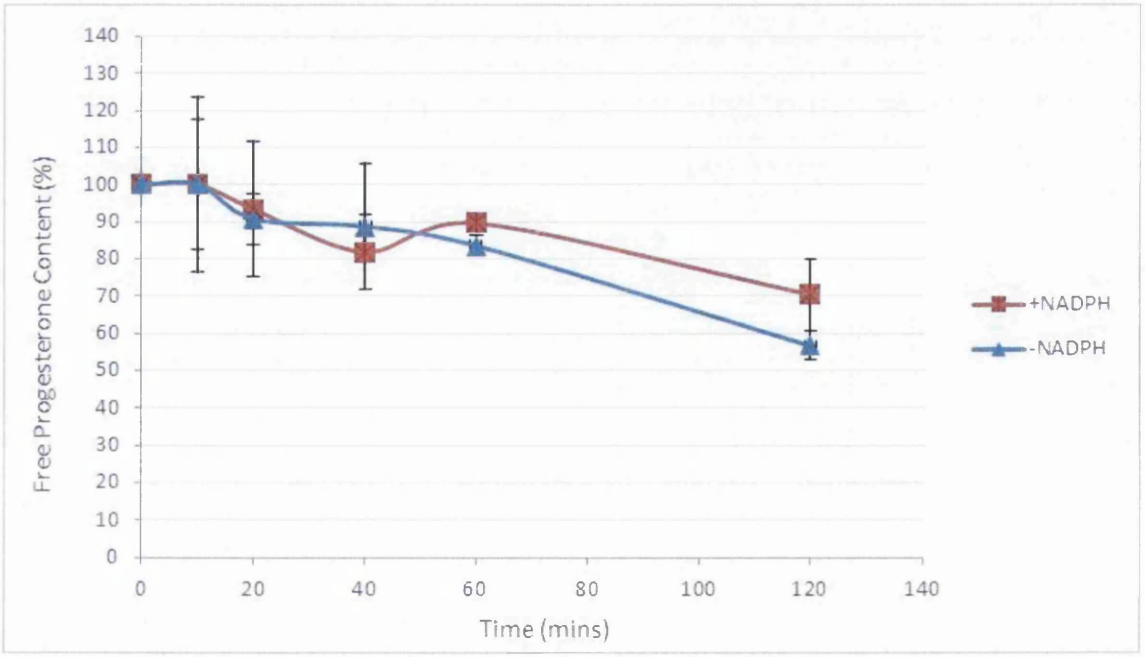


Figure 4.7: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with female rat distal small intestinal homogenate approximating the ileum in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

4.3.2.4 Progesterone metabolism by female rat large intestinal homogenates

Although the most likely route of metabolism in the large intestine is through bacterial enzymes, the presence of mucosal enzymes in this region has also been documented (Thörn et al., 2005). Thus the stability of progesterone to homogenates of the female rat large intestinal mucosa was determined. As with the distal small intestine, the addition of NADPH to the homogenate caused no significant change in progesterone depletion (Figure 4.8). The half-life of progesterone in colonic mucosa was found to be 139 minutes in the presence of NADPH and 142 minutes in its absence (Table 4.1 and 4.2).

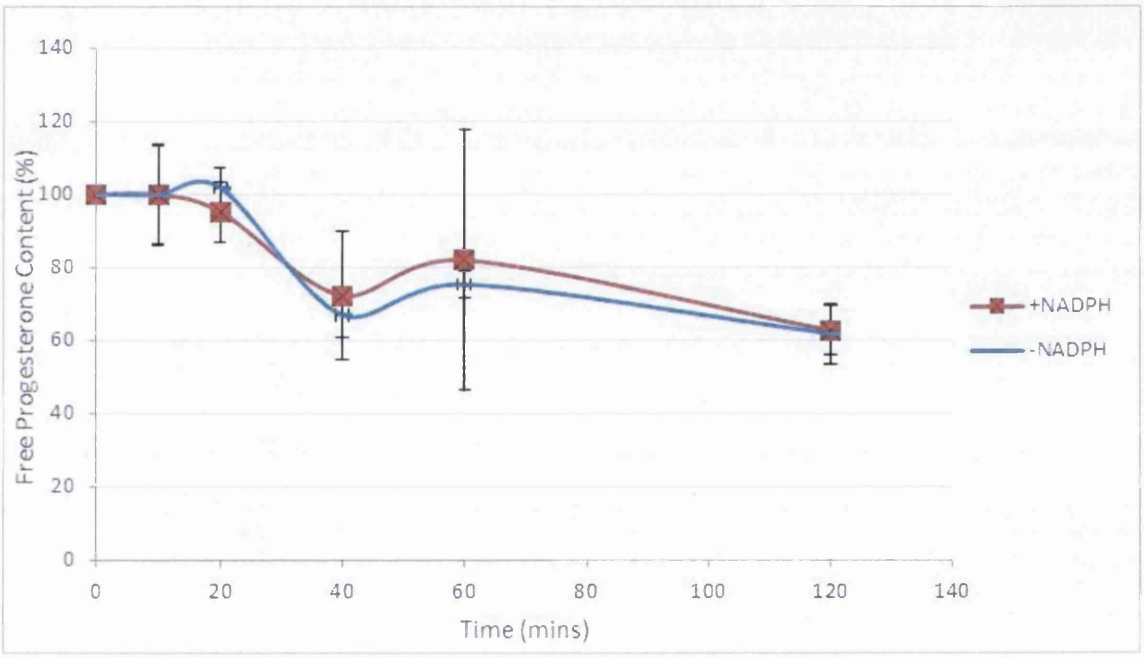


Figure 4-8. Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with female rat large intestinal homogenate (colon) in the presence (blue) and absence (red) of NADPH (n=3, error bars are +/- SD).

4.3.2.5 Where is progesterone most stable?

Data obtained from progesterone stability assays carried out in homogenates of different regions of the rat intestinal mucosa were statistically analysed (Figure 4.9).

Since the stability of progesterone was not deemed statistically different between male and female rats, these samples were pooled in order to uncover the region of least metabolism of progesterone. The rate constant of progesterone was found to be reduced in the rat distal small intestine (ileum) compared to other regions of the intestine in both the presence and absence of NADPH and in most cases this difference was found to be significant. Therefore, homogenates of the distal small intestinal mucosa appear to have the lowest impact on the rate of progesterone depletion.

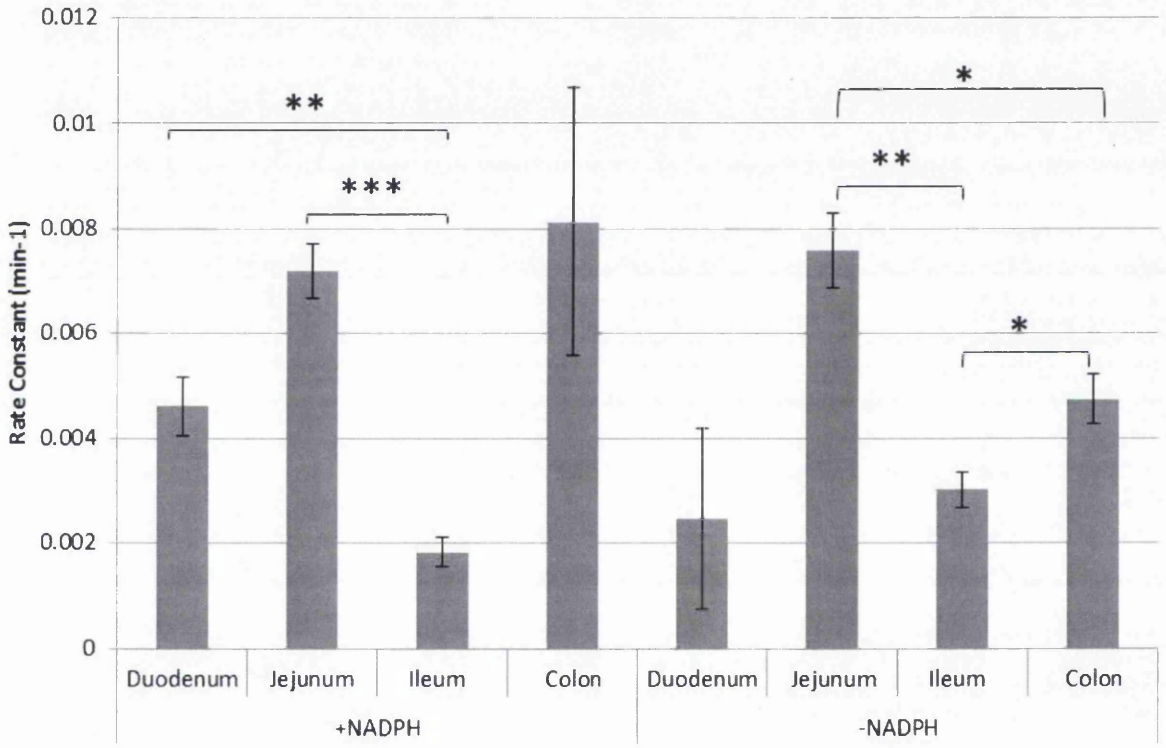


Figure 4.9: Rate constant (min^{-1}) of progesterone in homogenates of the rat intestine approximating the duodenum, jejunum, ileum and colon.

In order to summarise the data displayed graphically throughout this chapter the rate constant, half-life and percent drug remaining after 60 minutes are displayed in table 4.1 and 4.2.

Sample (+NADPH)	Rate constant (min^{-1})	Half-life(minutes)	% Drug remaining After 60minutes
Human Liver	-	3.55 ± 0.03	0
Human Intestine	0.00439 ± 0.00099	171.42 ± 5.85	77.13 ± 4.51
Rat Liver	-	3.71 ± 0.06	0
Rat Intestine	0.00445 ± 0.00061	248.08 ± 18.79	76.68 ± 2.76
Male Rat Proximal small intestine (Duodenum)	0.002697 ± 0.00037	184.76 ± 9.69	85.1 ± 1.88
Male Rat Proximal small intestine (Jejunum)	0.007202 ± 0.0003	134.78 ± 4.02	64.93 ± 1.15
Female Rat Proximal Small Intestine (Duodenum)	0.004616 ± 00056	153.65 ± 11.22	75.89 ± 2.5
Female Rat Distal Small Intestine (Ileum)	0.001832 ± 0.00027	143.45 ± 14.21	89.61 ± 1.46
Female Rat Large Intestine (Colon)	0.00813 ± 0.00254	138.63 ± 29.76	75.29 ± 2.16

Table 4.1: The mean (± S.D) degradation rate (k min-1), half-life (t1/2 min) and % drug remaining after 60 minutes for progesterone after incubation with rat and human tissue homogenates in the presence of NADPH.

Sample (-NADPH)	Rate constant (min^{-1})	Half life(minutes)	% Drug remaining After 60minutes
Human Liver	0.01397 ± 0.00106	80.91 ± 1.05	43.42 ± 2.8
Human Intestine	0.00224 ± 0.00084	166.56 ± 8.10	87.65 ± 4.32
Rat Liver	0.01293 ± 0.00014	77.38 1 ±.20	46.04 ± 0.4
Rat Intestine	0.00063 ± 0.00104	1491.08 ± 127.70	96.69 ± 6.2
Male Rat Proximal small intestine (Duodenum)	0.0031 ± 0.00053	184.54 ± 13.44	83.12 ± 2.6
Male Rat Proximal small intestine (Jejunum)	0.00759 ± 0.00041	132.37 ± 3.71	63.47 ± 1.58
Female Rat Proximal Small Intestine (Duodenum)	0.00246 ± 0.00172	186.71 ± 21.91	87.17 ± 8.83
Female Rat Distal Small Intestine (Ileum)	0.00301 ± 0.00033	204.32 ± 9.28	83.5 ± 1.64
Female Rat Large Intestine (Colon)	0.00474 ± 0.00047	141.9573 ± 15.54	73.64 ± 2.16

Table 4.2: The mean (± S.D) degradation rate (k min-1), half-life (t1/2 min) and % drug remaining after 60 minutes for progesterone after incubation with rat and human tissue homogenates in the absence of NADPH.

4.3.3 Progesterone Metabolism in Human Liver and Intestinal Cytosol

4.3.3.1 Progesterone metabolism in liver cytosol – NADPH dependent enzymes

To further investigate progesterone metabolism, the stability of this compound was determined in liver cytosol in the presence of specific enzyme inhibitors including AKRs inhibitors in the presence and absence of the NADPH co-factor.

Aldo-Keto Reductase Inhibitors

As discussed, enzymes of the cytochrome P450 family play a significant role in progesterone metabolism in the liver, particularly CYP3A4 (Yamazaki and Shimada, 1997, Szklarz and Halpert, 1997a, Williams et al., 2004). However, the role of aldoketo reductases (AKRs) in progesterone metabolism is also important. AKRs are a superfamily of NADPH-linked oxidoreductases involved in endogenous and xenobiotic metabolism (Penning and Drury, 2007). Their role in progesterone metabolism has been investigated previously (Penning et al., 2000, Charbonneau and The, 2001, Chen et al., 2011) but to a lesser extent than CYP enzymes. Hence the role of AKRs in progesterone metabolism was investigated herein. Enzymes of the AKR1C family have been shown to be involved in the oxidation of progesterone (Penning et al., 2000) and are highly expressed in the human liver (Palackal et al., 2002). Diazepam is a known inhibitor of all members of the AKR1C subfamily (Byrns et al., 2011, El-Kabbani et al., 2011) and is a more potent inhibitor of the AKR1C1, 1C2 and 1C4 isoforms relative to AKR1C3 (Byrns et al., 2011). However, no difference was observed in progesterone metabolism with Diazepam incubation in human liver cytosol compared to incubation with cytosol alone (Figure 4.10). The reason for no observed change is unclear; however, previous findings by Penning and colleges (2000) were based on incubations with recombinant human liver AKRs which may have yielded different results to those found within this study. The study by Penning and colleagues uses specific enzyme concentrations whereas the level of AKRs in the homogenates used in this study were not predetermined (Penning et al., 2000).

AKR1D1 is also known to metabolise progesterone. AKR1D1 is able to catalyse the conversion of progesterone to 5β-dehydroxy progesterone (Charbonneau and The, 2001, Chen et al., 2011). Finasteride is a synthetic drug used in the treatment of prostate hyperplasia and is a competitive inhibitor of AKR1D1 but with low micromolar affinity (Drury et al., 2009). Finasteride is also a 5α-reductase inhibitor (Tian et al., 1994) and progesterone is a known substrate of 5α-reductase (Azzouni et al., 2011). When incubated with intestinal cytosol and NADPH in the presence of Finasteride, the rate of progesterone degradation was reduced significantly (P=0.03), taking more than 120 minutes to fully metabolise compared to complete metabolism in less than 60 minutes when incubated with cytosol alone (Figure 4.10). It is not clear whether the inhibition of AKR1D1 or 5α-reductase had the largest impact on the rate of progesterone metabolism but as finasteride is a more potent inhibitor of 5α-reductase this is likely to have a substantial influence.

The final AKR enzyme investigated was AKR1B1. AKR1B1 has not been shown to be involved in the metabolism of progesterone and it is likely to play a role in the initiation of labour through metabolism of prostaglandins (Byrns, 2011). It has been reported that prostaglandins are involved in progesterone production and their inhibition can lead to a reduction in progesterone in uterine tissue (Elvin et al., 2000, Speroff and Ramwell, 1970). Although, there is no evidence suggesting this kind of reaction is catalised in liver cytosols. To investigate the role of AKR1B1 on progesterone metabolism Epalrestat was incubated with progesterone in the liver cytosol solution. Epalrestat is an AKR inhibitor shown to inhibit AKR1B1 (Zhang et al., 2013, Liu et al., 2009). Like Finasteride, Epalrestat significantly reduced the rate of progesterone metabolism (P=0.04). Eleven percent of progesterone could still be detected after 60 minutes of incubation compared to complete degradation of progesterone by this time-point in the absence of an inhibitor, suggesting AKR1B1 plays a previously unreported role in progesterone metabolism.

In the presence of all three inhibitors (AKR cocktail) the rate of progesterone metabolism was significantly reduced with more than 32% of free progesterone still detectable after 1 hour and almost 10% of free progesterone still detectable after 2 hours (Figure 4.10). The decrease in metabolism caused progesterone half-life to

increase significantly from 11 minutes in the absence of an inhibitor and to 52 minutes in the presence of the AKR cocktail ($P<0.01$). The decrease in progesterone metabolism in the presence of AKR inhibitors suggests a substantial role of AKRs in progesterone metabolism.

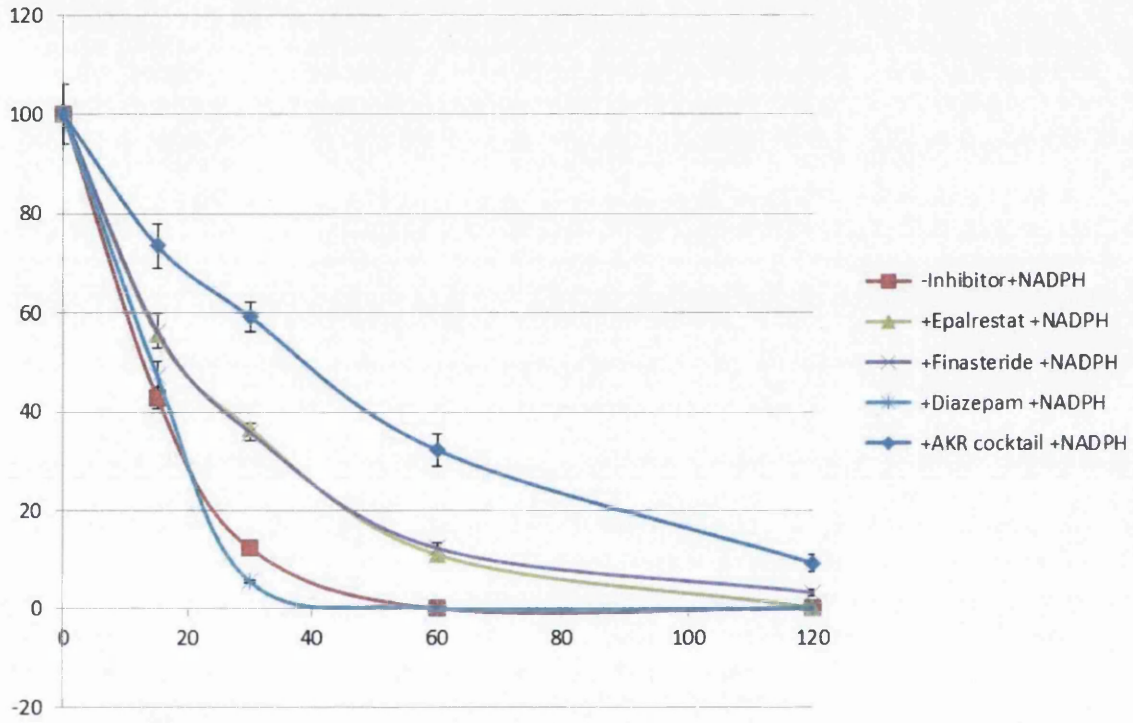


Figure 4.10: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human liver cytosol and 1mM of NADPH alone (red) or with NADPH and Epalrestat (Green), Finasteride (Purple), Diazepam (Light Blue) or a combination of all three (Dark blue) (n=3, error bars are +/- SD).

To confirm that the enzyme inhibitors were specifically inhibiting NADPH-dependent enzymes, progesterone was also incubated with all discussed inhibitors in liver cytosol in the absence of NADPH and, as expected, no change was observed (Figure 4.11).

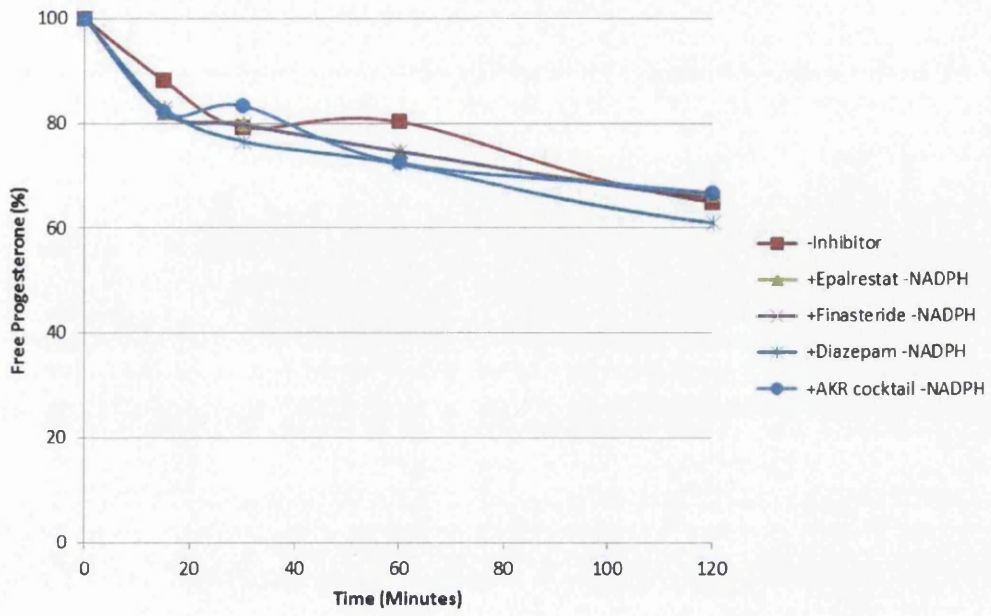


Figure 4.11: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human liver cytosol in the absence of enzyme inhibitor (red) or in the presence of Epalrestat (Green), Finasteride (Purple), Diazapam (Light Blue) or a combination of all three (Dark blue) (n=1).

4.3.3.2 Progesterone metabolism in liver cytosol – Non-NADPH dependent enzymes

As well as illustrating the role of different enzymes in progesterone metabolism, the work carried out in liver cytosol in the presence and absence of NADPH also showed that, without enzyme inhibitors, progesterone was still depleted to almost half its initial value in the absence of a cofactor (Figure 4.11, red line). There is very little information on progesterone metabolism by enzymes that do not require NADPH as an electron donor; therefore some key, non-NADPH, enzymes were investigated for their potential role in progesterone metabolism.

Aldehyde and Xanthine Oxidase Inhibitors

Aldehyde oxidases constitute a small sub-family of enzyme (Garattini et al., 2009) and are not known to be involved in progesterone metabolism. As one of the most

important non–P450 enzymes in drug metabolism (Beedham, 1997), the role of Aldehyde Oxidases in progesterone metabolism was assessed. Hydralazine and Chlorpromazine are potent inhibitor of aldehyde oxidase activity *in vitro* and *in vivo* (Johnson et al., 1985, Obach et al., 2004) and were thus incubated with progesterone in liver cytosol (Figure 4.12). It was confirmed that aldehyde oxidases do not affect progesterone degradation since no changes were observed in the rate of reduction of progesterone in the presence and absence of these inhibitors (Figure 4.12).

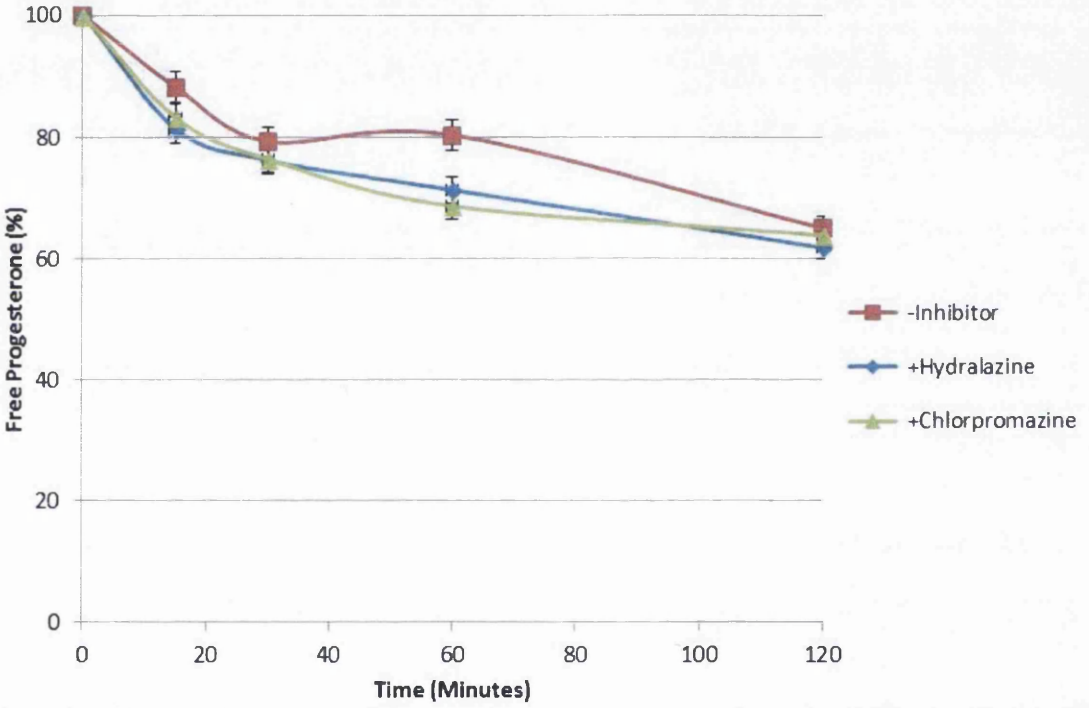


Figure 4.12: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human liver cytosol in the absence of enzyme inhibitor (red) or in the presence of Hydralazine (Blue) or Chlorpromazine (Green) (n=3, error bars are +/- SD).

Xanthine oxidases are structurally related to aldehyde oxidases (Garattini et al., 2009) and their role is generally accepted as a fundamental enzyme in purine catabolism, yet the structural complexity, low substrate specificity (Harrison, 2002) and specialised tissue distribution (Huh et al., 1976) indicate that other roles for these enzymes may yet to be identified. Xanthine oxidase has been reported to cause

inhibition of progesterone production in luteal cells (Gatzuli et al., 1991, Ota et al., 2001), but no direct interaction of xanthine oxidase and progesterone has been reported. Allopurinol is a commonly administered drug known to inhibit xanthine oxidase (Pacher et al., 2006, Massey et al., 1970). After, 2 hours of incubation in liver cytosol in the presence and absence of alluporinol no difference was observed in the rate of progesterone degradation (Figure 4.13) suggesting no direct interaction between xanthine oxidase and progesterone metabolism in the liver.

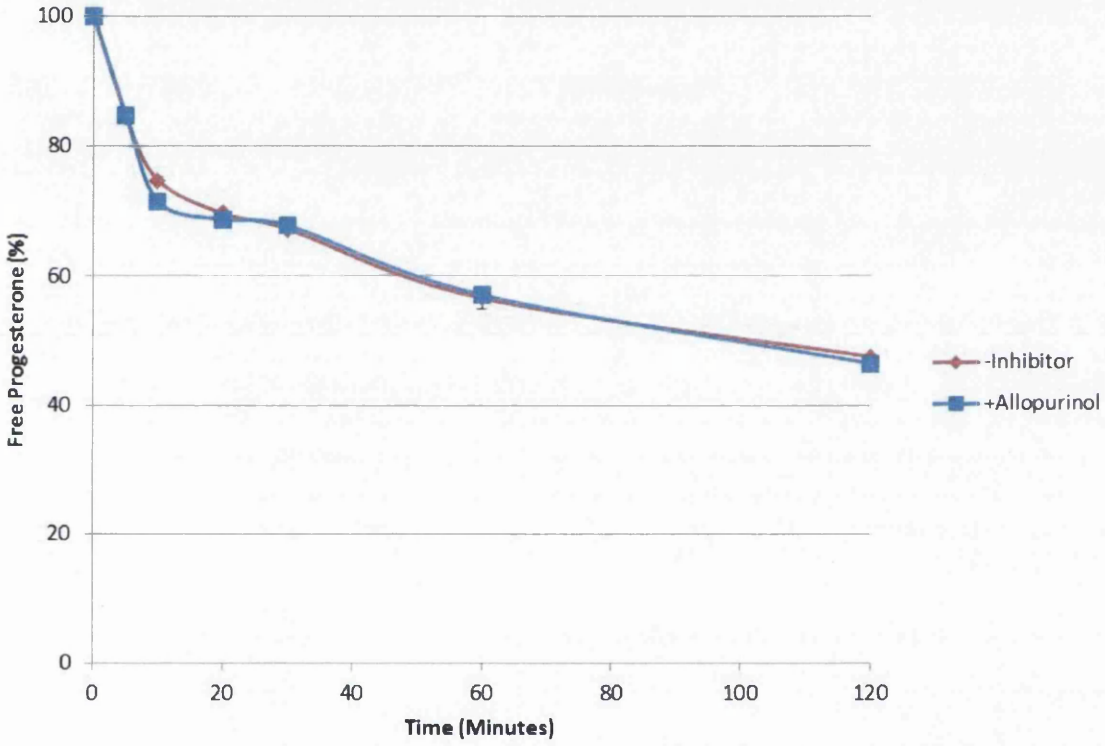


Figure 4.13: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human liver cytosol in the absence of enzyme inhibitor (red) or in the presence of Allopurinol (Blue) (n=1).

Like hydralazine and chlorpromazine, menadione is also an aldehyde oxidase inhibitor (Obach et al., 2004). In addition, menadione inhibits several enzymes including aniline-p-hydroxylase activity and can negatively affect CYP reductase activity (Floreani and Carpenedo, 1990). Therefore, progesterone was incubated with Menadione in liver cytosol in the presence and absence of NADPH (Figure 4.14).

Menadione significantly inhibited progesterone degradation at a similar level in both the presence (P=0.05) and absence of NADPH (P=0.002) suggesting menadione did not inhibit NADPH-dependent enzymes. Moreover, previous incubation of progesterone with the aldehyde oxidase inhibitors, hydralazine and chlorpromazine, did not affect the rate of progesterone degradation (Figure 4.12). This suggests menadione-induced aldehyde oxidase inhibition is not responsible for the decrease in the rate of progesterone metabolism. Menadione is also capable of inhibiting NRH:quinone oxidoreductase 2 (NOQ2) (Long et al., 2002). NOQ2 is a cytosolic flavoprotein that utilises dihydronicotinamide riboside (NRH) as an electron donor (Megarity et al., 2014) but this would require the presence of NRH in the liver cytosol which has not been confirmed. This data presents novel insight into progesterone metabolism in the liver suggesting a role for not only the well characterised NADPH-dependent enzymes but also for enzymes that do not require NADPH as a cofactor. The precise enzymes involved in the non-NADPH metabolism of progesterone requires further investigation but at least within this study their role has been shown to be significant.

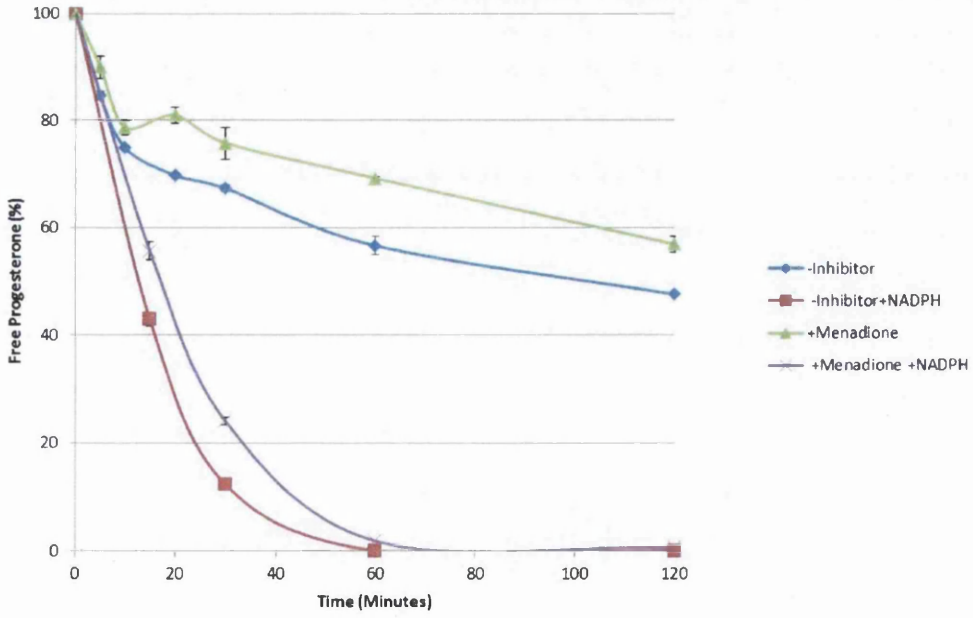


Figure 4.14: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human liver cytosol alone (blue) or with Menadione (Green) or incubated with human liver cytosol plus

1mM of NADPH (Red) or with human liver cytosol plus 1mM of NADPH with Menadione (Purple) (n=3, error bars are +/- SD).

4.3.3.3 Progesterone metabolism in intestinal cytosol

The stability of progesterone was also assessed in human intestinal cytosol in the presence and absence of NADPH. After 2 hours of incubation free progesterone was depleted to 57% of its initial value in the presence of NADPH and this was reduced to 72% in the absence of NADPH (Figure 4.15). This led to a significant difference in progesterone half-life (P>0.05); whereby in the presence of NADPH the half-life of progesterone was 131minutes and in the absence of NADPH this was reduced to 209 minutes. The occurrence of NADPH dependent enzymes, including CYPs and AKRs, is well documented in the human intestine (Thelen and Dressman, 2009, Barski et al., 2008) which is the likely reason for the increase seen in the presence of NADPH.

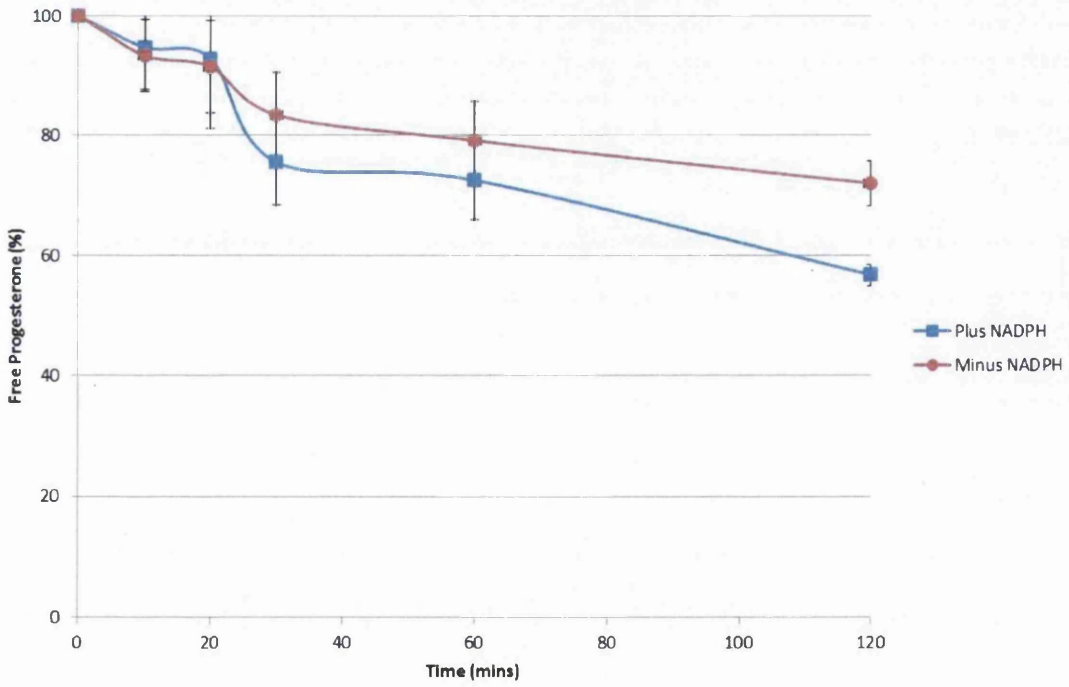


Figure 4.15: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human intestinal cytosol either alone (blue) or combination with 1mM of NADPH (red) (n=3, error bars are +/- SD).

As the largest amount of metabolism was observed in the presence of NADPH, it was decided to further investigate the role of NADPH-dependent enzymes only in progesterone depletion in intestinal cytosol. AKRs are found throughout the human gastrointestinal tract at varying levels (Palackal et al., 2002), with the highest levels being found in the proximal regions of the small intestine. Moreover the expression of distinct subtypes of these enzymes differs between regions of the small and large intestine (Barski et al., 2008). For example, enzymes of the AKR1C family are involved in the oxidation of progesterone and can be found at varying levels in small intestine with the exception of AKR1C4 which is found almost exclusively in the liver (Penning et al., 2000). Progesterone was incubated with intestinal cytosol in the presence of diazepam, a known inhibitor of the AKR1C subfamily (Byrns et al., 2011, El-Kabbani et al., 2011) as well as a combination of diazepam and flufenamic acid. Flufenamic acid is also a known inhibitor of AKR1C (Bauman et al., 2005, Adeniji et al., 2012).

After 2 hours of incubation in the absence of an inhibitor, free progesterone was reduced to 57%, however progesterone metabolism was significantly reduced to 65% in the presence of diazepam ($P>0.01$) and to 64% in the presence of AKR1C cocktail ($P>0.05$) (Figure 4.16). The addition of the AKR1B1 inhibitor, Epalrestat also significantly reduced progesterone depletion to 68% ($P>0.01$). Free progesterone was found to be 67% in the presence of the AKR1D1 inhibitor Finasteride after 2 hours of incubation which was, again, a significant reduction compared to the minus inhibitor sample ($P>0.01$). The reduction in progesterone depletion with the addition of AKR inhibitors in intestinal cytosol confirms that members of this family of enzymes play a role in progesterone stability in the human intestine and may influence the overall bioavailability of progesterone after oral administration.

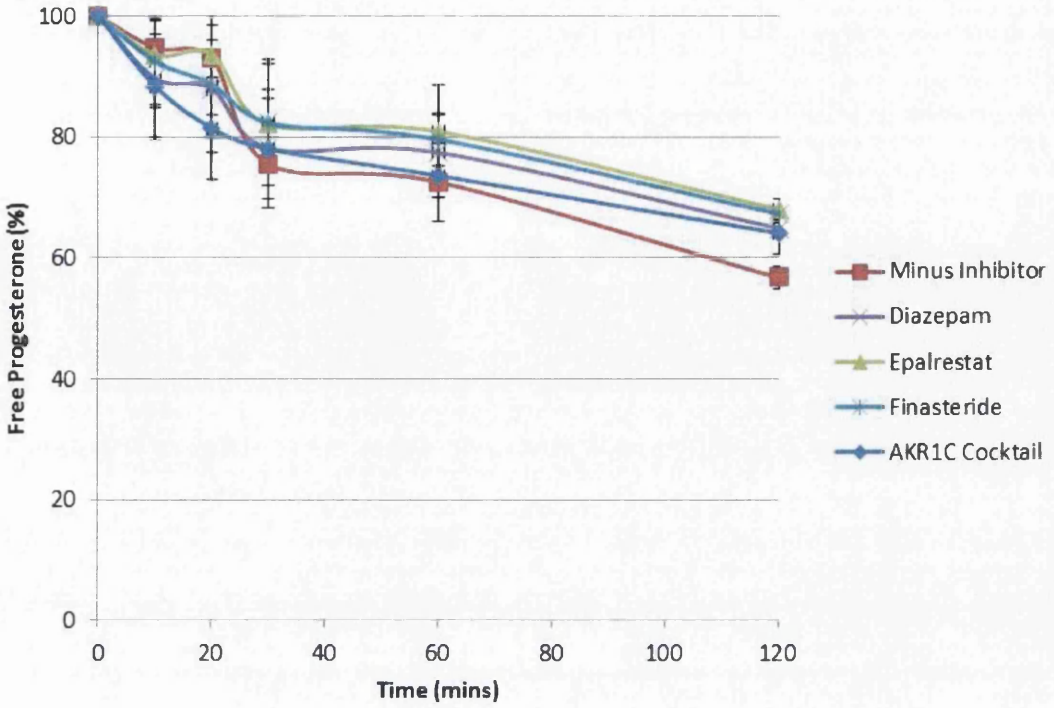


Figure 4.16: Concentration (expressed as percent of initial value) of progesterone as a function of time of incubation with human intestinal cytosol alone plus 1mM of NADPH in the absence of an inhibitor (red) or in the presence of Diazepam (Purple), Epalrestat (Green), Finasteride (Light Blue) or AKR1C cocktail containing Diazepam and Flufenamic acid (Dark Blue) (n=3, error bars are +/- SD).

4.3.4 Stability of Progesterone in Simulated Fluids of the Intestine

As well as intestinal mucosa, intestinal fluid is also capable of drug metabolism due to the presence of bacterial enzymes and enzyme secretions from the pancreas and may impact on drug stability and overall bioavailability. Progesterone, mainly as metabolites, has been recovered from the faeces and urine of primates after oral administration with over 40% of the total drug recovered reportedly being found in faeces (Wasser et al., 1994, Ziegler et al., 1989) thus showing that a large portion of orally administered progesterone reaches the ileo-colonic region of the gastrointestinal (GI) tract. It has been suggested that progesterone is able to undergo enterohepatic circulation (Symonds et al., 1994), if this is indeed the case then it may be that progesterone requires enzymatic metabolism in order to be re-absorbed. The available information on progesterone stability to colonic bacterial metabolism is

insufficient with the available studies providing no quantitative information but only highlighting the metabolites formed from such incubations. Furthermore the available studies were all carried out before 1980 (Schubert et al., 1964, Bokkenheuser et al., 1977, Eriksson and Gustafsson, 1970, Adlercreutz et al., 1979). Therefore, the stability of progesterone to metabolism by simulated intestinal fluids was assessed herein.

4.3.4.1 Solubility of Progesterone in Simulated Small Intestinal and Colonic Fluids

The method used to assess the stability of progesterone in simulated fluids involved incubating a solution of progesterone with simulated colonic fluid (SCF) and simulated intestinal fluid (SIF). However, seeing that progesterone is very poorly soluble there is a risk of precipitation on addition of the progesterone solution into the SIF/SCF.

Simulated fluids were prepared using a PBS phosphate saline buffer (see Materials and Methods Section 2.2.5), therefore the solubility of progesterone in PBS was determined and found to be >2μg/ml. Thus, the addition of different solvents and solubilisers were assessed as a means of enhancing progesterone solubility for this stability assay. Two solvents, DMSO and ethanol, were assessed in combination with the solublilisers Cremophor or Tween80. The findings suggested that the solubility of progesterone in PBS was enhanced most by the addition of DMSO and Tween80 (Figure 4.17). The solubility of progesterone in this solution was approximately 50μM.

The presence of proteins such as albumins in faeces is also likely to increase the solubility of progesterone. Hence the solubility of the progesterone solution was assessed in simulated colonic fluid (SCF) before running the experiment. It was observed that progesterone was soluble at a concentration of 65μM in this solution, thus this concentration was used for intestinal fluid stability assays. 65μM was deemed to be sufficient progesterone content to visualise degradation by UV-HPLC.

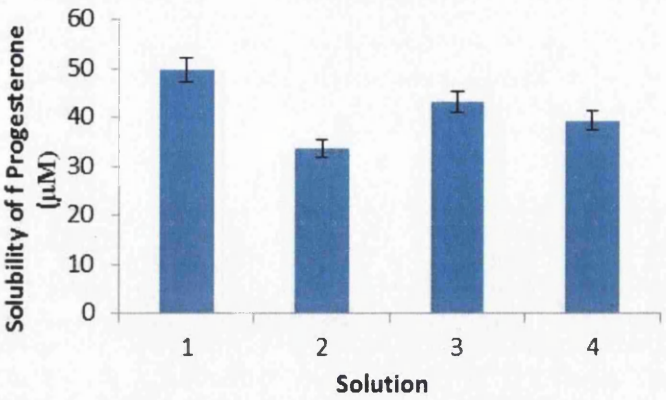


Figure 4.17: Solubility (µM) of progesterone in four solutions: 1. PBS with 1%DMSO and 1% Tween80, 2. PBS with 1%Ethanol and 1%Tween80, 3.PBS with 1%DMSO and 1% Cremophor and 4.PBS with 1% Ethanol and 1%Cremophor (n=3, error bars are +/- S.D.). Solubility of progesterone was determined as described in Section 2.2.4 (Materials and Methods).

4.3.4.2 Simulated Intestinal Fluid with Pancreatin

As small intestinal fluid is difficult to access, simulated fluids of the small intestine were used as an alternative. Simulated intestinal fluid (SIF) was produced with pancreatin as described in Materials and Methods Section 2.2.5. Pancreatin contains enzymatic components produced by the exocrine cells of the pancreas including trypsin, amylase, lipase, ribonuclease, and protease (Desnuelle and Rovery, 1961). 65µM of progesterone, dissolved in DMSO and Tween80 was incubated with SIF plus pancreatin for 24h (Fig 4.18). There was no change in progesterone concentration after 2 hours of incubation with SIF plus pancreatin. Further samples were taken at 6 and 24hours but no significant change in the level of progesterone was observed (Figure 4.18). The data suggests that the enzymes present in pancreatin are not able to metabolise progesterone. However, it is quite likely that bacterial enzymes present in the small intestine would play a far more significant role in progesterone degradation in natural human intestinal fluid.

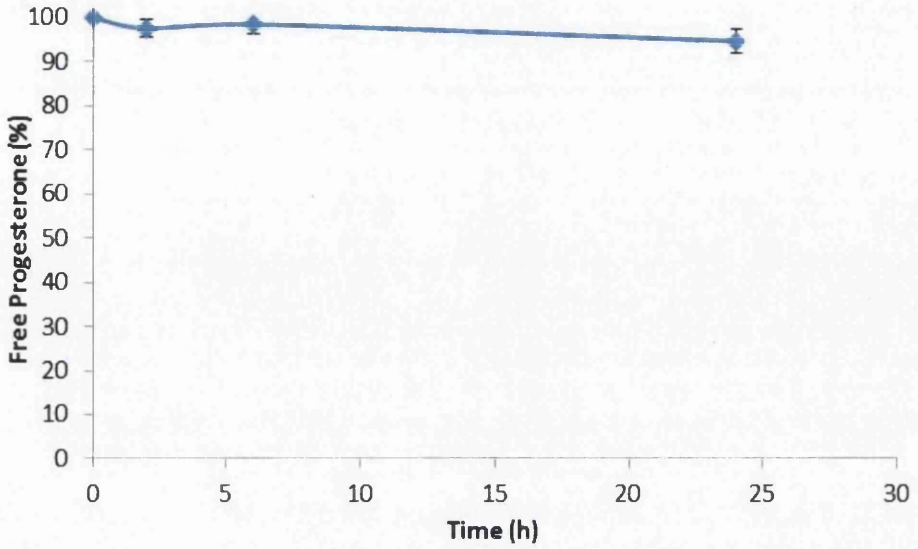


Figure 4.18: Stability of progesterone (concentration expressed as percent of initial value) in simulated intestinal fluid plus pancreatin measured by HPLC (n=3, error bars are +/- SD).

4.3.4.3 Progesterone Stability in Simulated Colonic Fluid

To assess the stability of progesterone in simulated colonic fluid (faecal slurry, SCF), the faeces of two healthy adult males were obtained and a slurry was produced as described in Materials and Methods Section 2.2.5. There are currently no commercially available mixtures of colonic enzymes which is due to the vast quantity of enzymes present in this fluid, some of which have not been identified. Therefore, the preparation of SCF from by suspending faeces in buffered solution is the best method for assessing colonic drug metabolism.

Before assessing the stability of progesterone to SCF, the amount of NADPH present in the SCF was determined, as the level of co-factor available to progesterone-metabolising enzymes can have a significant impact on the stability of the drug as observed in liver homogenates experiment described in this chapter (Figure 4.10).

The level of NADPH was determined by HPLC by comparing standards of known concentrations of NADPH to levels of NADPH found within the SCF. The SCF was also spiked with NADPH to confirm the peak observed at the correct retention time

was indeed NADPH and not contaminants from within the slurry. Figure 4.19 shows the HPLC chromatogram obtained for this experiment.

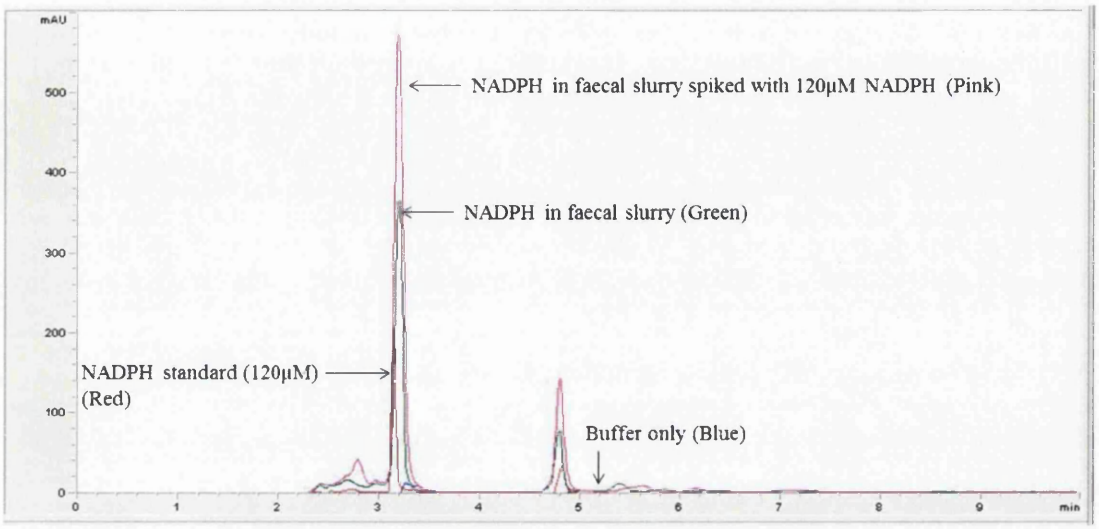


Figure 4.19: Chromatogram showing levels of NADPH in three solutions. NADPH standard containing 120µM NADPH in PBS (with 1% DMSO and 1% Tween) can be seen in red, NADPH levels in faecal slurry can be seen in green and faecal slurry spiked with 120µM of NADPH can be seen in pink. A blank sample containing buffer only (PBS plus 1% DMSO and 1% Tween) is also present on the chromatogram in blue with no visible peak.

Using the linear equation obtained from 5 standards of different concentration of NADPH, the final concentration of NADPH in SCF was determined to be 503.96µM at the time of the experiment. Therefore, NADPH was not added to the incubation of progesterone with SCF as sufficient amounts of this co-factor were already present (Figure 4.19).

4.3.4.4 Stability of Progesterone to SCF

The metabolism of progesterone by human colonic bacteria has been investigated previously (Schubert et al., 1964, Bokkenheuser et al., 1977, Eriksson and Gustafsson, 1970, Adlercreutz et al., 1979). Progesterone is reduced to tetrahydroderivatives when incubated with intestinal microorganisms (Schubert et al., 1964). In addition progesterone is transformed to pregnanedione when incubated

with the faecal bacteria *E.Lentum* and *C.Paraputrificum*, (Bokkenheuser et al., 1977). Other previous studies have also described the bacterial metabolism of progesterone (Adlercreutz et al., 1979, Eriksson and Gustafsson, 1970). These studies provide a good insight into the type of metabolism that may occur in colonic fluid but do not provide any information on the rate of progesterone degradation or its half-life in such conditions. As well as progesterone there is very little information on MPA and no data available on LNG metabolism in the colon. There is, however, a great deal of information available on the metabolism of MPA and LNG in the liver (Thigpen et al., 1999, Pannuti et al., 1982, Eriksson and Gustafsson, 1970, Fotherby, 1996, Hümpel et al., 1978, Grimmer et al., 1986, Back et al., 1987). MPA, like progersterone, has low oral bioavailability which estimated to be as low as 15% (Fotherby, 1996, Adlercreutz et al., 1983b, Pannuti F, 1982) whereas LNG has a very high oral bioavailability of more than 87% (Hümpel et al., 1978, Grimmer et al., 1986, Back et al., 1987). Due to the substantial differences seen in the oral bioavailability of progesterone, MPA and LNG as well as the lack of previous research, the stability of all three hormones was assessed in SCF.

The degradation of each hormone in SCF is represented in Figure 4.20A and an example chromatogram can be seen in Figure 4.20B. Progesterone was rapidly degraded within 2 hours of incubation whereas MPA and LNG were degraded at a slower rate. Within 60 minutes only 12% of progesterone remained detectable by HPLC whereas more than 80% of LNG and MPA could be detected at this time-point. Each drug was also incubated with buffer only for 24hours with >99% recovery confirming that the observed degradation was a result of faecal bacteria and enzymes (Appendix M).

LNG is derived from and structurally similar to testosterone but with the addition of an ethynyl group (HC≡C) to significantly reduce androgenic activity (Stanczyk, 2002). Unlike progesterone, the oral bioavailability of LNG is considered to be very high (>87%). However LNG has been shown to undergo extensive reduction at the α,β-unsaturated ketone in ring A and hydroxylation at carbons 2 and 16 (Stanczyk and Roy, 1990). Reduction is also likely to play a role in the degradation of LNG in

the colon seen within this study. Indeed enzymes from several bacteria strains have been implicated in the oxido-reduction of steroids (Aries and Hill, 1970).

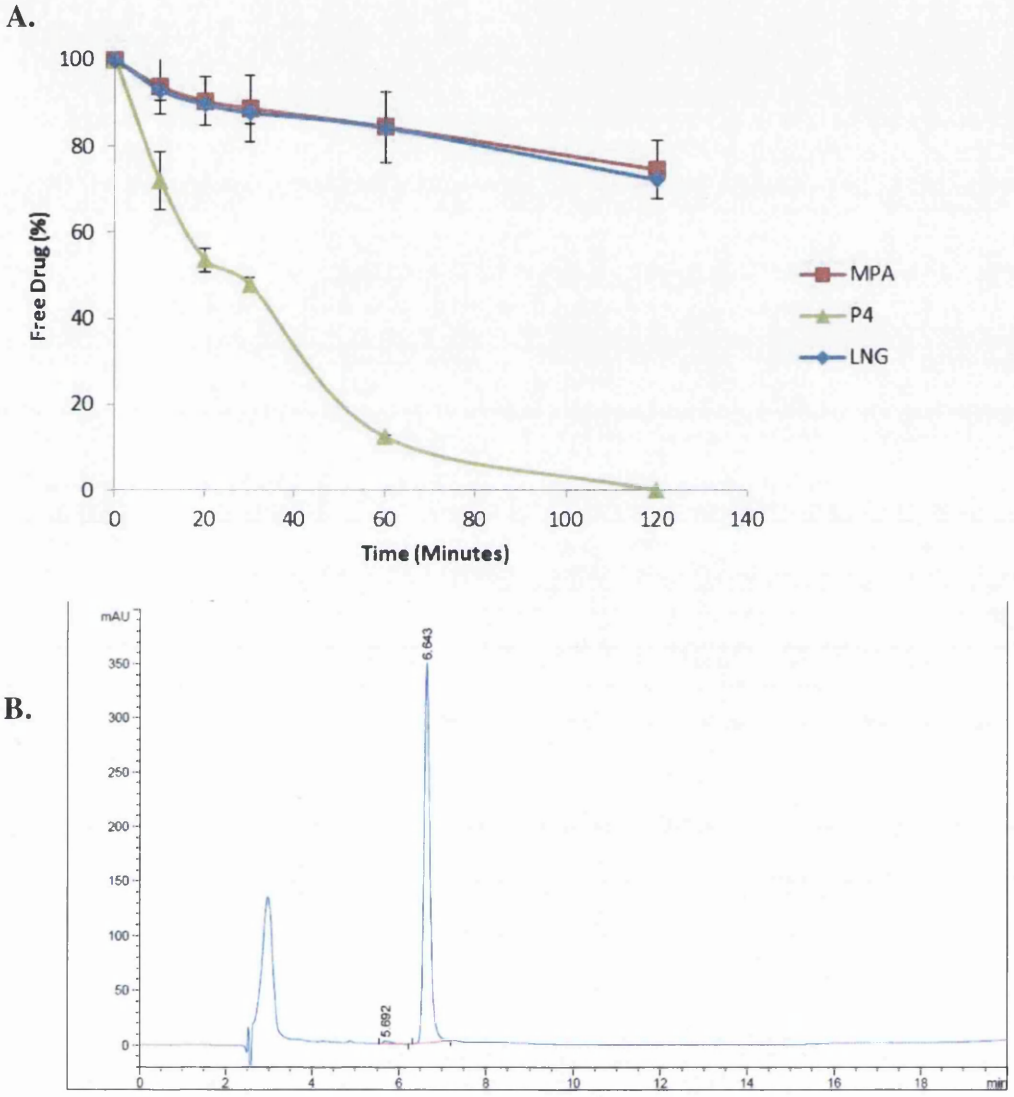


Figure 4.20 A: Stability of progesterone (concentration expressed as percent of initial value) in simulated colonic fluid measured by HPLC (n=3, error bars are +/- SD). B: An example chromatogram of progesterone at t-0 of study.

The data obtained in this study shows that LNG is susceptible to gradual degradation under colonic conditions over a period of at least 6 hours, with a half- life of 192 minutes. Similar to LNG, MPA was degraded gradually in the presence of colonic

bacteria but had a greater half-life of 327 minutes. MPA differs from progesterone by the addition of a hydroxyl group which has been acetylated at carbon 17 and the further addition of a methyl group at carbon 6 (Stanczyk, 2002). Like LNG, MPA also undergoes extensive metabolism by A-ring reduction but also undergoes hydroxylation at carbons 6 and 20 and conjugation (primarily glucuronidation) with subsequent elimination in urine (Stanczyk, 2002).

Some studies have looked into MPA metabolism by intestinal bacteria. In particular a study by Martin *et al* (1980), revealed that MPA is readily reduced in cultures of human faecal bacteria and rat caecal bacteria incubated aerobically or anaerobically. In both the human faecal and rat caecal bacteria, MPA was metabolised mainly to tetrahydro-derivatives but also dihydro-derivatives and the author suggests that A-ring reduction in both the liver and intestine are possible fates of MPA in humans (Martin et al., 1980). Despite the large amount of information provided on MPA metabolism, the percent of free parent drug remaining after incubation has not yet been explored. However, due to its reported susceptibility to intestinal reduction it was hypothesised that MPA would be rapidly and completely reduced in simulated colonic fluids yet more than half of free drug remained after 6h of incubation with >10% still detectable after 24hours. It has been previously suggested that the acetate group on carbon 17 of the MPA molecule may be involved in the reduced metabolism of this drug through steric hindrance, which may in part explain the reduced rate of MPA metabolism seen within this study (Stanczyk, 2003).

Unlike LNG and MPA, progesterone was rapidly metabolised in simulated colonic fluids. The rapid clearance of progesterone is in accordance with the previuos reports. Metabolism of progesterone by liver enzymes and intestinal bacteria after oral administration has been investigated in several previous studies and indeed many enzymes have been shown to metabolise this hormone (Williams et al., 2004, Yamazaki and Shimada, 1997, Szklarz and Halpert, 1997a, Swart et al., 1993b, Nebert and Russell, 2002). The presence of two ketone groups and a double bond on its molecule makes progesterone vulnerable to reduction and hydroxylation (Stanczyk 2003), which is the likely route of depletion in the colon as colonic

microbiota are known to be involved in *reductive* and *hydrolytic* reactions (Sousa et al., 2008b)

Based on the available data, it can be speculated that the colonic degradation of all 3 hormones is likely to involve the ring A structure. LNG has been shown to be reduced at the α,β-unsaturated ketone in ring A and hydroxylation at carbons 2 and 16. The two ketone groups and a double bond in ring A on the progesterone molecule allows reduction and hydroxylation reactions to occur. In addition, several studies have suggested MPA is highly susceptible to reduction at ring A. Yet, despite the reported susceptibility of all 3 drugs to metabolic degradation, there is great variability in their stability to colonic conditions.

4.4 Discussion

The aim of this study was to better understand the role of different regions of the intestines in the first pass metabolism of orally delivered progesterone, with a view to identifying an optimal site for the delivery of this compound. Insight into the intestinal metabolism should help to establish a region of the GI tract where progesterone is most stable and least susceptible to degradation, highlighting an optimal region of the intestine for targeted drug delivery. Information on progesterone metabolism was determined in human and rat liver and intestinal homogenates. Prior to this work, only a very limited amount of data was available on the progesterone-metabolising capabilities of the intestinal mucosa and fluids (Adlercreutz et al., 1979).

The data obtained within this study showed that, like many drugs, progesterone was rapidly degraded in liver homogenates of both human and rats in the presence of the electron-donor, NADPH (Figure 4.1 and 4.2) confirming previous finding that the liver plays a significant role in the metabolism of this drug (Rao and Taylor, 1965, Shirley and Cooke, 1968, Yamazaki and Shimada, 1997). However, homogenates of the human and rat pooled small intestine were also shown to have progesterone metabolising capabilities (Figure 4.3). It was found that within 60 minutes of incubation with intestinal homogenates, in the presence of NADPH; more than 20% of progesterone had been depleted. Intestinal metabolism has been shown to

contribute significantly to the overall metabolism and bioavailability of several drugs (Benet et al., 1996, Gomez et al., 1995, Hebert, 1997) and the degree of metabolism reported in this study suggests that the intestinal mucosa can influence the overall bioavailability of progesterone. This novel finding suggests that targeted delivery to an area of reduced progesterone metabolism may enhance the oral bioavailability of this compound.

Furthermore, until now, no direct comparison had been made between rat and human liver and intestines when assessing the degradation rate and half-life of progesterone, yet rats are the often the animal models of choice in such stability experiments (Rao and Taylor, 1965).

As a result of this research it was possible to assess the suitability of rats as a model species for progesterone stability. When comparing the data obtained from rat and human liver and pooled small intestine, similar results were observed. No significant differences were seen in the rate of progesterone metabolism between species with the exception of the human and rat pooled intestine in the absence of NADPH. Indeed there is a possibility that this difference was due to the presence of different enzymes in the mucosal lining of the rat and human intestine (Mitschke et al., 2008, Nishimura and Naito, 2006, Paine et al., 2006). Nonetheless, similar results were seen in progesterone depletion within the remaining samples. Based on these findings the rat appeared to be a good surrogate for assessing progesterone stability to degradation by mucosal enzymes throughout the GI. Therefore, the remainder of the experiments were carried out in rat intestinal segments of the proximal and distal small and large intestinal mucosa.

As the samples obtained for this study were from both male and female rats, the effects of gender on progesterone metabolism in the rat intestine were also investigated. Although similarities in free progesterone recovery have been reported in male and female rat livers (Rao and Taylor, 1965), no work has set out to compare differences in progesterone stability between male and female rat intestine. However, gender difference in the intestinal metabolism of some compounds has been previously documented (Liu et al., 2010a). Due to sample availability it was not

possible to directly compare each region of the male and female rat intestine. However, samples of the male and female rat proximal small intestine, approximating the duodenum, were obtained. With the limited data generated it was not possible to put forward a case for gender difference in rat intestinal metabolism. Therefore, gender was assumed to have only a minimal effect on this data-set but was still considered a potential limitation in the findings. The rat intestinal samples obtained for this study included male rat duodenal and jejunal intestinal homogenates and female rat duodenal, ileal and colonic homogenates.

At present there is very little data on progesterone stability in different sections of the GI tract. There is no data available whatsoever on the influence of the duodenum on the rate of progesterone metabolism and only a very limited amount of information available on progesterone metabolism in the jejunum, ileum and colon (Nienstedt and Hartiala, 1969, Harri et al., 1969). The rate of progesterone degradation (K) in rat intestinal homogenates differed significantly between certain samples with the highest rate of degradation being found in the jejunum. In particular the degradation of progesterone was most reduced in homogenates of the ileum (Figure 4.9). As described in Section 1.9, published data shows that the distribution of CYPs and AKRs varies throughout the GI tract (Thelen and Dressman, 2009, Mitschke et al., 2008, Chhabra, 1979), with some data showing that these enzymes are lower in the distal parts of the small intestine (Mitschke et al., 2008). This may be the reason a reduced rate of progesterone depletion was seen in this area whereas the highest was noted following incubation with jejunal homogenates.

To further investigate the first pass metabolism of progesterone and identify enzymes to which progesterone is substrate, the stability of this drug was also assessed in human liver and intestinal cytosol in the presence of NADPH dependent and independent enzyme inhibitors. Studies in cytosol further confirmed the rapid liver degradation of progesterone compared to the intestine and the significant impact of NADPH on progesterone metabolism. Moreover, in intestinal cytosol, more than 40% of progesterone was depleted in the presence of NADPH (Figure 4.15), further elucidating the importance of the intestine in progesterone bioavailability.

From the literature it is already clear that progesterone is highly sensitive to degradation by several members of the cytochrome P450 family (Yamazaki and Shimada, 1997). These enzymes are expressed throughout the length of the GI tract at varying levels (Thörn et al., 2005). Of particular interest is the elevated expression of CYP3A4 in the duodenum compared to the distal parts of the intestine (Thörn et al., 2005). CYP3A4 is a potent metaboliser of progesterone which may influence drug stability in this region.

As well as CYPs, AKRs are also known to be involved in progesterone metabolism (Penning et al., 2000, Charbonneau and The, 2001, Chen et al., 2011). Different AKRs are expressed at varying levels along the human GI tract (Pratt-Hyatt et al., 2013, Nishimura and Naito, 2006, Penning and Byrns, 2009), yet the role of these in progesterone metabolism is not clear. Thus the degradation of progesterone by AKRs was examined within this study.

The results suggest that progesterone is susceptible to degradation by AKR1B1 (Figure 4.16). The expression of AKR1B1 has been reported in the human small intestine but with no details on its expression level or location (O'connor et al., 1999). One study has found that the levels of this enzyme are low in the jejunum and ileum of the mouse and is virtually absent in duodenum and colon (Pratt-Hyatt et al., 2013). Thus the role of AKR1B1 could potentially affect progesterone stability in the gut but to what extent in the different regions of the human intestines is unclear.

Findings from this study also suggest that AKR1D1 is involved in progesterone metabolism. The precise location of this enzyme has not been studied though *in silico* research has suggested it is liver specific (Penning and Byrns, 2009). Nonetheless, data within this study showed that the rate progesterone degradation decreases when this enzyme is inhibited in intestine, suggesting its presence in this tissue.

As well as AKRs, novel findings from this work have indicated that enzymes inhibited by Menadione can affect progesterone stability. Menadione is known to inhibit aldehyde oxidases (AOXs) which are found at their highest concentration in the liver but are also expressed at low levels in the small intestine and colon

(Nishimura and Naito, 2006). However, Menadione is more likely to affect progesterone stability through its inhibition of NOQ2. The human tissue-specific expression of NOQ2 has not been determined though its expression has been reported in mouse colon (Long Ii and Jaiswal, 2000).

These unique findings not only help unpick the role of AKRs in progesterone metabolism, but also further reveal the potential of the intestines to considerably influence the oral bioavailability of progesterone.

The role of intestinal mucosa on progesterone metabolism is substantial; however, the influence of bacterial enzymes on drug metabolism must not be overlooked. Intestinal bacteria may indeed have an effect on progesterone stability; early studies have shown that some C21 steroid metabolites, found in rat and human faeces, are most likely the products of metabolism by intestinal bacteria (Gustafsson and Sjövall, 1968, Jänne et al., 1971). In these studies the dampening of bacterial metabolism through antibiotic administration greatly modified faecal steroid metabolite excretion.

To determine the role of intestinal fluids on the stability of progesterone, simulated fluids were produced. The stability of progesterone was first determined in simulated fluids of the small intestine containing pancreatic enzymes. However, no progesterone depletion was observed within this fluid. Conversely, when incubated with simulated colonic fluid containing human faeces, progesterone was completely degraded within 120 minutes. Despite the lack of data on progesterone stability in real fluids of the small intestine, it is likely that any metabolism taking place here is attributed to the presence of bacterial enzymes. This hypothesis is based on the fact that the non-bacterial enzymes present in the simulated fluid had no influence on progesterone stability (Figure 4.19).

It is worth noting that these experiments were carried out in simulated fluids of the human intestine only. Rats have been shown to have higher levels of bacteria throughout their GI tract suggesting their colonic metabolism is higher than in humans (Kararli, 1995) which may make rats poor models for assessing metabolism of progesterone in the intestine.

It is well established that the concentration of bacteria increases along the length of the GI tract (Jung and Kim, 2010, Hawksworth et al., 1971). Therefore, it is likely that fluids within earlier regions of the GI would have a lesser impact on steroid metabolism than fluids in the latter regions.

4.5 Summary

Altogether, the data obtained within this chapter has suggested that the rat is a good model for assessing the half-life of progesterone in homogenates of the intestine and liver and has provided new insight into the stability of progesterone in different regions of the GI tract of the rat. The susceptibility of progesterone to degradation by specific enzymes in the human intestine and liver has also been investigated. Novel findings associated with the activities of NAPDH dependent enzymes including CYPs, AKRs and AOXs was of particular interest and requires further research.

Finally, data from this chapter has highlighted the ileum as an optimal region of the intestine for the targeted delivery of progesterone. Therefore, the next chapter will focus on the development of oral formulations that have the potential to deliver progesterone specifically into the human ileum whilst increasing its solubility in the ileum fluids, thereby ensuring complete release and dissolution at this site. The ultimate goal is to enhance the oral bioavailability of progesterone as compared to that of immediate release formulations of these drugs.

CHAPTER 5

Production and Evaluation of Target Specific Delivery Formulations of Progesterone

5. Production and Evaluation of Target Specific Delivery Formulations of Progesterone

5.1 Introduction

A natural progesterone oral formulation for the treatment of endometrial hyperplasia and other forms of long-term oral dosing is not currently available. The development of such a pill has been hindered by the poor digestive absorption of progesterone due to its low aqueous solubility (8.81μg/ml) (Yalkowsky et al., 2010). Further to this, when administered orally, progesterone is highly susceptible to metabolism by hepatic enzymes (Yamazaki and Shimada, 1997). The combination of these factors means that the oral bioavailability of progesterone is extremely poor deeming it virtually inactive post-administration (Stanczyk, 2002). These limitations have been partly overcome in the form of micronized progesterone in peanut oil (Hsia et al., 2005) which has an oral bioavailability of approximately 10% (Stanczyk, 2002) but with significant intra- and inter- patient variability (Levine and Watson, 2000b). Other formulations containing micronized progesterone have also been explored with a view to improve bioavailability (Maxson et al., 1990, Chandler et al., 1997, Liu et al., 2010b), yet none have resulted in a commercially available, long-term, oral formulation.

As an alternative to the natural drug, several synthetic progestins have been manufactured (Schindler et al., 2008). Although the pharmacokinetic profiles of these drugs are favourable they each have adverse side effects which are brought about through undesirable binding and adverse signalling (Schindler et al., 2003). Synthetic progestins are also associated with increased incidence of breast and endometrial cancer (Fournier et al., 2005, Stanford et al., 1995, Seeger et al., 2003, Kelley et al., 1976, Kaaks et al., 2002). On the other hand, research shows that the use of natural progesterone has fewer side effects than synthetic progestins and brings about benefits such as improved lipid profiles and osteoporosis prevention (Sitruk-Ware et al., 1987, Murray, 1998, Holtorf, 2009). Moreover, recent research into the use of natural progesterone for the treatment of endometrial hyperplasia has

shown great promise (Chernukha et al., 2013, Marra et al., 2014, Stanosz et al., 2014).

It is clear that there is a preferred clinical profile for giving natural progesterone over synthetic progestins (Fitzpatrick 2000, Fitzpatrick 1999, Wood 2007 Valentine 1997). However, for such a drug to be successful there must be a viable market in which practitioners are keen to prescribe the drug and patients are willing to take it. With natural estrogen at least, a study of patients taking oral contraceptive revealed that >75% of women would consider switching to an oral formulation containing the natural compound (Genazanni 2013). It has also been reported that many women are opting for natural estrogen and progesterone products for hormone replacement therapy (Adams and Cannell, 2001, Ferguson et al., 1989, Holtorf, 2009). The attitude of women to taking natural hormones suggest a strong consumer market for a natural progesterone oral product, however, in order to produce such a product, the poor bioavailability of progesterone would need to be substantially improved.

As described in the previous chapter, the ileum appears to be the optimal site for progesterone delivery due the reduced rate of progesterone metabolism observed in this region. There are a number of ways in which drugs can be targeted to the ileum, for example, pH-sensitive or time-dependent polymer coatings can be used (Philip and Philip, 2010). Alternatively, a novel approach could be used whereby progesterone is formulated into amorphous solid dispersions (ASDs). Not only would this formulation travel quickly through the gut and dissolve at the ileal pH with great accuracy but it would also cause an increase in progesterone dissolution. Accurate drug release is of great importance; if progesterone is not specifically targeted and some of the drug reaches the colon then the bioavailability can be reduced. Indeed rapid progesterone metabolism by bacterial enzymes present in the human colon was observed within this study.

An amorphous solid dispersion (ASD) generally consists of a hydrophilic matrix and a hydrophobic drug whereby the drug is molecularly distributed within the inert matrix (Van Drooge et al., 2006). When in the amorphous state, poorly water soluble drugs exhibit an increase in apparent solubility and have an improved

dissolution rate as no energy is needed to breakdown the crystal lattice during drug release (Vasconcelos et al., 2007, Saffoon et al., 2011).

There are several methods available for the production of ASDs; the most commonly used methods include hot-melt extrusion, spray-drying and emulsion solvent evaporation which are discussed in detail in Section 1.10.1. Emulsion solvent evaporation will be the technique used within this study. With this method the polymer and drug are both dissolved in a solvent and gradually added to a liquid which is immiscible with the chosen solvent and contains a stabiliser. The addition of the dissolved drug and polymer is carried out under propeller stirring and as the stirring continues the solvent is removed slowly by evaporation at room temperature (Section 2.3.1 Figure 2.7). Eventually, a suspension of amorphous microparticles containing the drug and polymer is formed and can be separated from the solution by filtration (Kendall et al., 2009). The use of surfactants as stabilisers in the production process improves drug dissolution and can reduce drug precipitation or protect a fine crystalline precipitate from agglomerating into larger hydrophobic particles (Vasconcelos et al., 2007).

The key advantage to using emulsion solvent evaporation is its simplicity. The use of simple, inexpensive equipment reduces the production cost at lab-scale and the technique usually results in high loading efficiency (>70%). However, the technique has some draw-backs; despite the low cost, conductivity to industrial scale up can be challenging, as well as this, solvent residues may be an issue if evaporation is not fully complete (Freitas et al., 2005, Watts et al., 1989). Nonetheless, the simplicity and low cost of solvent evaporation makes this technique a good basis for investigation into the production of progesterone ASDs.

As mentioned, ASDs contain a drug dispersed in a polymer matrix. The main roles of the polymer are to stabilise against crystallisation and enhance dissolution by preventing crystallisation of the supersaturated solution. As ASDs are thermodynamically unstable they can recrystallize with time, negating any increase in apparent solubility (Lee et al., 2014), it is therefore important that they are

stabilised within the amorphous state. Further to improving apparent solubility, polymers can be designed to dissolve at a set pH for targeted delivery to the intestine.

For the production of any ASD it is important to select a suitable polymer. For the production of progesterone ASDs Eudragit L100 and hypromellose acetate succinate (HPMCAS) were investigated. The reason for selecting Eudragit for this study is due to its proven effectiveness in a clinical setting, as well as the successful use of Eudragit for the production of ASDs within our laboratory (Kendall et al., 2006, Kendall et al., 2009) providing a template for the production of progesterone ASDs. Moreover, the use of the method described in Chapter 2.3.1 was shown to be highly pH responsive for targeted drug delivery using Eudragit polymers (Kendall et al., 2009) and the patent for this method of ASD production has been licenced for use by Kuecept Ltd, the company sponsoring this project (Basit et al., 2013). However, Eudragit polymers are limited by their poor aqueous solubility (Evonik Industries AG) so the more soluble polymer, HPMCAS, was also investigated. HPMCAS polymers show enhanced solubility in water-immiscible solvents, a trait which allows the polymer to be used in a water-based solvent evapouration technique. This trait may be beneficial for the production of progesterone ASDs as progesterone is highly lipophilic and will not dissolve into water.

Following production, the progesterone ASD must adhere to a number of *in vitro* parameters. The microparticles should be uniform in size and ideally present a size below 500 μm to afford predictable and reproducible transit times through the GI tract. The drug contained within the microparticles should be amorphous and exhibit higher thermodynamic apparent solubility compared to the natural (crystalline) drug. High drug-loading efficiency is also important; poor loading efficiency is not only wasteful resulting in an expensive manufacturing process but will often result in excess polymer being present in the final dosage form with the potential to exceed the polymer's recommended daily intake dose.

The particles being developed are targeted for delivery to the ileum, thus dissolution of the ASD at the ileal pH is important. In its dissolved state, natural progesterone readily diffuses through the gut wall (Krishna et al., 2001, Rubas et al., 1993), it is

important to maintain the high permeability of progesterone in the new formulation in order to enhance bioavailability and this will be tested using a gut wall model (CACO-2). Finally, as shown in Chapter 3, synthetic progestins are able to adversely regulate target genes and proteins. The new progesterone formulation should not adversely regulate these targets and this will be assessed at gene and protein level.

Although previous research has looked into the production of progesterone microparticles (Jameela et al., 1998, Gangrade and Price, 1991, Royall et al., 2001, Kim et al., 2007, Hill et al., 1998, Miller et al., 2012), the use of Eudragit and HPMCAS polymers for oral delivery of progesterone in an amorphous dispersion has not been fully investigated.

Overall the aim of this chapter is to enhance the oral bioavailability of progesterone as compared to that of immediate release formulations by way of targeted delivery and enhanced apparent solubility and dissolution. To obtain this aim, progesterone ASDs will be produced using the solvent evaporation technique with Eudragit L100 or HPMCAS polymers.

5.2 Methods

The methods used within this chapter are described in detail in Chapter 2.3.1. Progesterone ASDs were produced using the solvent evaporation technique. The resulting microparticles were then subjected to a range of tests to assess their characteristics. The shape and size of the microparticles was determined by scanning electron microscopy, the crystallinity of the microparticles was assessed by x-ray powder diffraction and differential scanning calorimetry, whereas encapsulation efficiency and solubility and dissolution kinetics were evaluated by UV-HPLC. After assessing the characteristics of the progesterone ASDs, the signalling mechanisms of the progesterone ASDs were compared to non-formulated progesterone. This was carried out by treating endometrial cell lines with progesterone and progesterone ASDs and assessing changes in target gene and protein expression through RT-PCR, InCell Analysis, and ELISA. Changes in cell morphology were also assessed using light microscopy and ImageJ.

5.3 Results and Discussion

5.3.1 Development and Characterisation of Progesterone-Loaded Amorphous Solid Dispersions (ASDs/Microparticles) with a 10% drug-load

In order to improve the apparent solubility of progesterone, amorphous solid dispersions were produced using the solvent evaporation method with two distinct polymers; the methacrylic copolymer, Eudragit L100 and a cellulose derivative, HPMCAS. For the preparation of progesterone microparticles with Eudragit L100, an oil-in-oil method was used. The internal phase consisted of drug and polymer dissolved in absolute ethanol and the external phase consisted of liquid paraffin, containing 1% (w/w) sorbitan sesquioleate as previously described (Basit et al., 2013). For the preparation of progesterone microparticles with HPMCAS polymers an oil-in-water method was used. The internal phase consisted of progesterone and HPMCAS in ethyl acetate: dichloromethane (1:1 v/v) and the external phase consisted of deionised water containing 1% (w/w) polyvinyl alcohol as a dispersing agent.

5.3.1.1 Properties of Progesterone-Loaded Eudragit L100 Microparticles

The particle size and morphology of the progesterone-loaded Eudragit L100 microparticles was assessed by scanning electron microscopy (SEM). According to the SEM images the microparticles were highly spherical and smooth in appearance with no pores or perforations as seen in Figures 5.1 A-E. Additionally, by means of the scale bar, the size of the microparticles was estimated to be less than 40μm whereas unformulated progesterone has a particle size of approximately 150μm (Flores et al., 1994).

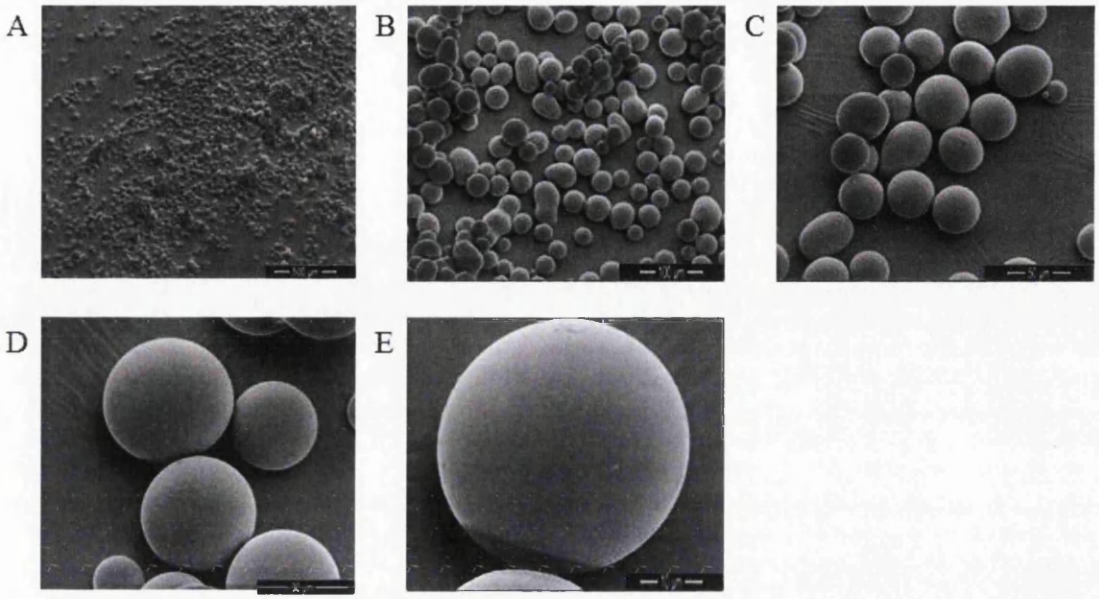


Figure 5.1 A-E: SEM micrographs of progesterone loaded eudragit L100 microparticles prepared by an O/O emulsification solvent evaporation technique, at different magnifications. A) 100x, B) 500x, C) 1000x, D) 2500x and E) 5000x

To assess the physical form of progesterone-loaded eudragit microparticles, powder X-ray powder diffraction (PXRD) was used. The PXRD pattern for natural progesterone can be seen in Figure 5.2 A, the pattern is characterised by sharp peaks representative of crystalline progesterone. The PXRD pattern obtained from progesterone-loaded Eudragit L100 microparticles was completely devoid of any diffraction peaks as compared to the crystalline pattern, indicating successful formation of an amorphous solid (Figure 5.2 B).

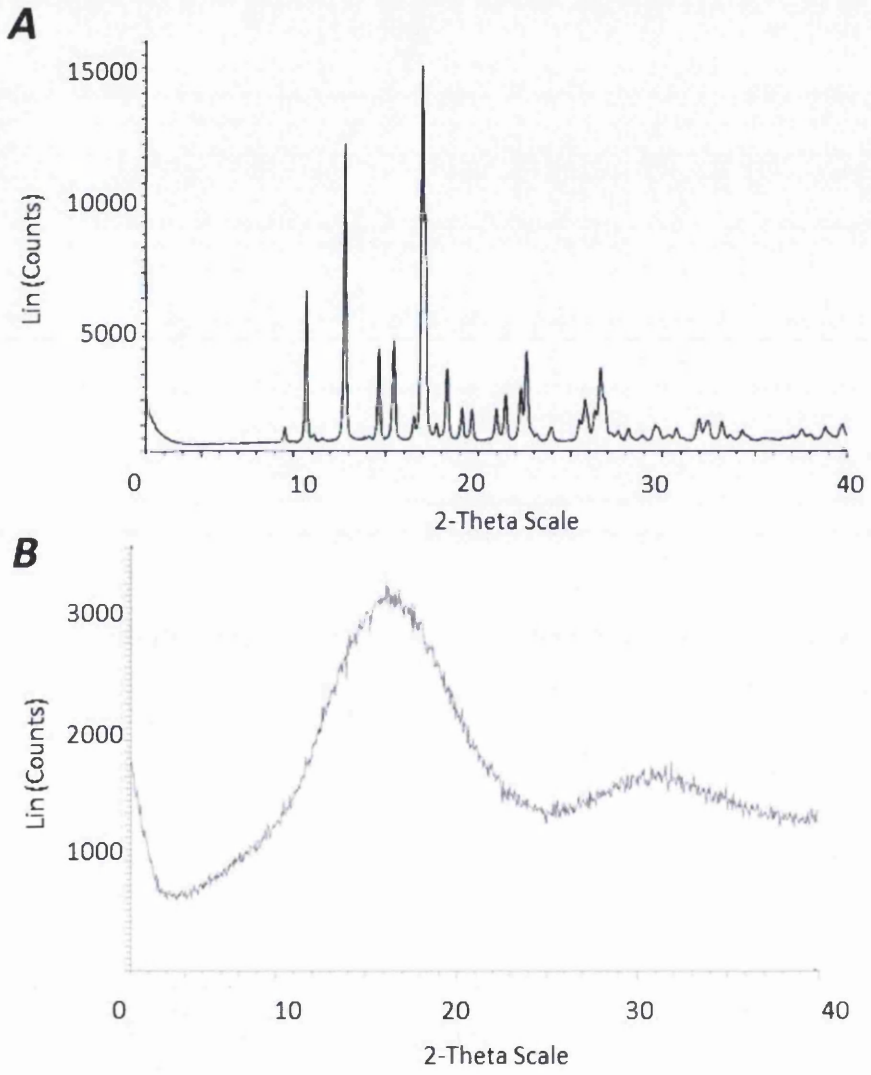


Figure 5.2: Graphical Representation of the Powder X-ray diffraction measurement of natural progesterone powder (A) and Progesterone-Loaded Eudragit L100 microparticles (B). Graph shows peak intensity (Lin Counts) vs. 2-theta scale.

Determination of progesterone encapsulation within the Eudragit L100 microparticles showed only 22% of the total drug added was successfully loaded into the microparticles (This can be seen in Figure 5.13, page 245). The poor encapsulation efficiency was likely due to the lipophilic nature of progesterone. Oil was used as the internal phase allowing progesterone to partition into the oil instead of into the polymer. Therefore, a water-based solvent evaporation technique would be a more efficient method for increasing encapsulation efficiency.

In order to use a water-based method with the Eudragit L100 polymer, a solvent immiscible with water, yet able to dissolve the polymer and drug, needed to be used. However, given the poor solubility of Eudragit L100 (practically insoluble in ethyl acetate, methylene chloride, petroleum ether and water (Evonik Industries AG), a water immiscible solvent able to dissolve this polymer was not obtainable. Therefore, the more soluble polymer, HPMCAS, was utilised as an alternative. As a result of the poor loading efficiency (<22%) no further characterisation was carried out with progesterone-loaded Eudragit L-100 microparticles.

5.3.1.2 Properties of Progesterone-Loaded HPMCAS Microparticles

Production of progesterone ASDs was carried out using a fine HPMCAS-HFwhich dissolves at pH >6.8. Progesterone loaded HPMCAS HF microparticles were produced as described in Section 5.3.1 As with the progesterone-loaded Eudragit L100 microparticles, the particle size and morphology was assessed by SEM as described in Chapter 2, Section 2.3.1.2 The SEM images of progesterone-loaded microparticles can be seen in Figure 5.3, the microparticles were spherical in appearance with no pores or perforations but the size of the microparticles was variable and drug could be seen on the surface which may be related to the drug load. Although the size of the microparticles was variable, they were estimated to be less than 100μm based on the scale bar, with the majority appearing to be less than 50μm.

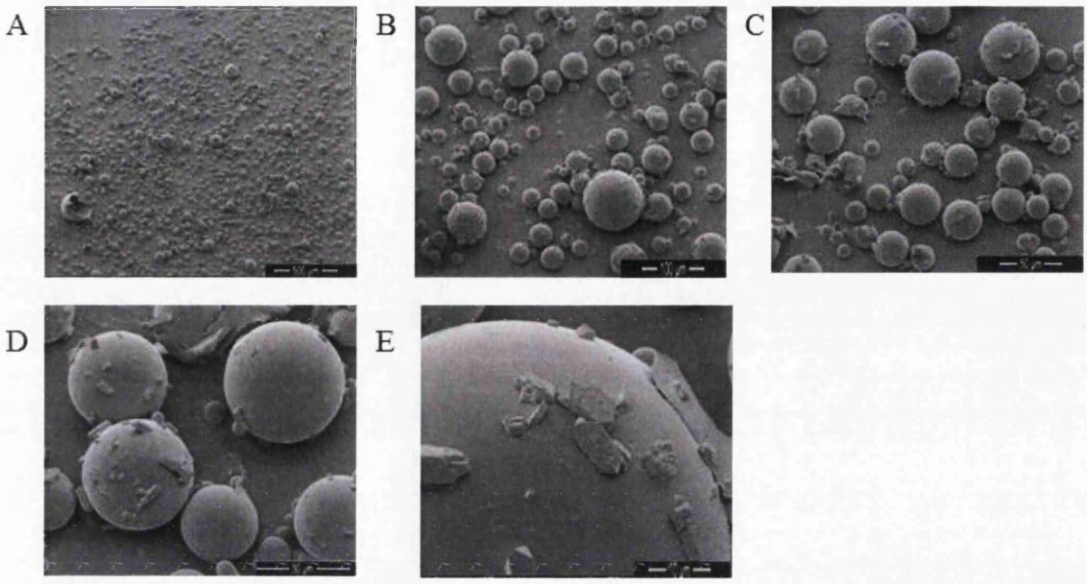


Figure 5.3: SEM images of progesterone loaded HPMC AS-HF microparticles. A) 100x magnification, B) 500x magnification, C) 1000x magnification, D) 2500x magnification and E) 5000x magnification

The PXRD pattern obtained for progesterone-loaded HPMCAS HF microparticles confirm that the drug was predominantly amorphous as seen by the absence of multiple diffraction peaks and presence of a characteristic amorphous halo (Figure 5.2A). To assess whether the microparticles were stable over a long period of time they were stored at room temperature in a sealed container for 2 years then again analysed by powder X-ray diffraction (Figure 5.4 B). The data obtained showed minimal recrystalisation within this time-scale suggesting the ASDs are highly stable.

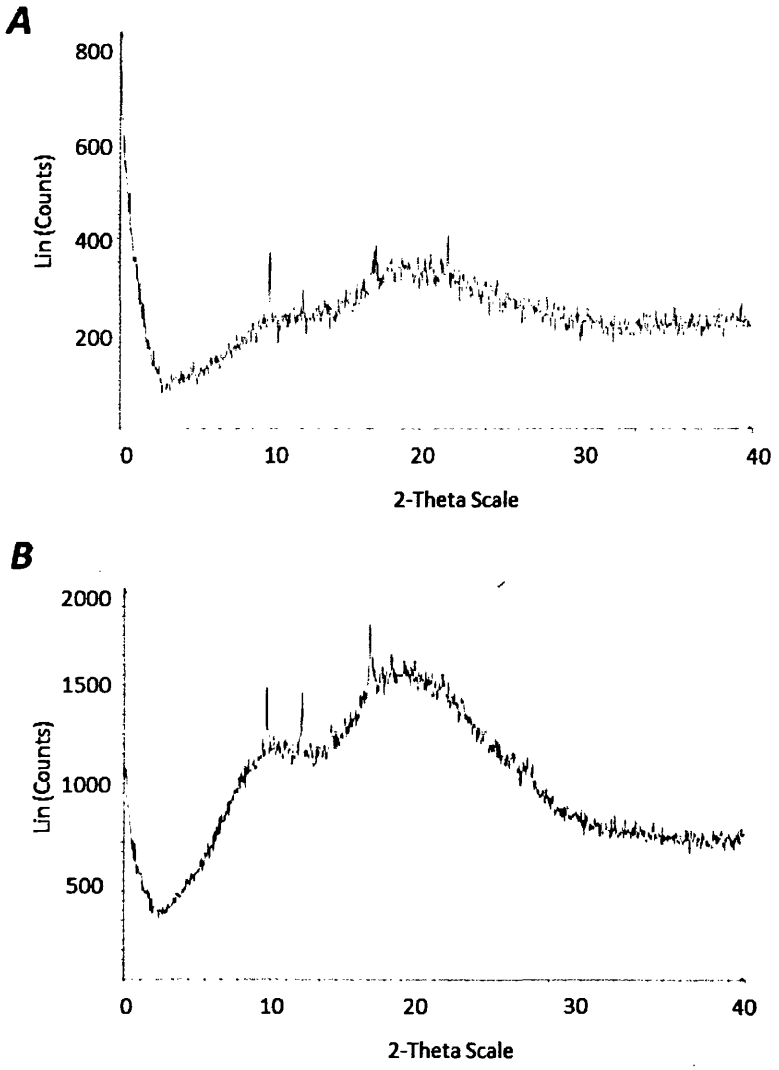


Figure 5.4: Graphical Representation of the Powder X-ray diffraction measurement of P_4-loaded HPMCAS HF microparticles A. At time of production and B. 2 years post-production. Graph shows peak intensity (Lin Counts) vs. 2-theta scale. Data shows progesterone is in an amorphous state.

To further test the physical form of the progesterone loaded into the HPMCAS HF microparticles, differential scanning calorimetry (DSC) was used. The thermogram obtained for the natural progesterone powder can be seen in Figure 5.5 A with a melting endotherm of 129.88°C, whereas the melting endotherm was absent in the microparticles (Figure 5.5 B) further confirming their amorphous nature.

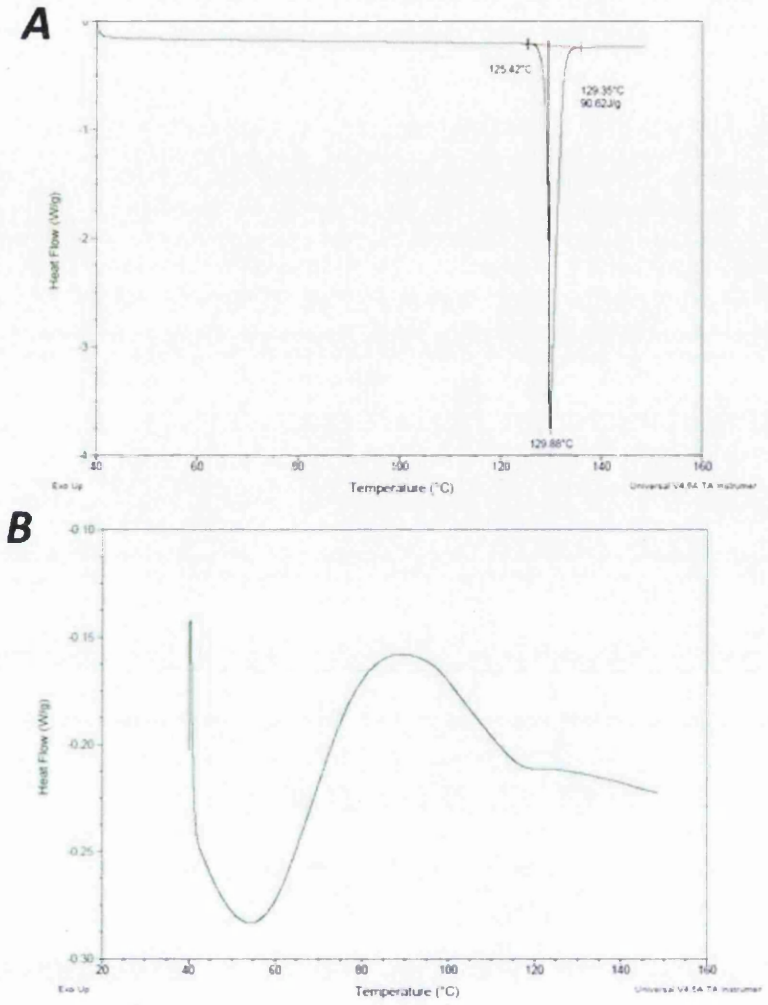


Figure 5.5 Differential Scanning Calorimetry of natural P_4 (A) and P_4-loaded HPMCAS HF microparticles. Data shows progesterone is in a crystalline state with a melting point of 129.35°C whereas progesterone-loaded HPMCAS microparticles are in an amorphous state.

Estimation of the progesterone content in the HPMCAS HF microparticles showed that drug loading efficiency was 89%. Thus, the encapsulation of progesterone within this polymer was dramatically improved compared to the Eudragit L100 polymer. This clear improvement in drug loading was also proven to be statistically significant by students t-test ($P<0.001$) (Figure 5.13).

As a result of the successful amorphous outcome and high loading efficiency with the HPMCAS HF polymer, progesterone was also loaded into HPMCAS polymers that dissolve at pH ≥5.5 (HPMCAS LF) and pH ≥6.0 (HPMCAS MF). These polymers were used in order to further characterise the use of HPMCAS for progesterone loading and also as controls for dissolution experiments.

Progesterone loaded HPMCAS-MF and LF microparticles were visualised by SEM. Figures 5.6 and 5.7 show SEM images of progesterone loaded HPMCAS MF and LF microparticles respectively. As with the progesterone-loaded HPMCAS HF microparticles, the particle size was variable and even though the surface layer of the microparticles appeared smooth, excess drug or polymer fragments could be seen on the surface.

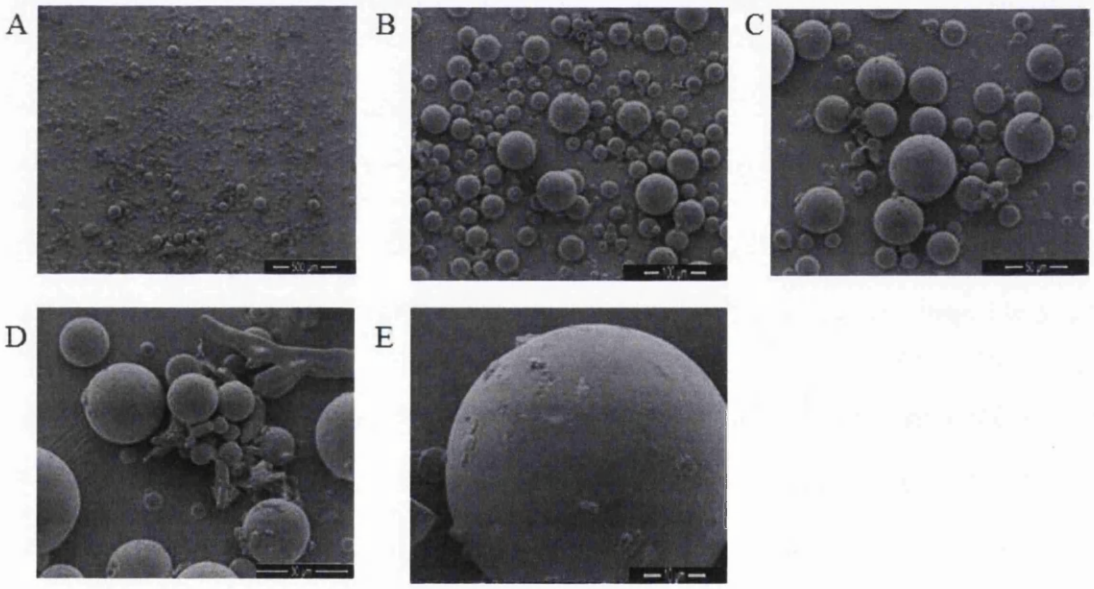


Figure 5.6 SEM micrographs of progesterone loaded HPMC AS-MF microparticles. A) 100x magnification, B) 500x magnification, C) 1000x magnification, D) 2500x magnification and E) 5000x magnification

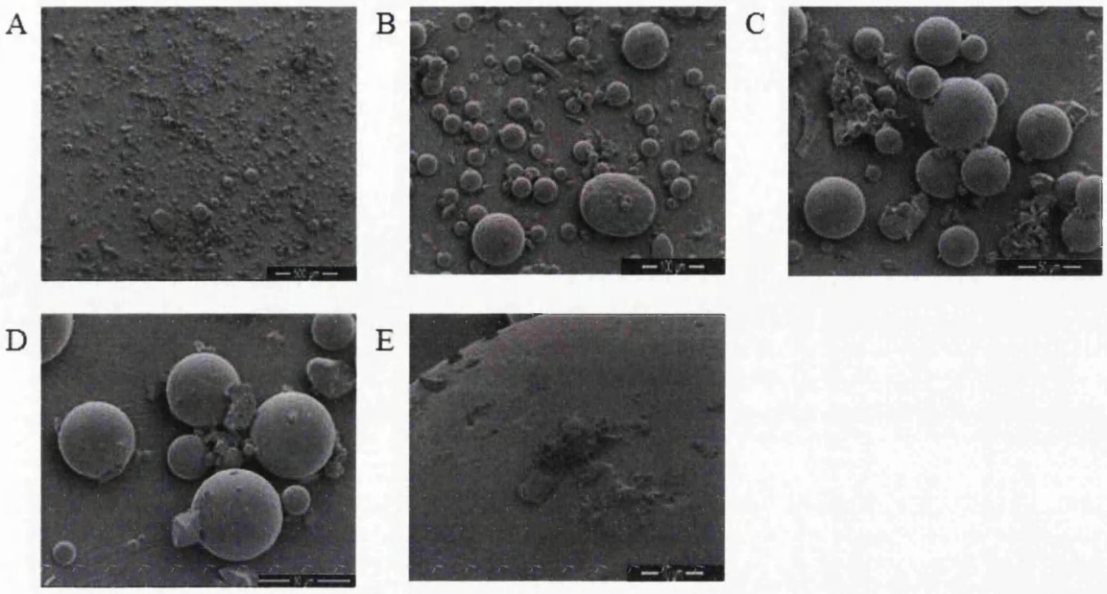


Figure 5.7 SEM micrographs of progesterone loaded HPMC AS-LF microparticles. A) 100x magnification, B) 500x magnification, C) 1000x magnification, D) 2500x magnification and E) 5000x magnification

The crystallinity of the progesterone-loaded HPMCAS MF and LF microparticles were once again characterized by PXRD and DSC. The PXRD pattern of the MF microparticles was devoid of any diffraction peaks compared to the crystalline pattern of the commercially available progesterone powder (Sigma Aldrich), indicating successful formation of amorphous progesterone (Figure 5.8A). Furthermore, very little recrystallization had occurred over a 2 year time-period suggesting the microparticle are highly stable (Figure 5.8B). As further evidence of amorphicity, the DSC thermogram of the MF microparticles showed the melting endotherm of crystalline progesterone was absent (Figures 5.9).

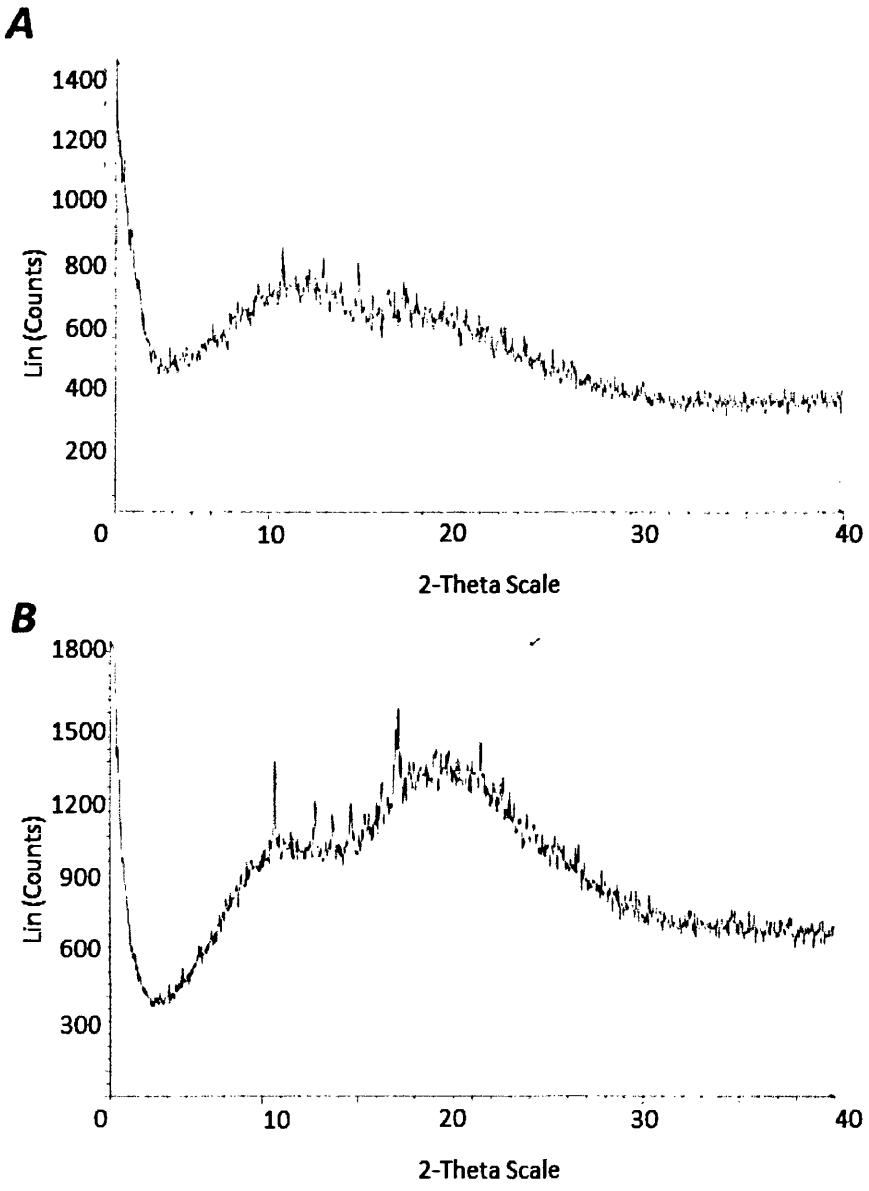


Figure 5.8 Powder X-ray diffraction of P_4-loaded HPMCAS MF microparticles. A. At time of production and B. 2 years post-production.

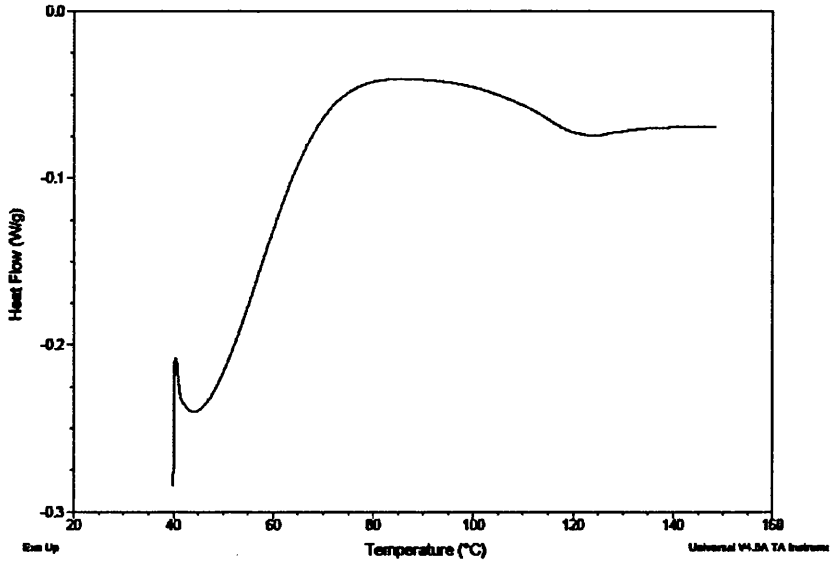


Figure 5.9 Differential Scanning Calorimetry of P_4-loaded HPMCAS MF microparticles. Data suggests microparticles are in an amorphous state.

Unlike the previous samples, progesterone microparticles with HPMCAS LF polymer showed some degree of crystallinity displaying two peaks. The formulation showed peaks at 2-theta values of 10.6° and 21.4° which corresponded with the peaks found in crystalline progesterone (Figure 5.10A). However, after 2 years of storage, here was little change in the PXRD pattern (Figure 5.10B)., suggesting that the microparticles were stable in this state Despite peaks being seen with PXRD, the melting endotherm of pure progesterone was absent from the LF microparticles on the DSC thermogram (Figure 5.11).

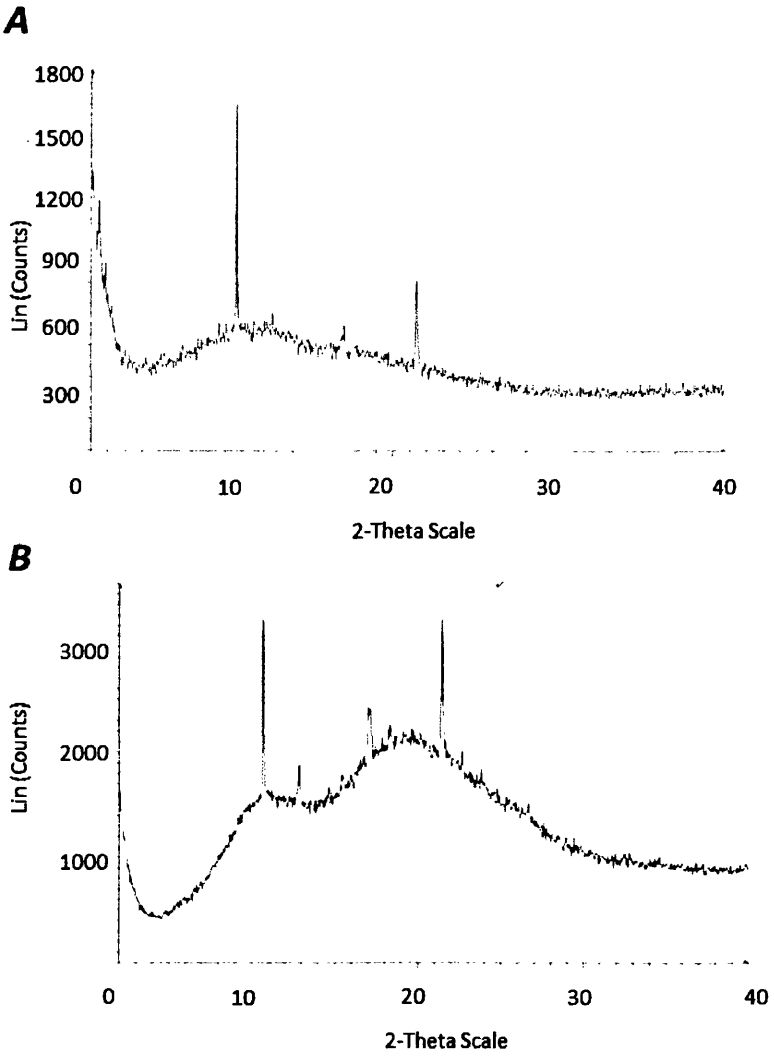


Figure 5.10 Powder X-ray diffraction of P_4-loaded HPMCAS LF microparticles. A. At time of production and B. 2 years post-production

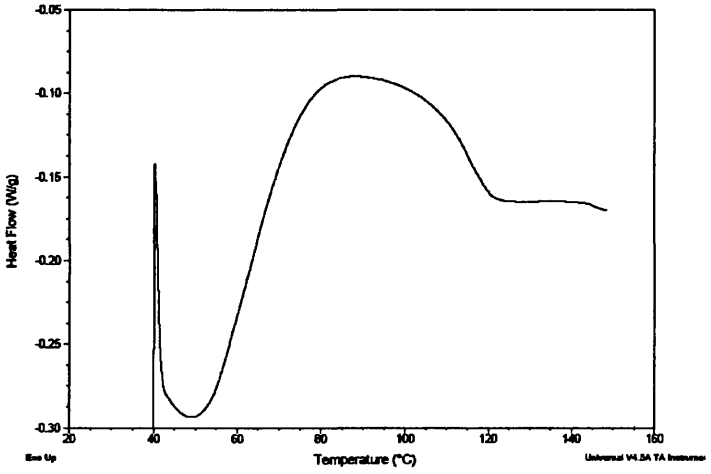


Figure 5.11 Differential Scanning Calorimetry of P_4-loaded HPMCAS LF microparticles. Data suggests microparticles are in an amorphous state.

Estimation of the progesterone content in the HPMCAS LF microparticles showed a high loading efficiency of over 90% whereas the encapsulation of progesterone in the HPMCAS MF was slightly lower at 70%. Nonetheless both were significantly improved compared to Eudragit ($P<0.001$). The chromatograms obtained for the encapsulation efficiency of progesterone within each polymer are shown in Figure 5.12; this data is depicted graphically in Figure 5.13.

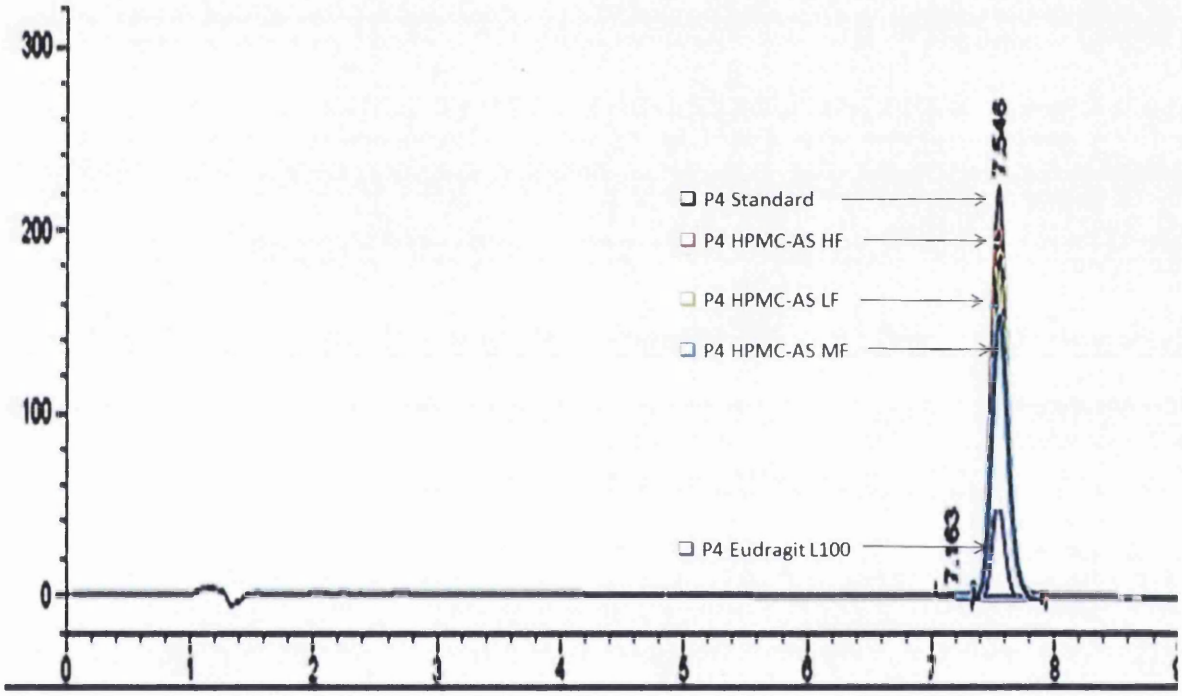


Figure 5.12: HPLC chromatogram obtained to assess encapsulation efficiency of progesterone in different polymers. Samples were eluted isocratically using a mobile phase consisting of 0.1% TFA in 50:50 acetonitrile and water. The column used was Zorbax 150 x 4.6 mm with 3.5µM particle size at 25^{0}C and the UV detector wavelength was set at 242 nm with a flow rate of 1ml/min. 100ul of sample was injected for each run.

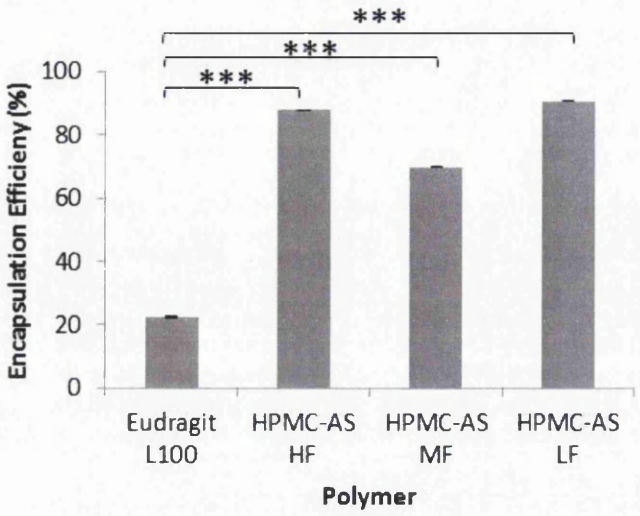


Figure 5.13: Encapsulation Efficiency (%) of progesterone in different polymers. Results represent average of 3 experimental repeats, error bars are +/- SD. Statistical analysis was performed using a Student T test ($p \leq 0.001$*)**

5.3.1.3 *In Vitro* drug release from progesterone-loaded HPMCAS microparticles.

The HPMCAS polymers utilised within this study each dissolve at a different pH; HPMCAS HF dissolves at pH 6.8, HPMCAS MF dissolves at pH 6 and HPMCAS LF dissolves at pH 5.5. Therefore, the release profiles of progesterone from HPMCAS HF, MF and LF was determined using the method described in Section 2.3.1.5 The dissolution of progesterone-loaded Eudragit L100 particles was not tested due to the poor encapsulation efficiency.

Release of progesterone into acid medium was well controlled from each ASD with less than 7% of the total drug content being released into the media after 120 minutes at pH 1.2 (Figure 5.14). Upon changing the pH to 5.5, 6.0 and 6.8 for HPMCAS LF, MF and HF microparticles respectively, rapid release of progesterone was achieved within 30 minutes of the change (Figure 5.14). For HPMCAS MF and HF microparticles drug release was high, with >85% of drug being detected after 2 hours in the alkaline medium. However, drug release form HPMCAS LF microparticles was significantly lower at only 59% ($P<0.001$), which could be related to the presence of crystalline drug within these microparticles (Figure 5.10). The limited

release of progesterone from these microparticles was not investigated further and the remaining characterisation of HPMC microparticles was carried out in HPMCAS MF and HF polymers.

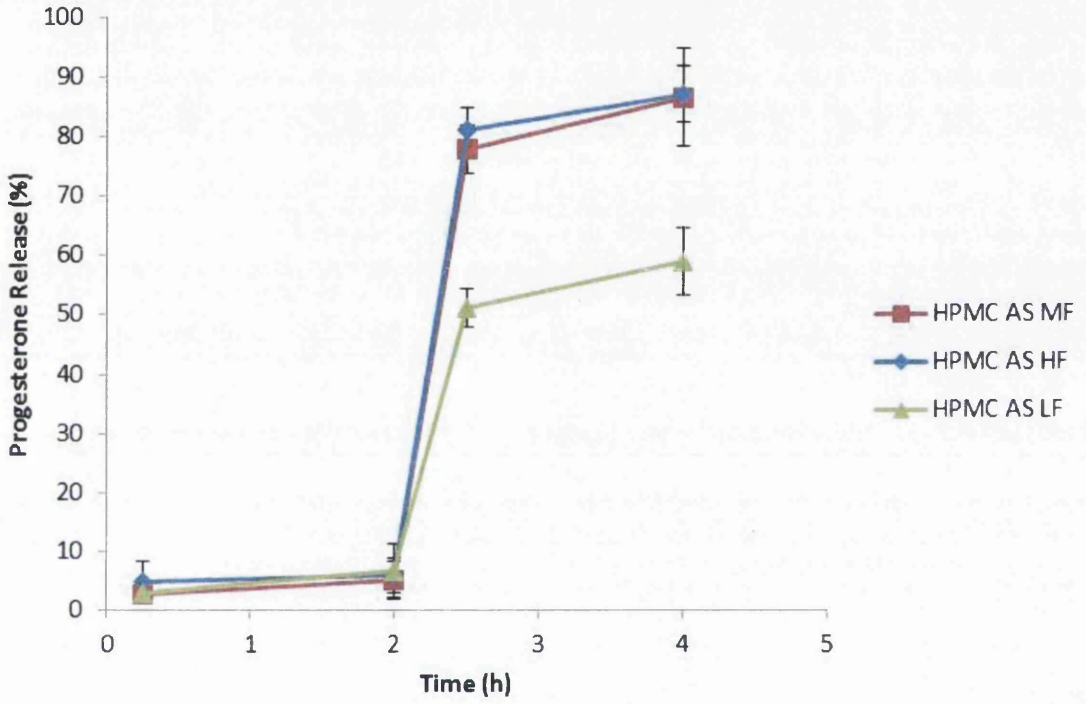


Figure 5.14: *In Vitro* release of progesterone from HPMCAS microparticles with time, using a pH change dissolution method (drug:polymer ratio 1:10) (n=3, error bars are +/- SD). pH was maintained at 1.2 for 2hours, this was then increased to 5.5, 6.0 or 6.8 for HPMCAS LF, MF and HF polymers respectively.

5.3.1.4 In Vitro permeability of progesterone and progesterone loaded microparticles

A number of different delivery approaches have been developed with an aim to improve the apparent solubility of lipophilic compounds but the use of many such approaches often cause a reduction in permeability. The use of surfactants, for example, can significantly decrease the free fraction of drug available for intestinal permeation (Miller et al., 2011). Such solubilisation methods rely on enhancing the

equilibrium solubility of the drug but, unlike other formulations, ASDs enable an unstable supersaturated solution to be attained. This difference leads to an increase in apparent solubility without compromising intestinal permeability (Miller et al., 2012).

To ensure the permeability of progesterone was not compromised during microparticle production, the permeability of natural progesterone and the progesterone loaded HPMCAS HF and MF microparticles was evaluated *in vitro* using the CACO-2 permeability assay as described in Section 2.3.3. Figure 5.15 shows the percentage of progesterone recovered from the basolateral compartment of the CACO-2 transwell at different time-points for up to 120 minutes and no statistically significant differences were observed between progesterone and progesterone loaded microparticles. Percentage was calculated based on the concentration determined in the apical compartment of the transwell at 0 minutes (16μM). As well as calculating the appearance of progesterone in the basolateral compartment of the CACO-2 transwell, the percentage of progesterone in the apical compartment was also calculated at 120 minutes and the data obtained was consistent between the natural product and the ASDs (Figure 5.16). Determining progesterone concentration in the apical and basolateral compartment allowed quantification of total progesterone recovery which was found to be more than 80% with each sample. Any progesterone not recovered was most likely retained in the CACO-2 cells.

In line with these findings, Yu and co-workers have shown that progesterone travels at a higher rate from the apical to basolateral compartment of the CACO-2 membrane than from the basolateral to apical. The groups suggested that this were due to the absence of P-glycoprotein driven efflux in the CACO-2 membrane (Yu et al., 1997). This may also explain why more than 50% of progesterone entered the basolateral compartment breaking the concentration gradient.

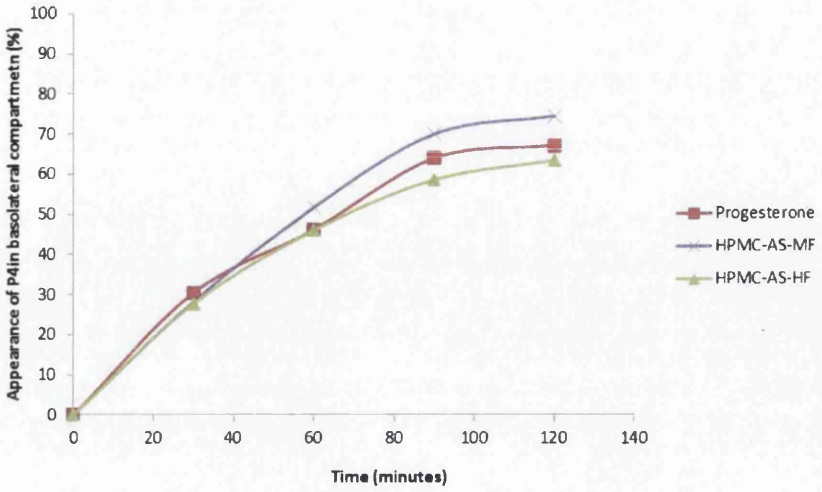


Figure 5.15. Appearance (%) of progesterone in basolateral compartment of CACO-2 cell line at several time-points for up to 120 minutes (n=3). CACO-2 cells were grown to confluency on transwell units and permeability to progesterone was assessed by HPLC. Error bars not shown for clarity but are detailed in Appendix N.

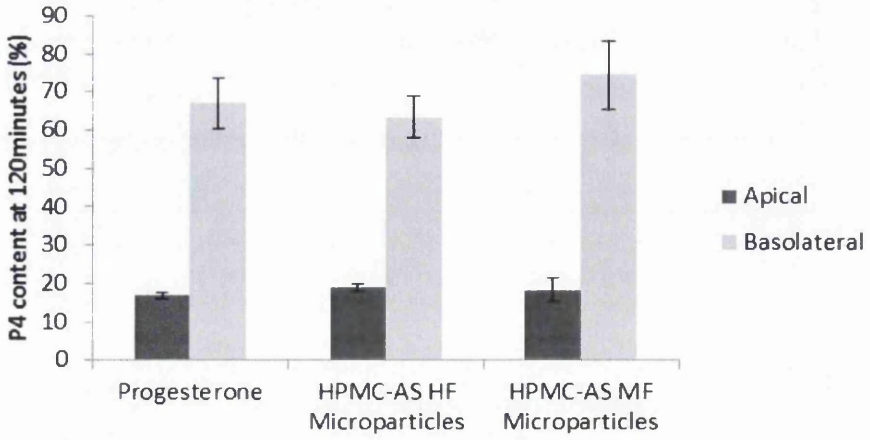


Figure 5.16. Percent of progesterone remaining in the apical compartment (dark grey) and percent appearance of progesterone in the basolateral compartment (light grey) of the CACO-2 transwell after 120 minutes of incubation (n=3, error bars are shown as standard deviation from the mean). Students t-test was used to assess for statistical differences between progesterone and progesterone loaded microparticles (P>0.05).

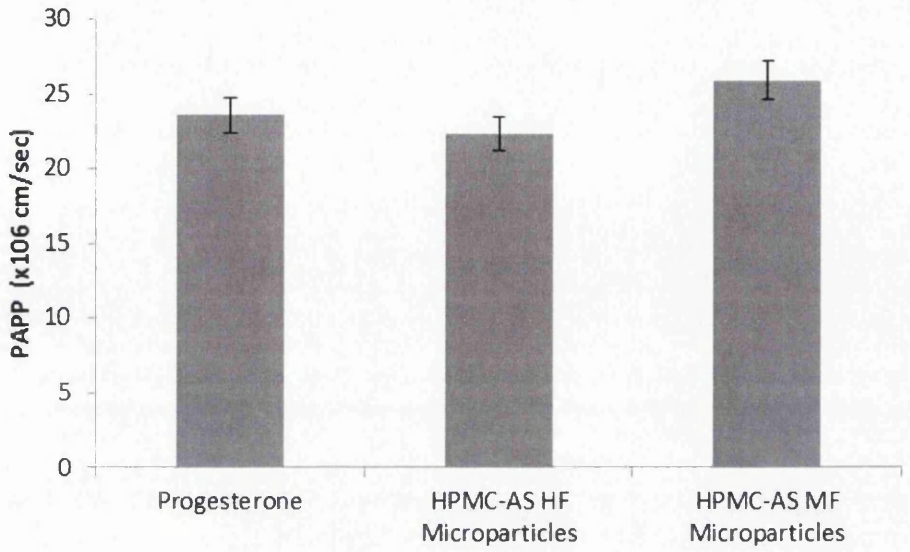


Figure 5.17. Apparent permeability (PAPP) of progesterone and progesterone-loaded HPMCAS HF and MF microparticles across the CACO-2 cell monolayer (n=3, error bars are shown as standard deviation from the mean). Students t-test was used to assess for statistical differences between progesterone and progesterone loaded microparticles (P>0.05).

To further investigate the permeability of progesterone, the data obtained was used to calculate the apparent permeability (P_{app}) as described in Section 2.3.3.8. The P_{app} of progesterone and progesterone loaded microparticles can been seen in Figure 5.17 and was found to be in line with findings reported by Krishna *et al* who also used the CACO-2 cell line to investigate the permeability of progesterone (Krishna et al., 2001). Moreover, no significant differences were observed when comparing progesterone and progesterone-loaded microparticles (P>0.05).

5.3.1.5 Apparent solubility of progesterone compared to progesterone loaded microparticles

It has been previously documented that amorphous solid dispersions can enhance the oral bioavailability of poorly water soluble drugs through enhanced apparent solubility (Leuner and Dressman, 2000, Vasconcelos et al., 2007). To assess whether progesterone microparticle produced with HPMCAS HF polymer caused an increase in apparent solubility, the shake-flask method was used to determine the apparent

solubility of progesterone compared to the progesterone-loaded microparticles (Section 2.3.4). The experiments were carried out in simulated intestinal fluids representing the fasted and fed state (FasSIF and FesSIF). After one hour in FasSIF the apparent solubility of progesterone was significantly increased from 25 to 30µg/ml (Figure 5.18B) and this was maintained for up to 24 hours. After 1 hour in FesSIF the apparent solubility of progesterone was significantly increased in the ASD from 85 to 129µg/ml (Figure 5.18A). Despite the increase in progesterone apparent solubility being significant an even greater increase that is in line with or more than currently available formulations would have been desirable to further improve oral bioavailability.

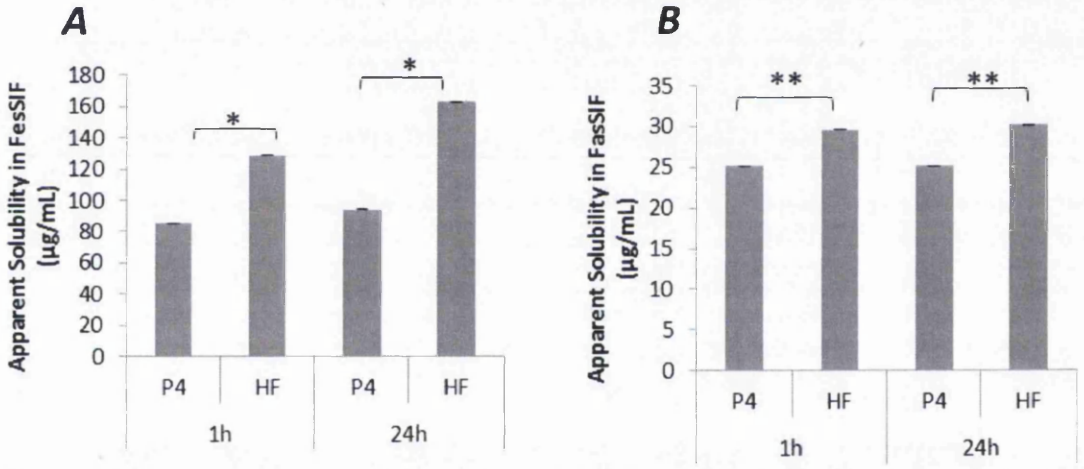


Figure 5.18: Apparent solubility of progesterone (P_4) and progesterone-loaded HPMCAS HF microparticles (HF) in simulated intestinal fluids of the A) fasted and B) fed state. Apparent solubility of progesterone was determined as described in Section 2.3.4. The solublity of unformulated (natural) P_4 was determined in the presence of an equivalent amount of drug polymer. N=3, error bars are shown as standard deviation from the mean.

During this study the apparent solubility of progesterone was improved through the production of progesterone amorphous solid dispersions. Previous work has suggested that alterations in the physical and chemical properties of a drug after formulation may have quality and clinical issues (Rahman et al., 2014). For example, if a solid dispersion formulation is not correctly prepared or properly stored the drug may begin to degrade, increasing the risk of impurities which can potentially lead to

unfavourable biological effects. As well as this, it is possible for excipients to interact either chemically or physically with the drug which can lead to alterations in the chemical nature of the drug and as a consequence alter there their therapeutic safety (Bharate et al., 2010). Although this process is unlikely to occur and any particulates are unlikely to reach the target cells after oral dosing it is still important to assess any differences in gene regulation by the formulated drug.

Therefore, the biological effect of P_4 ASD, its basic signalling mechanisms and gene regulation was compared to the unformulated drug. Thus, the remainder of the chapter will assess the regulation of progesterone target genes and proteins comparing P_4 ASD to unformulated P_4. This will also further confirm the findings obtained in chapter 3 of this study.

5.3.2 Regulation of Genes and Proteins by Progesterone and Progesterone-Loaded Microparticle

To establish whether target gene and protein regulation is affected by progesterone loaded ASDs, their regulation was analysed in the Ishikawa and HESC cell line after incubation with progesterone and progesterone-loaded HPMCAS HF microparticles. The target genes and proteins to be assessed are described in detail in chapter 3, the known progesterone-regulation of these targets will, therefore, only be discussed briefly within the context of this chapter. Furthermore, the data obtained within this section will not be critically analysed this has been done in Chapter 3. The purpose of this section is to compare gene and protein regulation between P_4 and the P_4 ASD.

5.3.2.1 FOXO1 is up-regulated by P4 and P4-ASD in the Ishikawa and HESC cell line

Progesterone-induced FOXO1 up-regulation has been well characterised, and is shown to occur specifically through progesterone binding to the PR-B (Kyo et al., 2011, Wang et al., 2009). Data obtained in Section 3.3.3. confirmed that FOXO1 is significantly up-regulated by progesterone in the Ishikawa cell line and this was again replicated in this chapter whereby PR RNA was induced 1.9 fold ($P<0.05$) with natural P_4 treatment (Figure 5.19 Panel B). Treatment of Ishikawa cells with

progesterone ASD also caused a statistically significant, 1.9 fold increase in FOXO1 gene expression (Figure 5.19 Panel B). Thus both P_4 and the P_4 ASD regulate the FOXO1 gene in a similar manner. This is also the case in the presence of E_2 whereby there was a 1.6 fold increase in FOXO1 gene expression after both treatments (Figure 5.19 Panel B). Akin to FOXO1 RNA, FOXO1 protein in Ishikawa cells was also upregulated after treatment with P_4 or P_4 ASD in the presence and absence of E_2. However, at protein level the up-regulation of FOXO1 was significant after each treatment and in each case the up-regulation was found to be between 1.6 and 1.7 fold (Figure 5.19C). Crucially, there was no statistical difference in FOXO1 regulation between P_4 and P_4 ASD.

Since FOXO1 is able to translocate in and out of the nucleus (Lengyel et al., 2007, Ward et al., 2008), the cellular location of this protein was also analysed after treatment with P_4 and P_4 ASD in the presence and absence of estrogen. The cellular location of the FOXO1 protein was similar between all four treatment regimens, whereby FOXO1 staining was much higher in the nucleus than in the cytoplasm (Figure 5.19D). The nuclear position of FOXO1 could also be visualised in the InCell images (Figure 5.19A)

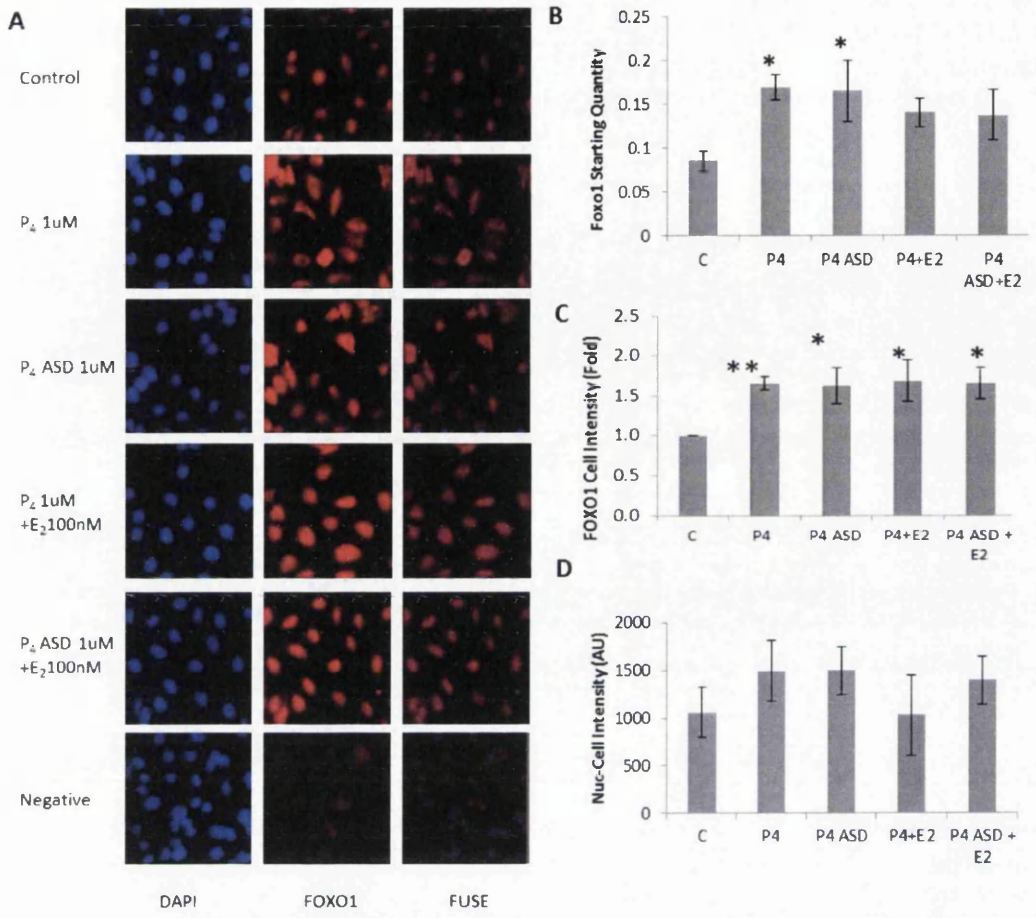


Figure 5.19. FOXO1 gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only.

A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-

Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

The regulation of FOXO1 RNA in HESC cells was also similar between P_4 and P_4 ASD whereby neither treatment alone upregulated FOXO1 in the HESC cell line. However, when combined with cyclic AMP, FOXO1 RNA was up-regulated 3-fold or more (Figure 5.20 B). A similar pattern was seen with FOXO1 protein whereby no change was observed after treatment with P_4 or P_4 ASD alone, but after the combined treatment with cyclic AMP, FOXO1 was significantly induced by 1.4 ($P<0.01$) and 1.7 ($P<0.02$) fold by P_4 and P_4 ASD respectively (Figure 5.20 C). The induction of FOXO1 can be visualised in the images obtained from the InCell Analyser in Figure 5.20 A. Unlike the Ishikawa cell line, the intensity of the FOXO1 protein stain appeared to be across the whole cell as opposed to just in the nucleus (Figure 5.20A). To determine whether this was true the average intensity for the whole cell was subtracted from the nuclear intensity. Although, there was some elevation in nuclear protein compared to cytoplasmic protein, this increase was small in both the control and progestin treatments. Moreover, the protein position was similar after P_4 and P_4 ASD treatment both in the presence and absence of cyclic AMP (Figure 5.22 D).

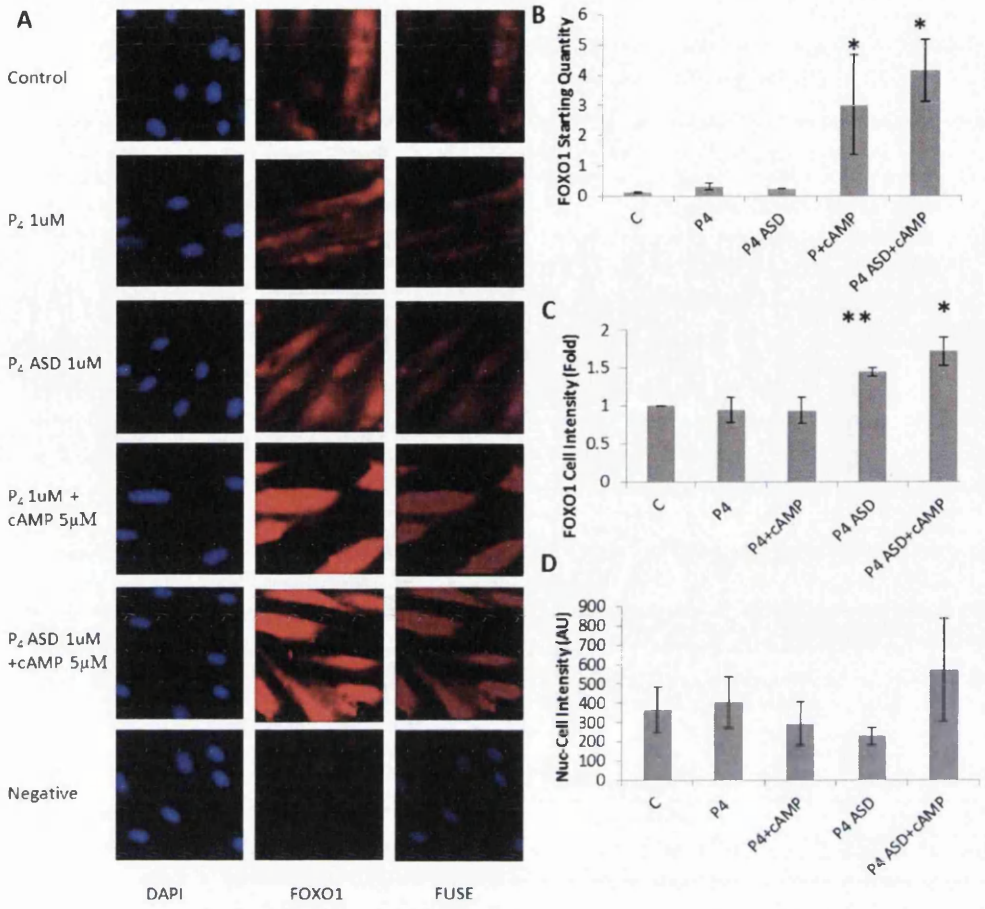


Figure 5.20 FOXO1 gene and protein expression in the HESC cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained FOXO1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of FOXO1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

5.3.2.2 P4 and P4-ASD similarly regulated the Progesterone Receptor in the Ishikawa and HESC cell lines.

Progesterone has been widely reported to down-regulate the PR (Graham and Clarke, 1997b) whereas estrogen has been reported to up-regulate the PR in human endometrial epithelium (Hata and Kuramoto, 1992, Jamil et al., 1991). Work carried out in Chapter 3.3.3 contradicted the literature whereby PR was up-regulated by 4 fold or more by progesterone in the endometrial cancer cell line. This finding was also significantly different to PR regulation by the progestins. In this chapter both P_4 and P_4 ASD significantly upregulated PR RNA; whereby an induction of 5-fold or more was seen in the absence and presence of E_2 (Figure 5.21 B). PR protein was also highly up-regulated after treatment with P_4 or P_4 ASD by 6.1 and 4.5 fold respectively. A more modest induction was seen after combined treatment of P_4 or P_4 ASD with E_2 whereby PR protein was upregulated by 2.6 and 2.9 fold respectively (Figure 5.21C).

Like FOXO1, PR is also a transcription factor able to shuttle in and out of the nucleus (Leonhardt et al., 2003a), therefore the cellular location of this protein was also analysed after treatment with P_4 and P_4 ASD in the presence and absence of estrogen.The cellular location of the PR protein also changed after P_4 treatment whereby there was a greater difference between cell and nuclear intensity after treatment and this was the case with each treatment regimen (Figure 5.21 D). Interestingly, the loss of PR is associated with poor prognosis in endometrial cancer, which is, in turn, is treated with synthetic progestins which serve to down-regulate this hormone receptor (Jänne et al., 1979). However, there was an increase in PR protein after P_4 and P_4 ASD treatment in Ishikawa cells, thus suggesting that natural progesterone treatment could have greater benefits in the treatments of some endometrial cancers, further promoting the need for a natural compound and a place in the market for the formulation presented herein.

Thus, in the Ishikawa cell line, P_4 ASD acts in a similar manner to the natural drug, unlike the synthetic progestins analysed in Section 3.3.4.2.

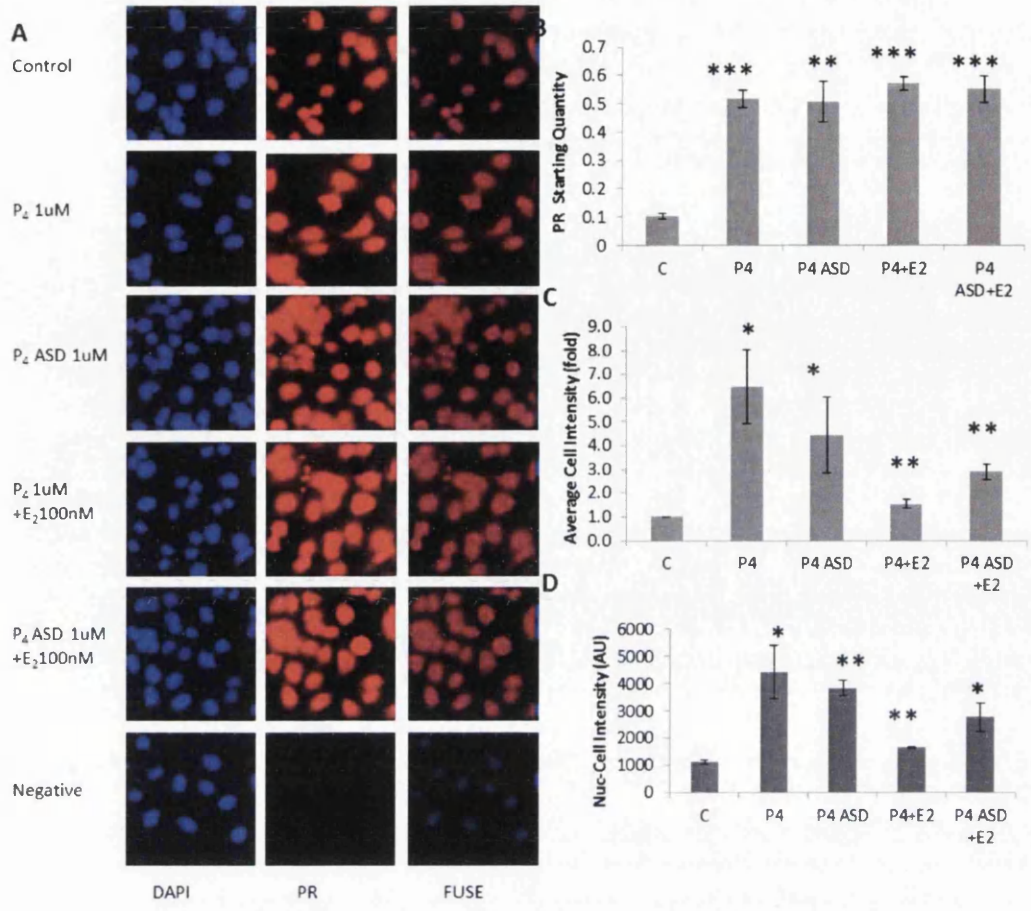


Figure 5.21. PR gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

Previously documented work has shown that PR regulation in stroma differs from in epithelial cells whereby an induction of up to 10-fold has been reported (Tseng and Zhu, 1997). Akin to data obtained in Section 3.3.5.2, a 1.6 fold increase was seen in PR RNA after treatment with P_4 in HESCs and similarly, a 1.9 fold increase was observed after treatment with the P_4 ASD in HESCs. Although there was no significant difference between the treatments themselves, the PR up-regulation instigated by P_4 ASD was significant compared to control unlike the treatment by natural P_4 (Figure 5.22 B). This induction, however, was not observed at protein level (Figure 5.22 A and C).

Data obtained in Chapter 3 revealed that P_4 is able to significantly up-regulate PR in HESC cells in the presence of cyclic AMP. This observation was further confirmed in this chapter whereby PR RNA was up-regulated 1.9 fold and 2.3 fold after treatment with cyclic AMP+P_4 and cyclic AMP+P_4 ASD respectively (Figure 5.22 B). When assessing the PR protein after the same treatment a smaller but significant increase in PR was detected whereby P_4 combined with cyclic AMP upregulated PR by 1.5 fold ($P<0.01$) and P_4 ASD plus cyclic AMP upregulated PR by 1.4 fold ($P<0.01$). Although PR protein increased, the intensity of PR stain in the nucleus did not change with any treatment suggesting that progesterone had no effect on PR cellular location in the HESC cells (Figure 5.22 D).

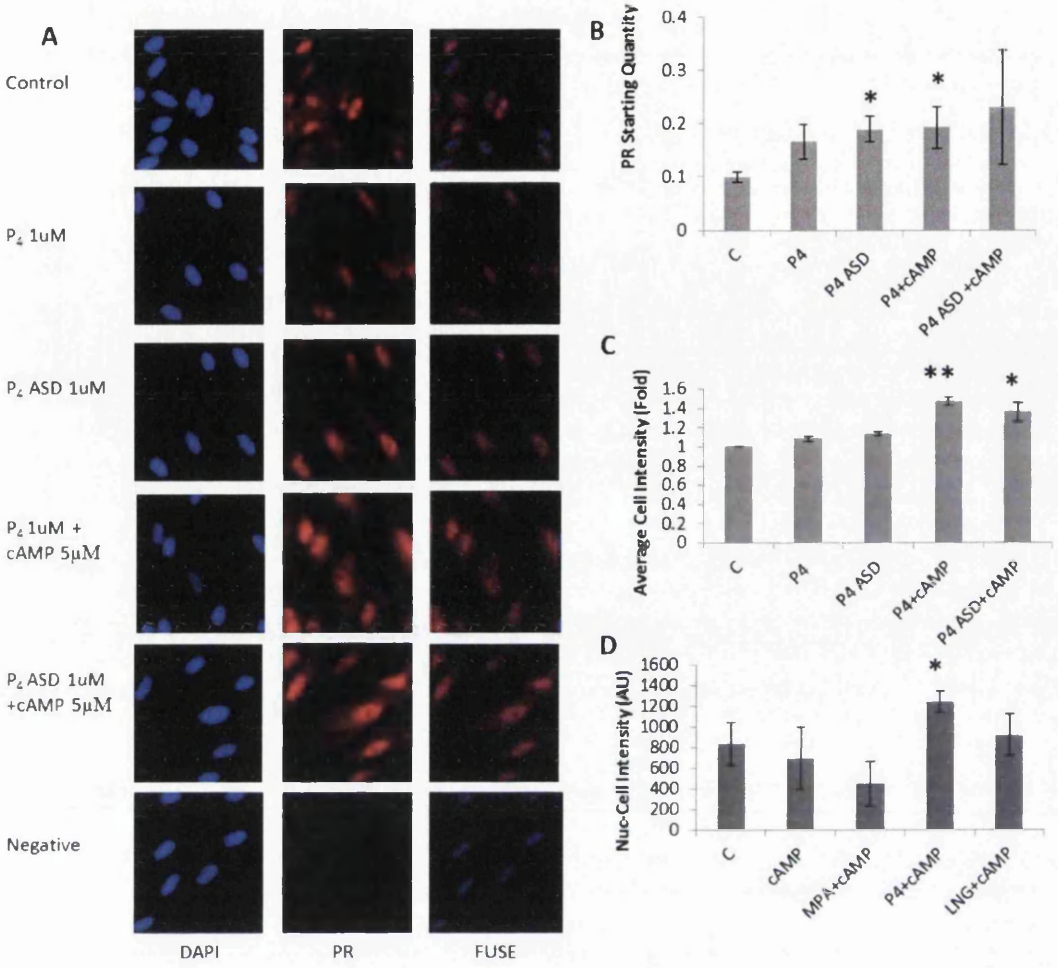


Figure 5.22. PR gene and protein expression in the HESC cell line following hormone treatment for 48h. A) Fluorescent images showing PR fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained PR protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of PR normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. D) Cell and Nuclear Intensity. Cell intensity accounts for the average intensity of the whole cell whereas nuclear intensity accounts for the intensity found above the DAPI stain only. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

5.3.2.3 Differential regulation of Lactoferrin in the Ishikawa cell line by P4 and P4-ASD was observed at RNA level but no difference was observed at protein level. There was no difference in P4 or P4 ASD-induced lactoferrin regulation in the HESC cell line.

Work carried out in Section 3.3.4.4 showed Lactofferin is significantly down-regulated in the Ishikawa cell line after progesterone treatment and this finding was in line with previous documentation (Kurita et al., 2000, Conneely and Jericevic, 2002). The P_4 induced down-regulation of LF was again confirmed in this chapter whereby P_4 significantly down-regulated LF RNA by 0.01 fold ($P<0.001$). P_4 ASD also significantly down-regulated LF RNA in Ishikawa cells ($P<0.01$), however, this reduction was by 0.17 fold which was significantly less than the down-regulation recorded after treatment with unformulated P_4 ($P<0.05$) (Figure 5.23 B). The combined treatment of P_4 or P_4 ASD with E_2 also caused LF RNA to be reduced but there was no difference between the treatments in this instance (Figure 5.23 B). Furthermore, differences seen between P_4 and P_4 ASD at gene level were not seen at protein level (Figure 5.23 A and C), this may have been down to the sensitivity of the antibody used for the InCell analysis or possibly due to the enhanced down-regulation of LF gene by P_4 not being great enough to cause a significant difference in the levels of LF protein.

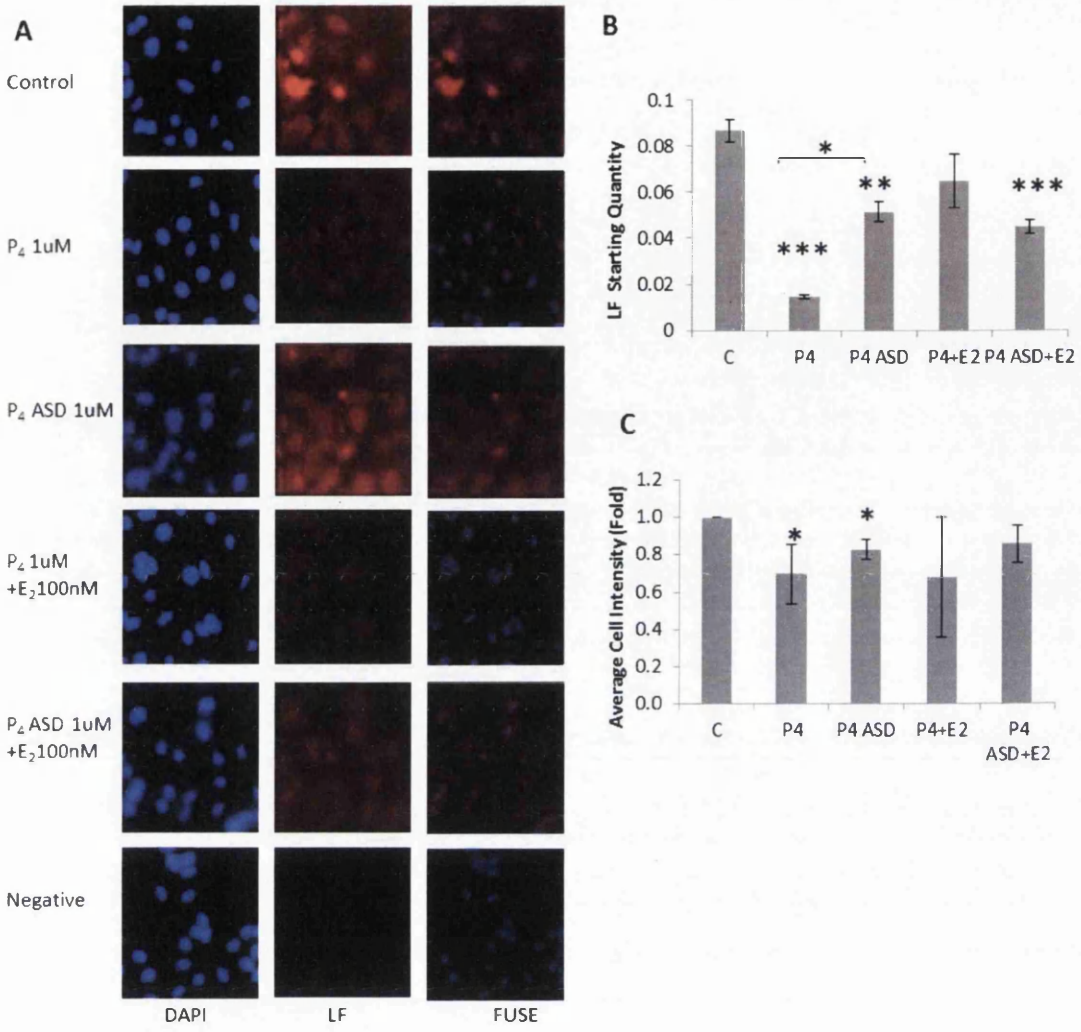


Figure 5.23. LF gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

In the HESC cell line P_4 caused a significant down-regulation of LF RNA by 0.6 fold whereas P_4 ASD caused a 0.55 fold down-regulation of LF but this was not significant compared to control (Figure 5.24 B). When observing the amount of LF protein after the same treatments it was found that both P_4 and the P_4 ASD significantly down-regulated LF at similar levels (Figure 5.24 A and C). The addition of cyclic AMP to P_4 and P_4 ASD enhanced the overall down-regulation of LF RNA (Figure 5.24 B) but such a drastic change was not observed with LF protein (Figure 5.24 C). The regulation of LF by cyclic AMP has not been previously reported and cyclic AMP alone was shown not to influence LF in HESC cells in Chapter 3.3.5. Suggesting synergistic action between progesterone and cyclic-AMP in the regulation of this target.

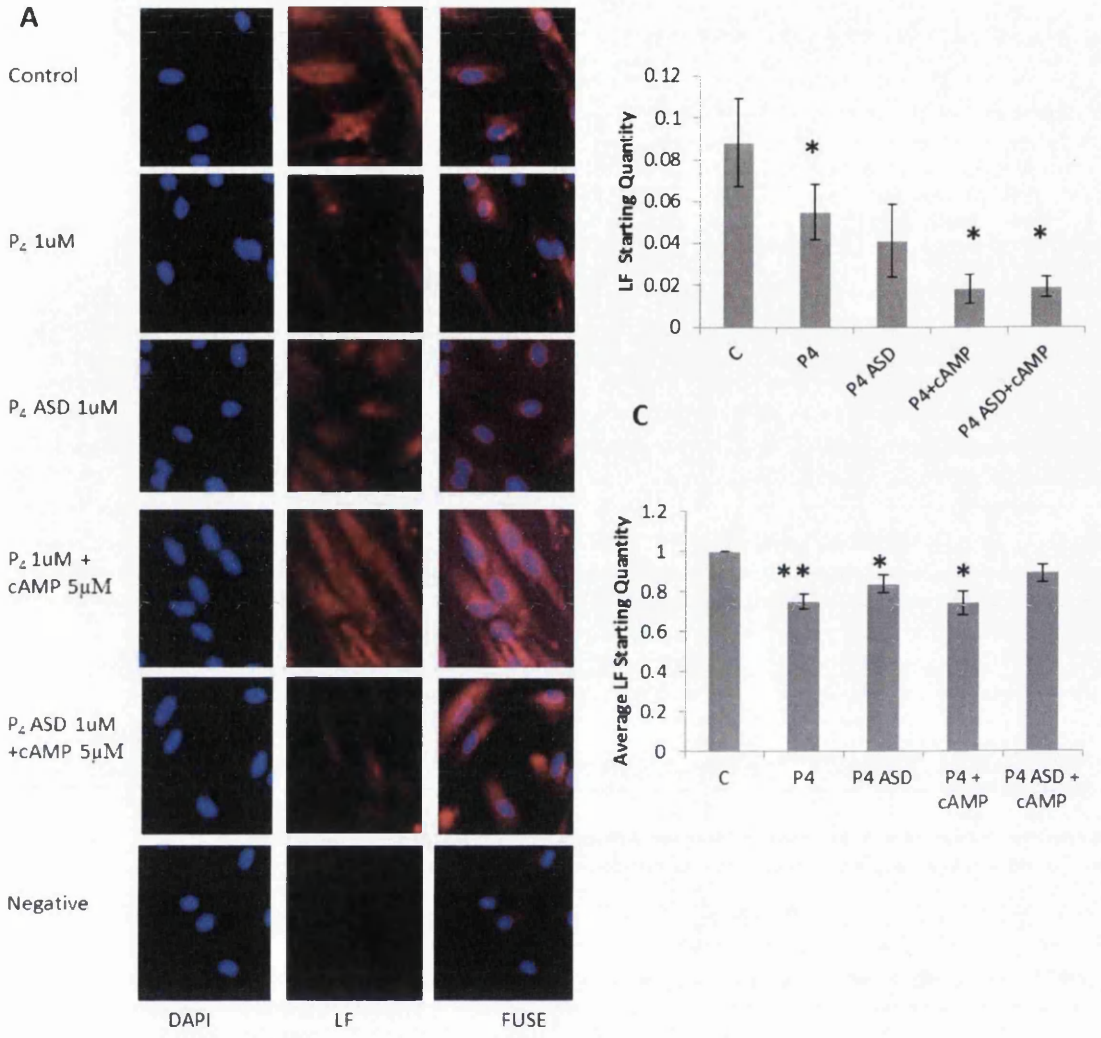


Figure 5.24: LF gene and protein expression in the HESC cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained LF protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of LF normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A. InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

5.3.2.4 P4 and P4-ASD do not effect AREG expression in the Ishikawa cell line.

Although a rise in AREG expression has been reported in the human endometrium with rising levels of P_4 (Gui et al., 2008a), no significant change in AREG RNA or protein was observed in the Ishikawa cell line after treatment with P_4 or P_4 ASD in both the presence and absence of estrogen (Figures 5.25). This was also the case with progesterone and the progestins in Chapter 3.3.3. It is worth noting that Gui and colleagues described AREG expression in healthy endometrium whereas this study investigates AREG regulation by PR in an endometrial cancer cell line, therefore, differences observed may be attributed to healthy versus disease models.

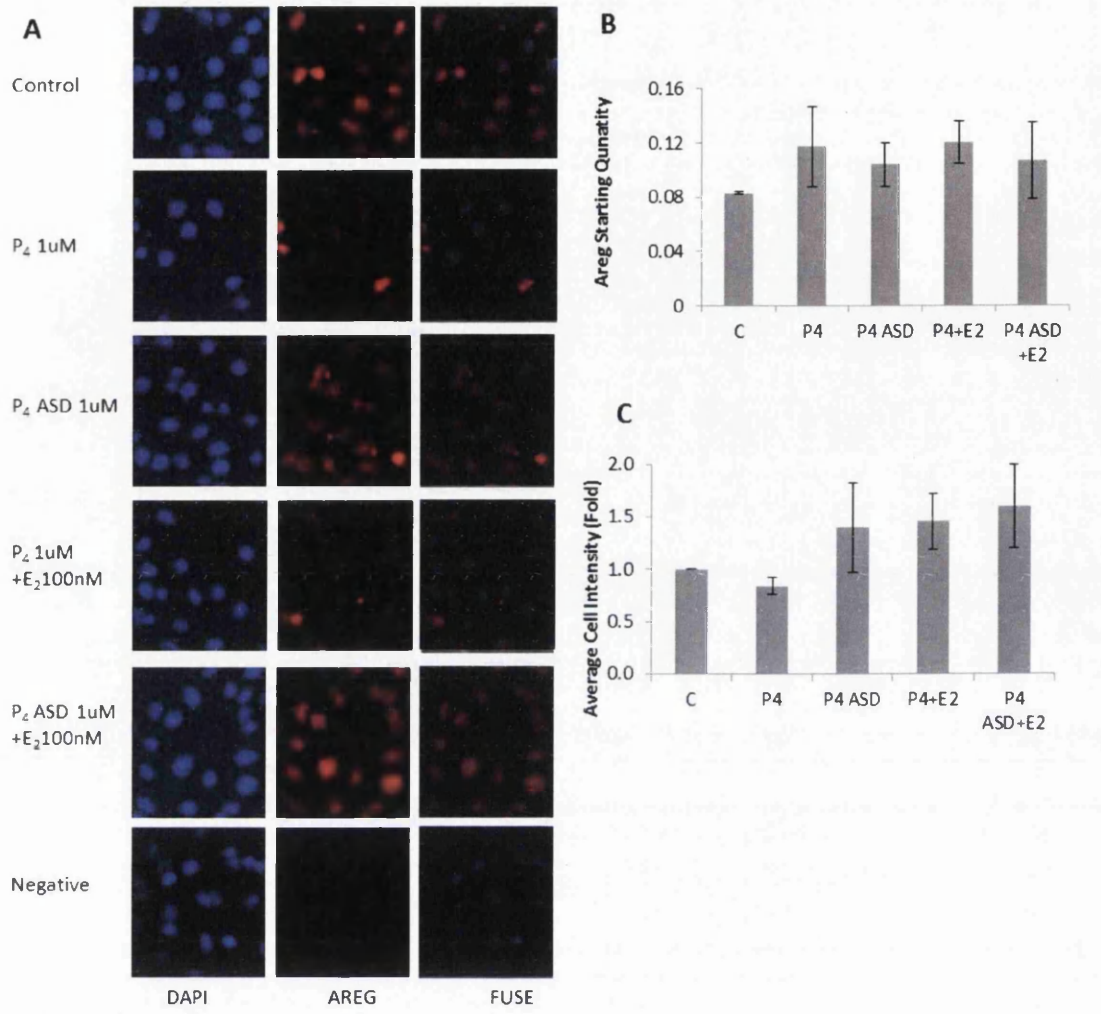


Figure 5.25. AREG gene and protein expression in the Ishikawa cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained AREG protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of AREG normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

5.3.2.5 GREB1 RNA and protein are significantly altered by P4 and P4-ASD in the presence of cyclic AMP in the HESC cell line.

Work carried out in Section 3.3.5.4 showed that GREB1 is significantly up-regulated by progesterone in combination with cyclic AMP. To see whether the same effect occurs after treatment with P_4 ASD, HESC cells were incubated with P_4 or P_4 ASD in the presence and absence of cyclic AMP. In the absence of cyclic AMP no change was observed in GREB1 RNA or protein, however, in the presence of cyclic AMP GREB1 RNA was upregulated 3.2-fold by P_4 and 2.5-fold by P_4 ASD (Figure 5.26 B) though this was only significant with P_4 ($P<0.05$). At protein level a significant increase in GREB1 was observed (Figure 5.26 A and C) after combined treatment of cyclic AMP with both P_4 and P_4 ASD. The increase was similar with both treatments though in contrast to RNA it was more significant after P4 ASD ($P<0.01$) (Figure 5.26 B). As seen in Figure 3.29, cyclic AMP alone also caused a significant induction of GREB1 making it unclear what influence progesterone has on this target. Regardless of this, both P_4 and P_4 ASD did not differentially regulate the GREB1 gene or protein.

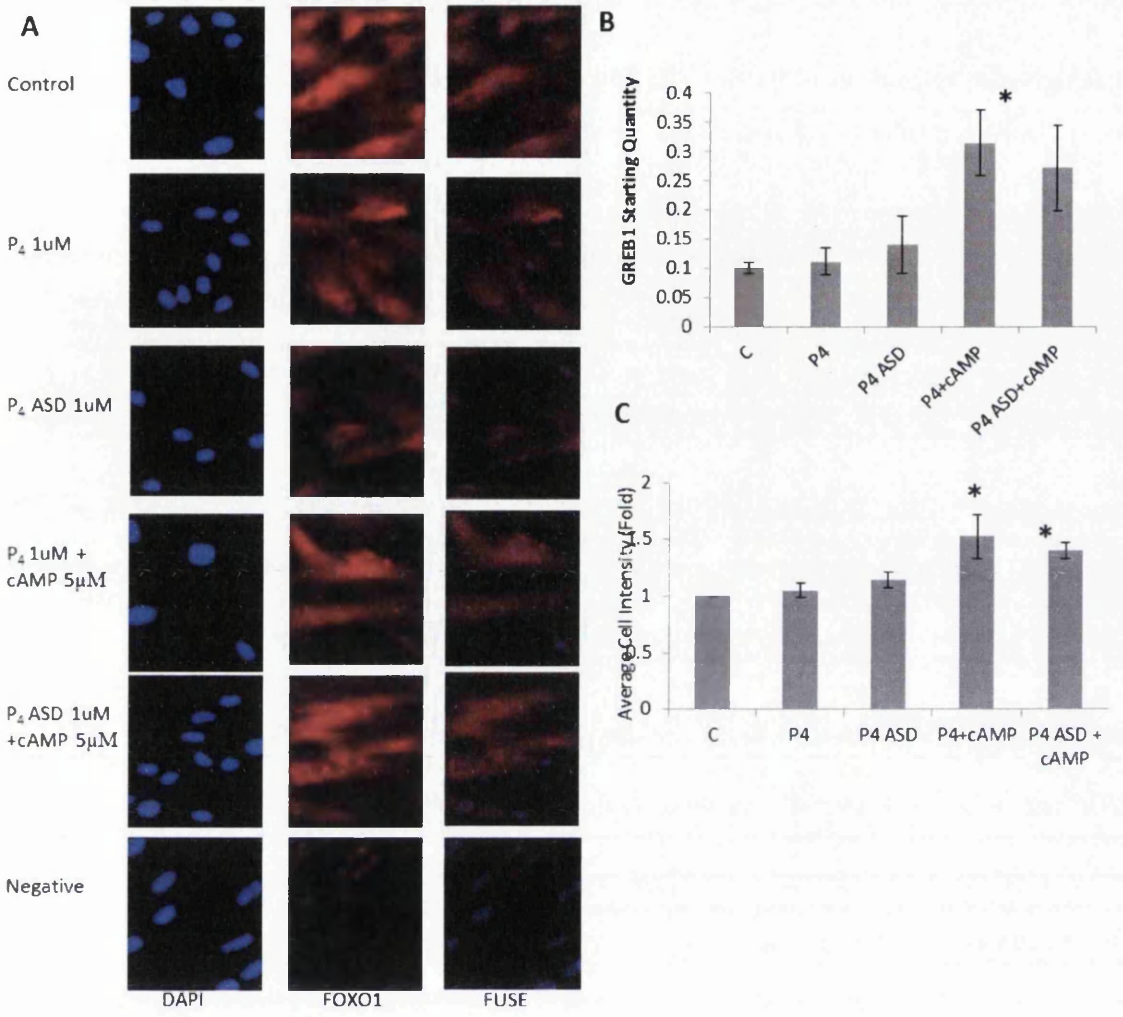


Figure 5.26: GREB1 gene and protein expression in the HESC cell line following hormone treatment for 48h. A) Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained GREB1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. B) RT-PCR data showing basal transcript level of GREB1 normalised to GAPDH. C) Cell intensity data obtained from InCell Analyser Workstation based on an average of 3 repeats observing at least 3 fields of view within each repeat. Data is represented as fold. A negative control containing Ishikawa cells incubated with secondary antibody only can be seen in Figure A; InCell Analyser Data is normalised to background based on this negative. Error bars represent standard deviation from the mean of 3 repeats. Data was analysed using a two-tailed students T-Test (*P<0.05, **P<0.01, ***P<0.001). All data presented in this section was subjected to the same analysis.

5.3.2.6 Decidual PRL is similarly regulated by P4 and P4 ASD in the presence and absence of cyclic AMP in HESC cells.

Progestogens alone are very weak inducers of the PRL gene, yet, the role of progestogens in PRL regulation is important as they synergistically enhance cAMP-induced PRL expression (Gellersen et al., 1994, Telgmann et al., 1997, Brosens et al., 1996, Brar et al., 1997, Tang and Gurpide, 1993). Therefore PRL gene expression was quantified after treatment with P_4 or P_4 ASD in the presence and absence of cyclic AMP. After progestogen treatment in the absence of cyclic AMP no change in PRL was observed as expected (Figure 5.27 A). However, the addition of cyclic AMP caused PRL gene expression to increase by more than 5700 fold with P_4 and by almost 4000 fold with P_4 ASD, which was highly significant by t-test in both incidences ($P<0.001$) (Figure 5.27 B).

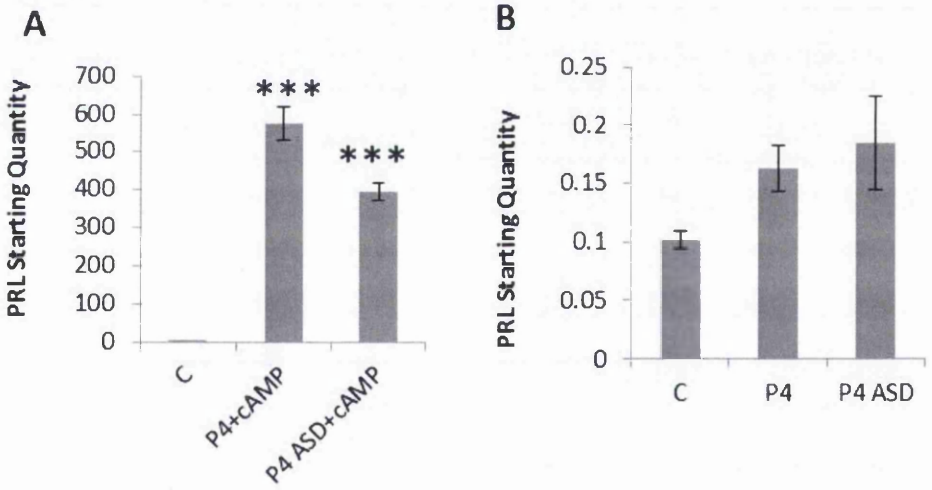


Figure 5.27. RT-PCR data showing basal transcript level of PRL normalised to GAPDH in HESCs after 48h of 1µM of P_4 treatment in A) the absence and B) presence of 5µM of cyclic AMP.

As the resulting PRL protein is secreted into surrounding media, ELISA was used for protein detection. In line with findings at RNA level, PRL was also significantly induced by P4 and P4 ASD in the presence of cyclic AMP but remained unchanged in its absence (Figure 5.28).

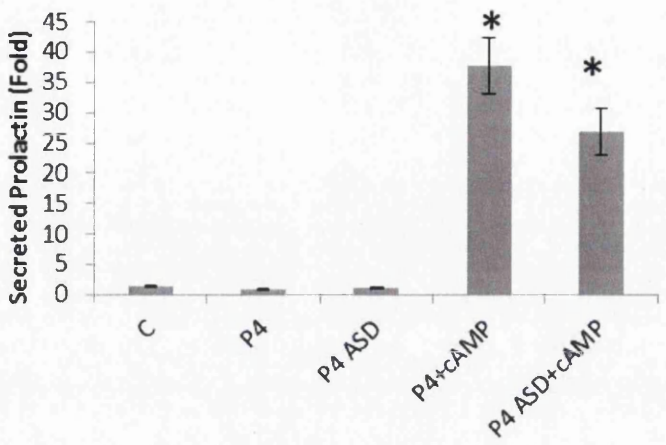


Figure 5.28: Secreted PRL levels in HESC cells in response 48hours of treatment.

The ELISA technique was conducted to measure hormone-stimulated levels of secreted PRL in HESCs. Values are given as average and standard deviation. Student's t-test was used to test significance. Significant changes are represented by * (*, P≤0.05 **, P≤0.01).

5.3.2.7 The HESC cell line decidualises after treatment with P4 and P4 ASD combined with cyclic AMP but not with P4 or P4 ASD treatment alone.

As described in Chapter 3, the increase in PRL expression is indicative of stromal decidualisation, therefore the extent of decidualisation was visualised using light microscopy and further confirmed by calculating the level of cellular roundness using Image J where a value of 1 represents a perfect circle and this value decreases with a reduction in circularity.

After treatment with either P_4 or P_4 ASD, no change in HESC decidualisation could be observed visually and this was further confirmed with image J (Figure 5.29). However, the addition of cyclic AMP to both treatments caused HESC cells to decidualise at a similar level. Cellular decidualisation can be seen in Figure 5.29. A change in cellular-roundness, indicative of decidualisation, was also confirmed with image J and was found to be significant and at a similar level after both combined treatments.

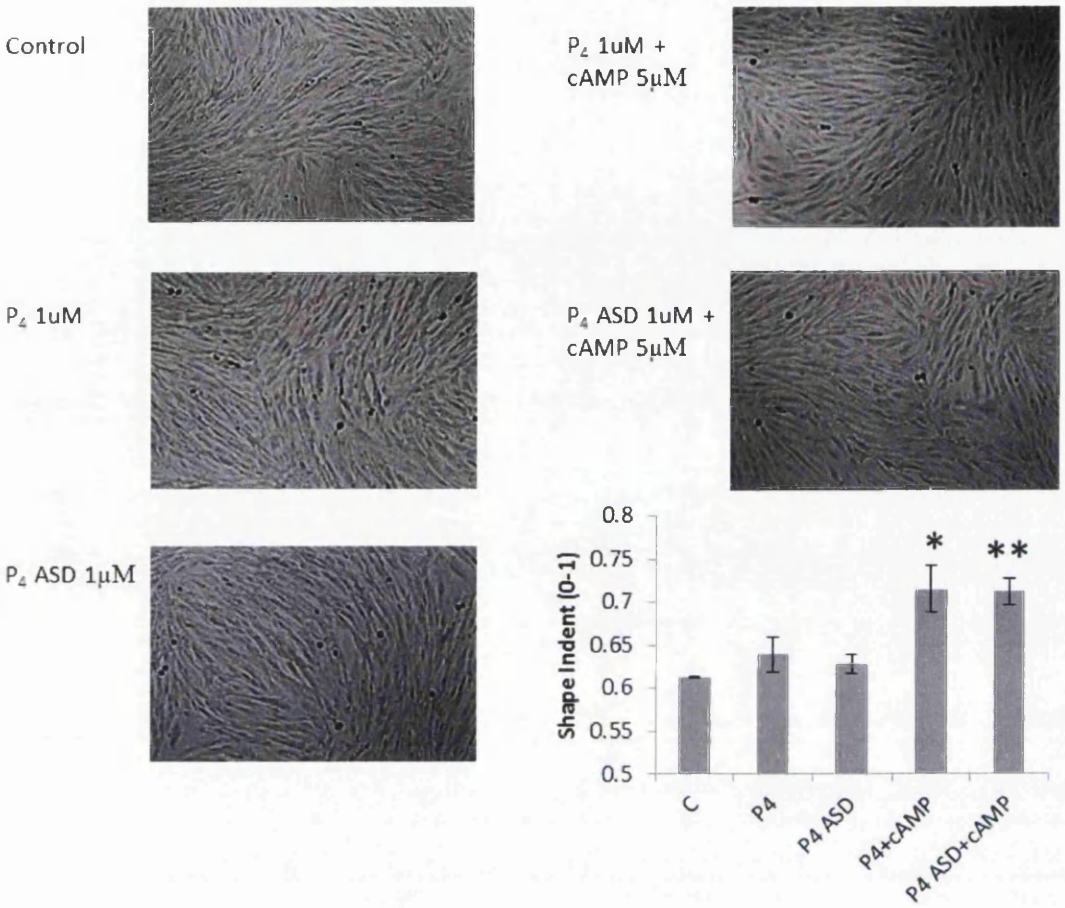


Figure 5.29. Light microscope images and graph showing extent of HESC cell roundness after 48 hours of treatment. Cell roundness was calculated using ImageJ software.

5.4 Discussion

At present there is no natural progesterone formulation available commercially for the treatment of endometrial hyperplasia. Progesterone is an excellent candidate for such a treatment as it occurs naturally in the body at high concentrations and therefore does not pose any toxicity problems unlike synthetic progesterone derivatives (Hapgood et al., 2004, Birrell et al., 2007). The absence of an oral progesterone formulation is, in part, due to its low aqueous solubility and poor dissolution kinetics, which results in incomplete digestive absorption. As well as this, when administered orally progesterone is subject to extensive first pass metabolism

in both the liver and intestines as detailed in Chapter 4. This combination of factors means that progesterone is pharmaceutically inactive when delivered orally.

In order to enhance the oral bioavailability of progesterone, several delivery methods have been patented. For example, Maxson *et al* (1990) dissolved micronized progesterone in glycerides and polyunsaturated fatty acids and Bensins and Besse (2008 and 2012) invented a pharmaceutical composition comprising micronized progesterone, soya bean lecithin and one of a number of naturally occurring oils (Besins and Besse, 2012, Maxson et al., 1990, Besins and Besse, 2008). The use of micronized progesterone in the current patents serves to enhance the dissolution rate of progesterone due to the smaller size particles. Yet none of these patents has resulted in a commercially available, long-term, oral formulation.

During this study, a unique drug delivery platform was developed for the oral delivery of natural progesterone. The current delivery system is a novel, patented technology (Basit et al., 2013) with a number of advantages. First of all, because this system is a multi-unit dosage form with a size below 1mm, transit time through the GI tract is expected to be rapid, reliable and reproducible with minimum retention in gastric contents of the fed or fasted state. These parameters lead to less pharmacokinetic variation, which is a problem often seen with single unit dosage forms particularly dose of larger dimensions (Davis et al., 1986) Furthermore, microparticles can be tailored for rapid particle dissolution at site specific regions of the GI tract. The improved GI targeting results in more reproducible pharmacokinetic profiles compared to conventional methods (Kendall et al., 2009). As seen in Chapter 4, the intestines can have a major influence on the degradation of progesterone and the rate of this depletion is reduced in the distal small intestine. Thus, the new formulation is designed for ileum targeting in order to evade some early first pass metabolism. This approach is unique and has not been previously investigated.

Another advantage of the current formulation is its potential to increase the apparent solubility of progesterone in the intestinal fluids. The current formulation contains the drug in an amorphous state stabilised by polymers allowing for supersaturated solutions to be attained and maintained until absorption occurs (Miller et al., 2012).

The manufacturing process itself also has a number of benefits; the process is simple with no need for high temperatures which reduces the potential of thermal incompatibility issues between drug and polymer. There are also no post manufacturing processes required (e.g densification or milling) and the resulting formulations are homogenous products and tend to have a high drug yield (Kendall et al., 2009).

Initial work within this chapter focused on the Eudragit L100 polymer; an anionic copolymer derived from methacrylic acid and methyl methacrylate. Eudragit was selected initially because of its excellent pharmaceutical profile (Sonje and Chandra, 2013, Ammar and Khalil, 1997) and its continuous use within our laboratories. The microparticles produced with Eudragit L100 using an oil in oil emulsification process were highly satisfactory in size, shape and amorphicity. However, when this approach was used, the encapsulation efficiency progesterone was very low (<25%) possibly due to siginificant partitioning of the drug into the external phase. This poor drug-loading efficiency resulted in an actual drug load of <2.5% making this oil in oil approach unsuitable for lipophilic drugs such as progesterone.

The system was therefore altered to use an oil in water approach which enabled the use of HPMCAS, a polymer that has been shown to have excellent solubility enhancement properties (Konno et al., 2008, Tanno et al., 2004) and shows resistance to to the absorption of water enhancing its long-term stability (Rumondor et al., 2009). At 10% theoretical drug load, an encapsulation efficiency of a least 70%, depending on the grade of polymer used, was achieved. This is considerably higher than that previously achieved with the Eudragit based microparticles and confirms the suitability of this approach for the encapsulation of progesterone. SEM analysis confirmed that some of the drug was not encapsulated (Figures 5.3, 5.6 and 5.7) yet all encapsulated drug was amorphous as confirmed by DSC and PXRD analysis.

Although the progesterone microparticles displayed an improvement in solubility compared to the non-formulated and crystalline drug (Figure 5.18), a greater enhancement has been achieved by Miller et al (2012). Miller and colleges showed

that spray-dried, progesterone loaded, HPMCAS microparticles, had a four fold higher aqueous solubility than progesterone and that the drug remained in solution for at least 10 hours (Miller et al., 2012). However, Miller and colleges used a drug load of only 5% as opposed to the 10% drug load used in this study which may be the reason for the observed differences. If the issue with solubility is indeed related to drug load then decreasing the drug to polymer ratio is certainly possible, especially as HPMCAS has low toxicity and is very well tolerated meaning increased polymer consumption would not pose a risk to the user (FDA, 2006). Failing this, it may be possible to improve microparticle topography by changing the production technique; for example solvent extraction can produce particles with a more regular shape and narrower size distribution (Pavanetto et al., 1992).

In this chapter HPMCAS polymers with 3 different dissolution profiles were used for the production of progesterone-loaded microparticles. In the case of progesterone loaded HPMCAS HF and MF ASDs, microparticles were amorphous, however LF microparticles showed some degree of crystallinity. It is unclear why these differences were observed. Nonetheless, in each incidence, progesterone ASDs dissolved at the expected pH (Figure 5.16). Moreover, ASDs are thermodynamically unstable and can recrystallize with time yet progesterone loaded HPMCAS microparticles were stable in ambient conditions for up to 2 years. This level of stability has not been previously shown with the current method in published research or within our own laboratories promoting the present formulation as an excellent option for progesterone delivery.

Previous research has shown that conventional solubility-enhancing methods can compromise intestinal permeability (Miller et al., 2011). However, progesterone ASDs have been specifically shown not to alter intestinal absorbance (Miller et al., 2012). In line with this research, the intestinal permeability of the current progesterone formulation did not differ from the natural compound (Figure 5.18).

As well as establishing the pharmacological characteristics of progesterone ASDs, the biological effects of the formulated drug compared to the natural drug were also assessed. It has been previously documented that polymers can interact with the APIs

with which they are formulated (Byrn et al., 2001, Bharate et al., 2010) yet the regulation of a subset of target genes by progesterone ASDs was similar to the natural drug. As well as this, protein regulation did not differ between the two samples nor did cell morphology. In fact both protein regulation and cell morphology were highly similar between the treatments. Work carried out earlier in this study showed that target gene and protein regulation differed substantially between natural and synthetic progestogens making the natural drug a preferable choice for oral delivery.

Although the present progesterone delivery system is not completely optimised, the *in vitro* data obtained in this study highlight the possibility of constructing progesterone ASDs as a viable option for improved oral bioavailability.

Despite some minor drawbacks, this study has demonstrated that amorphous solid dispersions are a promising option for the future delivery of progesterone. As well as increasing solubility and dissolution, the formulation is targeted to the ileum where progesterone was shown to be most stable. Furthermore, the progesterone ASDs performed similarly to non-formulated progesterone *in vitro,* this was crucial as the rationale behind producing a natural oral formulation is that adverse signalling is diminished compared to synthetic compounds. Although further optimisation followed by *in vivo* testing needs to be carried out the current formulation shows great promise for the future of progesterone oral delivery.

5.5 Summary

To summarise, the work carried out within this chapter has explored ASDs as a possibility for the oral delivery of progesterone with improved bioavailability and equivalent endometrial activity. The data suggests that, with some optimisation, ASDs containing the polymer HPMCAS are a suitable option for the production of progesterone microparticles. Further work to establish changes in bioavailability *in vivo* needs to be carried out to establish the full potential of progesterone ASDs for oral dosing, particularly for the treatment of endometrial hyperplasia.

Chapter 6

General Discussion

6. General Discussion

Hormone therapy is widely used by women of all ages for diverse reasons, ranging from oral contraception and ovulation stimulation to hormone replacement therapy in menopause, to adjuvant therapy of tumours of the breast and uterus. Progesterone is a steroid hormone that is produced primarily by the corpus luteum during the luteal or secretory phase of the reproductive cycle. This steroid hormone exerts its action in tissues expressing progesterone receptor, including the breast, ovaries and the uterus (Deligdisch, 1999). Progesterone plays an important role in controlling proliferation and differentiation of the human endometrium. Continuous exposure to sex steroid imbalances, where there is insufficient progesterone or excessive estrogen, can cause hyperplasia of endometrial tissue with the potential to progress to hyperplasia with atypia and endometrial carcinoma.

Clinically the treatment for endometrial hyperplasia varies in relation to the grade of the disease; treatment can be medical, with progestins in the hyperplasia without atypia or surgical offering patients hysterectomy in hyperplasia without atypia (Clark 2006). However, a surgical procedure is not always feasible; younger patients often wish to avoid this procedure in order to preserve their fertility while many older patients are unsuitable for surgery because of other medical problems (Gotlieb 2003, Armstrong 2012). To tackle this disease non-surgically, synthetic progestin therapy is prescribed which involves the oral delivery of medroxyprogesteorne acetate (MPA) or the local delivery of levonorgestrel (LNG). Although progestin treatment is highly effective and often leads to regression of hyperplasia (Vereide et al., 2006),significant is the occurrence of side-effects, some of which are considered serious (Cummings 2002, McCann 1994).

The occurrence of hyperplasia is most common in post-menopausal women (Lacey et al., 2010, Rakha et al., 2012) and is strongly correlated with high levels of serum estradiol (Baskin et al., 2002, Mutter et al., 2007). In the normal post-menopausal uterus ovarian progesterone production ceases and only low levels of estradiol remain in circulation, causing cell proliferation and differentiation to stop, leaving the endometrium thin and atrophic (Figure 6.1A) (Deligdisch et al., 1978). However, in some women, high levels of estrogen persist after menopause leading to

endometrial proliferation and growth through alterations in gene expression (Woodruff et al., 1994). An example of an adversely regulated gene is FOXO1, a transcription factor involved in endometrial cell cycle regulation (Takano et al., 2007). FOXO1 was found to be significantly downregulated in endometrial hyperplasia both with and without atypia (Section 3.3.1.2). Other such genes are also adversely regulated in the hyperplastic endometrium resulting in abnormal cell proliferation and endometrial thickening (Figure 6.1B). In order to restore the atrophic endometrium, in clinical practice, patients are treated with MPA (Figure 6.1C). However, as well as restoring the altered expression of some genes, MPA, also regulates a number of other genes in a different manner to the natural compound. For example, in Chapter 3, MPA did not alter LF protein expression *in vitro* and up-regulated it *in vivo* whereas P_4 caused its down-regulation, LNG also down-regulated this target. Malignant transformation of the human endometrium has been associated with the overexpression of LF RNA and protein (Walmer et al., 1995), thus its down regulation by progesterone may be important in maintaining a healthy endometrium and avoid relapse, which can occur in 38-40% of MPA treated patients (Ushijima et al., 2005; Ushijima et al., 2007).

Expression of PR is another example of adverse MPA gene regulation. One of the main issues with the PR is its down-regulation by synthetic progestins *in vivo* as seen within this study and reported in the literature (Vereide 2006, Alexander 1989). As progestin therapy relies on the presence of the PR in order to work, the level of endometrial PR strongly influences clinical outcome (Ehrlich 1988). However the benefits of treatment are typically of short duration as the resulting drop in PR limits the treatment efficacy (Satyaswaroop 1992, Mortel 1990). Yet, in the Ishikawa cell line, the PR protein was up-regulated by both natural progesterone and the progesterone ASD but not altered by the synthetic progestins. Although this data is based on a singular cell line, the findings are still important and supportive of clinical use of natural progesterone. If the presence of PR is maintained with natural progesterone treatment, then, in theory the treatment should remain effective with long term use. It would be naïve to assume that in vivo the levels of PR would change radically after natural progesterone treatment compared to synthetic but it

certainly validates further research in this area and promotes the need for a natural progesterone formulation.

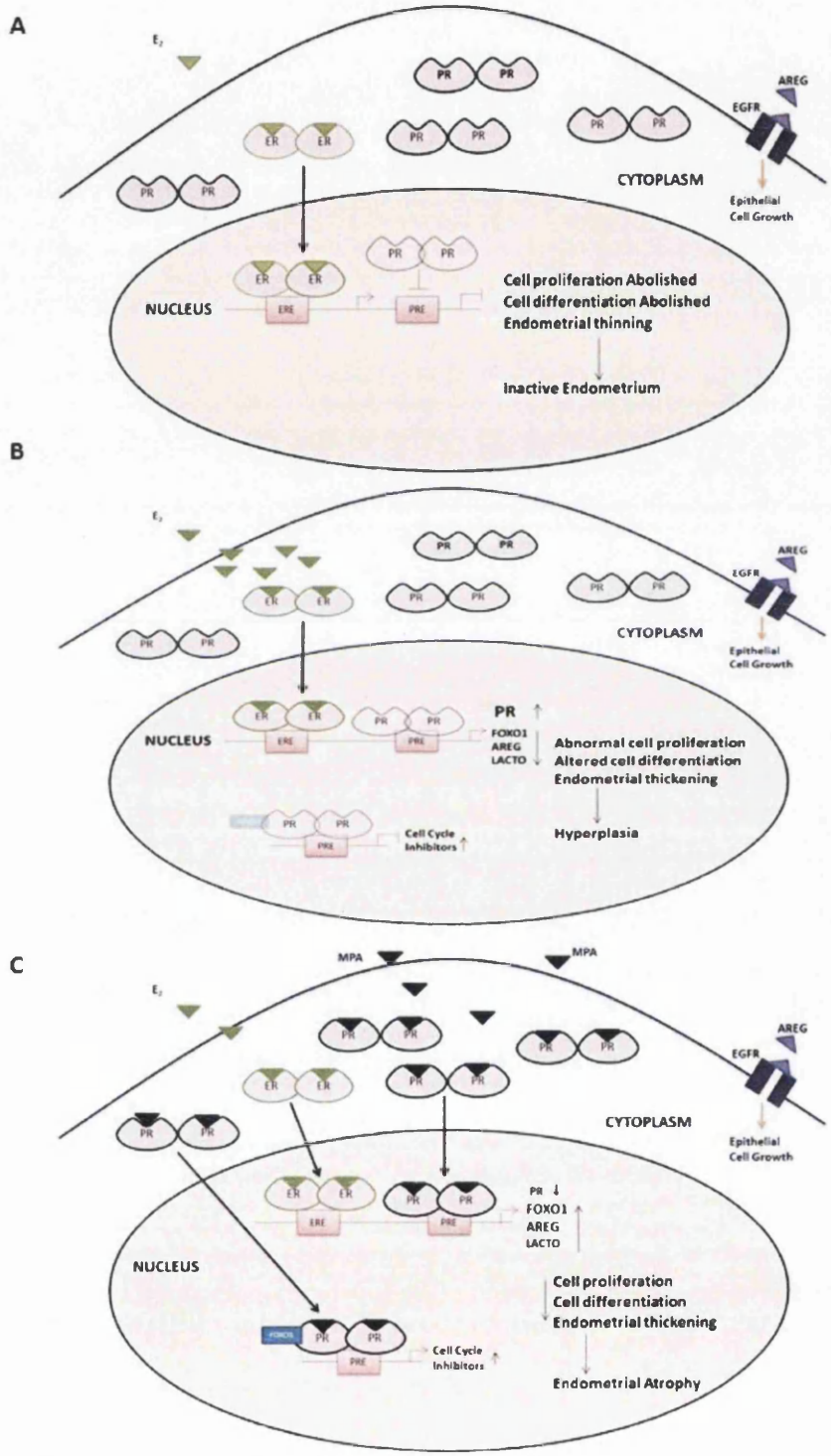


Figure 6.1. Hormone-Induced Gene Regulation in A. Normal post-menopausal endometrium. B Endometrial Hyperplasia and C. Endometrial hyperplasia with MPA treatment.

In the healthy postmenopausal endometrium only low levels of circulating estrogen are present and progesterone is absent resulting in a thin atrophic endometrium. In endometrial hyperplasia an influx of estrogen stimulates genes involved in cell proliferation and differentiation and inhibits genes associated with cell senescence. Absence of progesterone to oppose the estrogenic action, leads to endometrial thickening and hyperplasia. MPA treatment restores the atrophic endometrium but also adversely regulates a number of genes, which can result in disease recurrence.

Unlike synthetic progestins, natural progesterone has very little side effects post-administration (Cummings and Brizendine, 2002) and, although it's not used in clinical practice, the use of locally delivered progesterone was shown to be successful in the treatment of women with endometrial hyperplasia (Baker 2012, Affinito 1994). Moreover, recent research has looked into the oral delivery of micronized progesterone for the treatment of endometrial hyperplasia and the treatment has shown some success (Baker 2012, Chernukha 2013, Marra 2014). However, micronized progesterone is limited by its reduced endometrial control (Tasci 2014) and wide inter and intra-patient variability (Levine and Watson, 2000b, Nahoul et al., 1987).

The main hindrance in developing a suitable natural progesterone oral formulation is its poor bioavailability which is due to extensive first-pass metabolism and poor solubility. There is a great deal of literature available on progesterone metabolism by the liver; however, there is limited information available on progesterone metabolism in the intestines. The intestines are likely to be the most significant site of drug biotransformation outside of the liver due to the presence of metabolising enzymes such as Cytochrome P450s and aldo-keto reductases (Thelen and Dressman 2009, Barski 2008).

Therefore, the stability of progesterone was analysed in homogenates of intestinal mucosa and liver of rats and humans with a view to develop the current understanding of progesterone first pass metabolism and to establish the role of the intestine in this process. As well as confirming the susceptibility of progesterone to liver metabolism, work carried out in Chapter 4 also showed that the intestines make

an important contribution to progesterone depletion. Homogenates of human intestinal mucosa were shown to deplete progesterone by more than 20% within 60 minutes of incubation. The importance of drug metabolism by the intestinal wall has been previously reported; in the case of the synthetic estrogen, ethinylestradiol, gut wall metabolism plays a vital role in its systemic bioavailability (Back 1982). Back *et al* reported that the gut wall is almost twice as effective as the liver in conjugating ethinylestradiol (Back 1982). Thus, targeted delivery may substantially improve the levels of systemic progesterone after oral administration. To determine an optimal region for progesterone delivery rat intestinal segments were used. Segments of male and female rat intestinal mucosa were incubated with progesterone and the stability of progesterone was determined therein. Based on the data obtained, it was possible to identify the mucosa of the distal small intestine as the region where progesterone is most stable and least metabolised. Furthermore, progesterone metabolism by colonic fluid was assessed and rapid depletion of progesterone by gut bacterial enzymes was observed. The level of gut bacteria is reduced in the ileum compared to the colon, therefore, the ileum presents not only as a region where mucosal metabolism is less, but also a region where bacterial metabolism is reduced compared to the large intestine.

This information prompted us to consider options for targeted progesterone delivery to the ileum with a view to enhancing its oral bioavailability as compared to that of current immediate release formulations. In order to achieve this, a novel approach was used whereby progesterone was formulated into amorphous solid dispersions (ASDs). ASDs pose a great opportunity for progesterone oral administration as they not only travel quickly through the gut and rapidly dissolve at the ileal pH with great accuracy but also improve drug solubility and enhance the rate of dissolution.

Previous patents have focused on immediate release formulations containing micronized progesterone (Besins 2012, Maxson 1990, Liu 2010, Chandler 1997). Micronized progesterone has a reduced particle size, which enhances the dissolution rate and absorption and, by virtue, improves oral bioavailability (Hargrove 1989, Simon 1993 Fitzpatrick 1999). However, as well as having a reduced particle size, the current delivery system offers a unique platform for the oral administration of

natural progesterone by means of ileum targeting and improved solubility. As well as this, ASDs contain progesterone in an amorphous form, which leads to an improved dissolution rate as no energy is needed to break down the crystal lattice during drug release (Vasconcelos 2007, Saffoon 2011).

During this thesis two polymers were investigated for the production of progesterone ASDs. The first polymer, Eudragit L100, was used to formulate progesterone using an oil-in-oil method. However, with this method the encapsulation efficiency was poor. Therefore, an oil-in-water method was used with the polymer HPMCAS which drastically improved encapsulation efficiency. In their current state, the progesterone loaded HMPCAS microparticles are not fully optimised. At 10% drug load, excess crystalline drug was observed on the microparticle surface (Figure 5.3) and only minor improvements in solubility were obtained in relevant fluids (Figure 5.18). Yet, there is potential to improve the current profile of these microparticles. For example, a study conducted by Miller *et al* (2012) showed that, at a drug load of 5%, the solubility of progesterone was improved to more than four times that of the natural compound (Miller 2012). Furthermore, Miller and colleagues produced their progesterone microparticles using the spray-drying technique which may have influenced the characteristics of the resulting microparticles. Nevertheless, progesterone-loaded HPMCAS microparticles show excellent potential as a future option for the oral delivery of natural progesterone.

The rationale behind opting for a bioidentical formulation is the reduced side-effects which, as described in Chapter 3 of this study, come about as a result of adverse gene and protein regulation by synthetic progestins. Data shown in this study (Chapter 5) supports further refinement of progesterone ASD formulations given the similar regulation of progesterone targets by natural progesterone compared to the progesterone ASD.

6.1 Conclusions

The main aim of this study was to produce a natural progesterone formulation with enhanced bioavailability and equivalent endometrial activity compared to the natural

drug as a proposed treatment for endometrial hyperplasia, a condition currently treated with synthetic progestins.

To achieve this aim, a progesterone amorphous solid dispersion was produced. The dispersion was shown to have both enhanced solubility and equivalent endometrial activity compared to the unformulated drug, although the level of increase in solubility did not meet expectation. However, the progesterone ASD similarly regulated protein targets that were differentially altered by the synthetic progestins MPA and LNG in endometrial cell lines, highlighting natural progesterone as a preferred treatment option for endometrial hyperplasia than the currently available synthetic counterparts.

As a consequence of the work carried out in this thesis a number of novel findings came to light. Firstly, when assessing the basal expression levels of a number of proteins in endometrial hyperplasia, changes in levels of a panel of biomarkers were noted, suggesting their potential as candidates for determining endometrial hyperplasia presence and progression. Secondly, GREB1, a gene of unknown function, was shown to be up-regulated by progesterone in endometrial epithelial cells and also shown to be a marker of cellular decidualisation; these findings have not been previously reported in the literature. Finally, a number of previously unreported enzymes were found to metabolise progesterone in human liver and intestinal cytosol. Of particular interest was the discovery of the Menadione-induced reduction in progesterone metabolism in liver. Menadione is known to inhibit aldehyde oxidases, NRH:quinone and oxidoreductase 2, none of which have been previously implicated in progesterone metabolism.

Overall, the work carried out in this thesis has obtained the following major outcomes.

1. This study has identified PR targets FOXO1, Amphiregulin, PR and Lactoferrin, as putative biomarkers for endometrial hyperplasia with a potential use in clinic for diagnosis and monitoring response to treatment.

2. A differential regulation of important progesterone target genes and proteins and their differential control by the synthetic progestins MPA and LNG compared to natural progesterone is observed in human endometrium.
3. This study has provided previously unreported insight into the role of the intestine in progesterone metabolism with the ileum appearing to metabolise progesterone the least
4. The work presented herein has highlighted progesterone ASDs as an attractive option for the future production of a natural progesterone oral formulation with equivalent endometrial activity to that seen for natural progesterone.

6.2 Future Prospects

The data presented within this thesis presents ileum-targeted amorphous solid dispersions as an interesting option for the future delivery of natural progesterone. However, it was beyond the scope of this study to fully optimise the progesterone ASDs. Therefore, this study ends with the presentation of an excellent prospect for future development. The continuation of this work should attempt the same method of producing progesterone ASDs, but with a reduced drug-load to improve encapsulation and further enhance solubility. Alternatively, a range of techniques and excipients could be employed in order to find a method for producing 10% microparticles with improved characteristics. Further work should also include progesterone stability assays in intestinal segments of humans, particularly females, as the current work was carried out in both male and female rat models. Stability assays in human intestine would ensure the correct target region has been selected.

The ultimate goal for the future of this work would be to test the optimised progesterone amorphous solid dispersion in human volunteers with endometrial hyperplasia. This work would help establish whether the oral bioavailability is truly improved with the progesterone ASDs and whether the new formulation is sufficient to transform the hyperplastic endometrium.

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Appendices

Appendices

A: RNA Protocol for RNeasy Kit Qiagen

1. Cells grown in a monolayer (do not use more than 1 x 107 cells): Cells can be either lysed directly in the cell-culture vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis.

2. Disrupt the cells by adding Buffer RLT. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT (see Table 5). Vortex or pipette to mix, and proceed to step 3.

3. Pipette the lysate directly into a QIAshredder spin column placed in a 2mL collection tube, and centrifuge for 2min at full speed. Proceed to step 4.

4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

5. Transfer up to 700µL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000*x*g Discard the flowthrough.

6. Add 700µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 8000*x*g to wash the spin column membrane. Discard the flow-through.

7. Add 500µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 8000*x*g to wash the spin column membrane. Discard the flow-through.

8. Add 500µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2min at 8000*x*g to wash the spin column membrane.

9. Optional: Place the RNeasy spin column in a new 2mL collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1min.

10. Place the RNeasy spin column in a new 1.5mL collection tube (supplied). Add 30–50µL RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1min at 8000*x*g to elute the RNA. DNase digest protocol

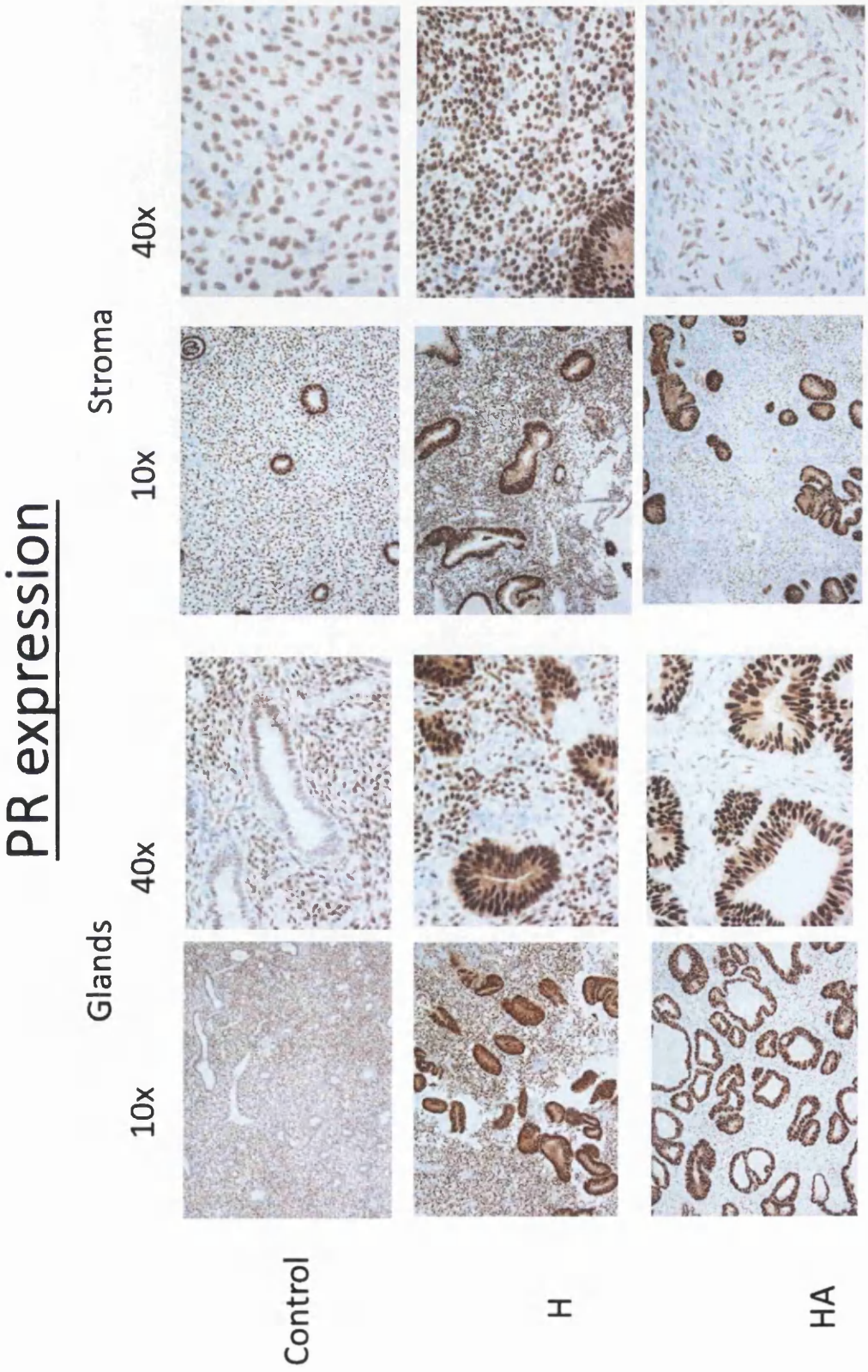
B: Liver Homogenate Donor Demographics.

Gender	Age	Race	Blood Gp	Smoker	Date/Time of Death	Cause of Death	Medical History	Allergies
M	38	Cauc	O	No	10/03/2001 02:55	Intracerebral haemorrhage	Hypertension	None
M	64	Cauc	A	Yes	03/09/2004 02:50	Subarachnoid haemorrhage	No PMH	None
M	33	(Unknown)	(Unknown)	Yes	21/12/2005 16:30	Road Traffic Accident	None	None
M	58	Cauc	O	Yes	24/02/2006 00:54	Subarachnoid haemorrhage	unremarkable Renal stones 20yrs ago	None
M	28	Cauc	A	No	10/02/2003 18:56	Exacerbation of cystic fibrosis Resp failure NHB	Hep B surface antigen negative Hepatitis B core antibody positive Non heart beating donor, 7 minutes arm Ischaemia. Patient had been hypoxic for 1 week but prior to this - reasonably well.	None
M	37	Cauc	O	Yes	20/03/2004 15:46	Road Traffic Accident	None	None
M	54	Cauc	A	No	08/07/2003 00:10	Intacranial Heamorrhage	None	None
M	41	Cauc	A	Yes	14/02/2006 05:59	Subarachnoid haemorrhage	Hypertension	None
M	53	Cauc	(Unknown)	Yes	20/04/2007 21:15	Brain Stem Infarct	Unremarkable	None
F	37	Cauc	(Unknown)	No	11/10/2005 03:10	Subarachnoid haemorrhage	Renal Transplant 1984, Failed in 1989, Dialysis to present Hypertensive 2 Abnormal Smears 2005	Penicillin
F	63	Cauc	O	Yes	09/02/2006 00:04	Intracerebral haemorrhage	Hysterectomy - Adenocarcinoma of Cervix	None
F	38	Cauc	A	Yes	21/04/2005 09:15	Brain Tumour	Migraine Insulin Dependent Diabetic Smoker	None

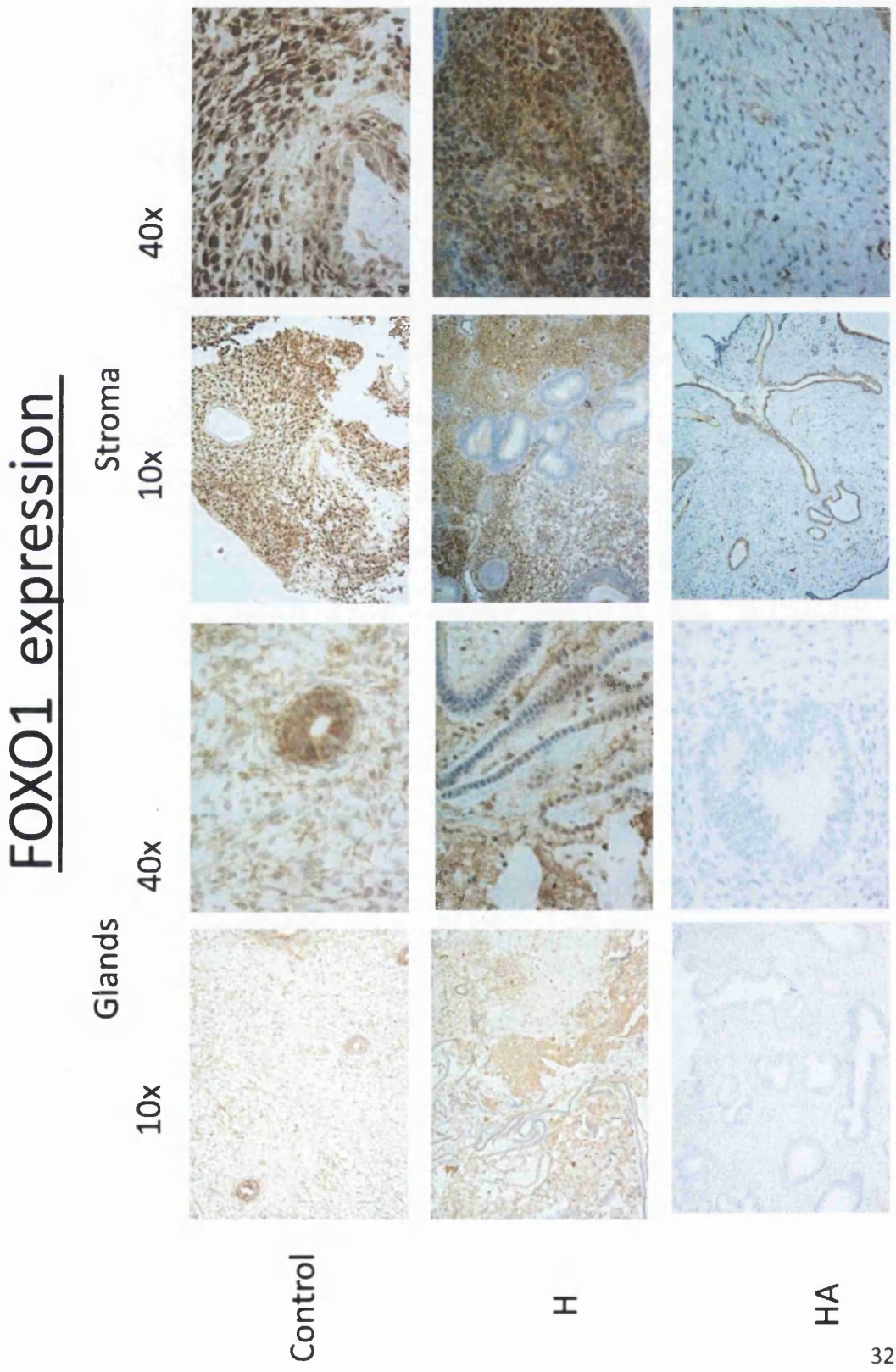
C: Intestinal Cytosol Donor Demographics.

Age:	Race:	Cause of death:	Height:	Weight:	Social history:	Medical history:	EBV	CMV	Hepatitis B	Hepatitis C	HIV
30M	C	Head trauma; 2nd to motorcycle accident	73"	73 KG	ETOH: 6 pk/day x 10 yrs; Tobacco: 1ppd x 15 yrs; Drugs: Marijuanna daily x 10 yrs	Professional tatoos, tennis elbow. Meds: none	Pos	Pos	Neg	Neg	Neg
36M	B	CVA	70"	341 LB	ETOH: rarely; No tobacco or drug use.	HTN x 11 yrs – noncompliant with meds; Meds: clonidine, hydromax, lodrare, aldactazide, creon, exforge, lexapro, tramadol, labetalol, parafon forte, legstrol	Not reported	Pos	Neg	Neg	Neg
48M	A	Anoxia; 2nd to MI	67"	73 KG	ETOH: 2-3 beers per year; Tobacco: unknown amt – quit; Drugs: Marijuana x 2 yrs	Cardiac arrest, T2D x 15 yrs, HTN x 2 yrs, end stage renal disease x 2 yrs w/ dialysis. Meds: unknown	Pos	Pos	Neg	Neg	Neg
52F	C	GSW to head	67"	60 KG	No ETOH or drug use; tobacco: 1 ppd x 40 yrs	20-30% mild LAD stenosis, chronic Chron's disease, blood hemodialated, hysterectomy, chronic back pain; Meds: unknown	Not reported	Pos	Neg	Neg	Neg
49F	C	ICH	62"	97 KG	No ETOH, Tobacco or drug use	HTN x 6 yrs – compliant w/meds, bacl surgery to correct scoliosis, vaccinated fo Hep B, osteoporosis	Pos	Neg	Neg	Neg	Neg
60F	B	CVA	72"	103 KG	No ETOH, Tobacco or drug use	High blood pressure/ HTN x 10 yrs – compliant; Meds: HTN meds (names unknown) TOXO Pos.	Pos	Pos	Neg	Neg	Neg

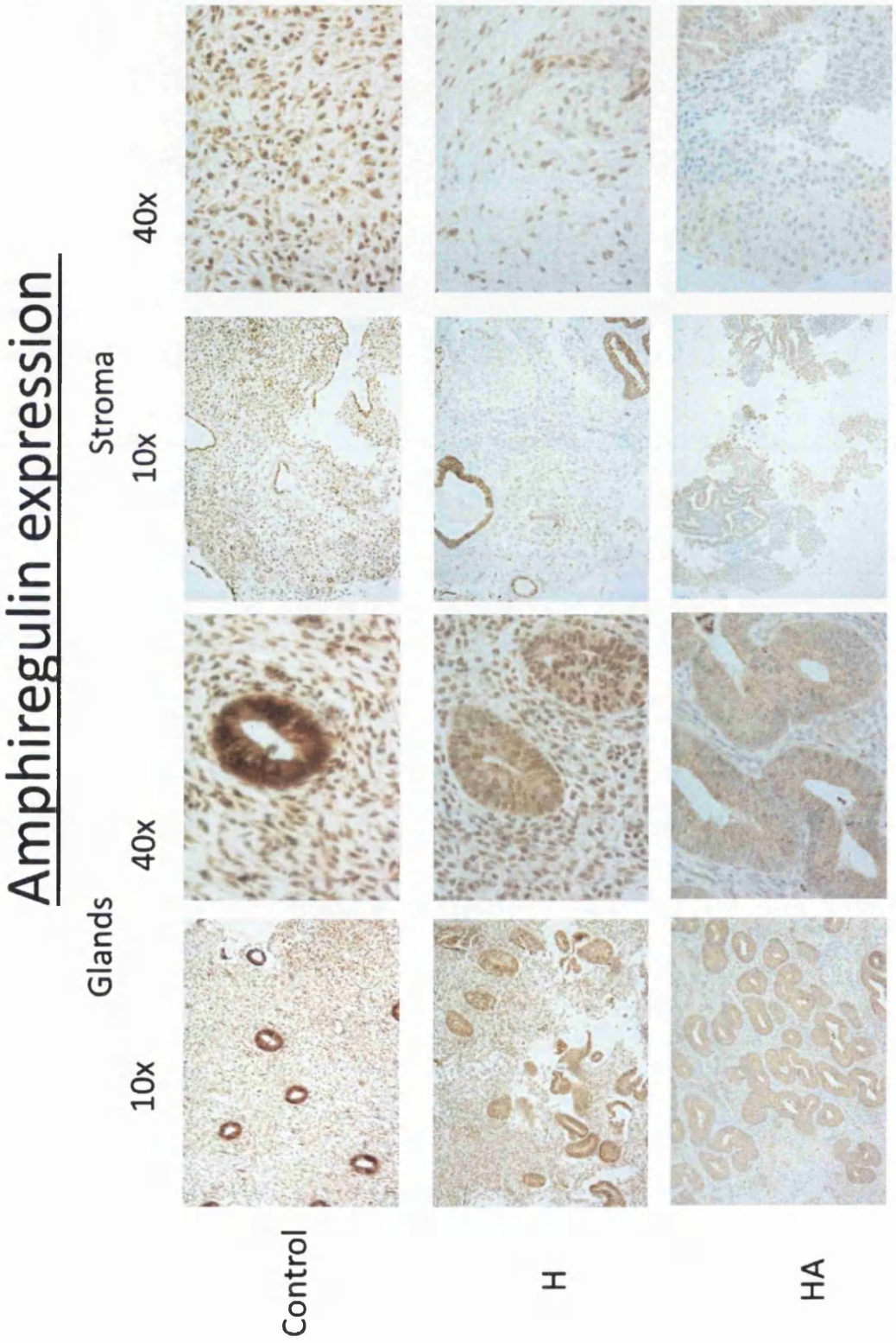
Appendix D: FFPE samples obtained from healthy (controls), patients diagnosed with hyperplasia without atypia (H) and hyperplasia with atypia (HA) were stained with a mouse anti-PR monoclonal antibody. Progesterone Receptor expression was identified in glands and stroma. Representative images (10x and 40x magnification) per group are shown.



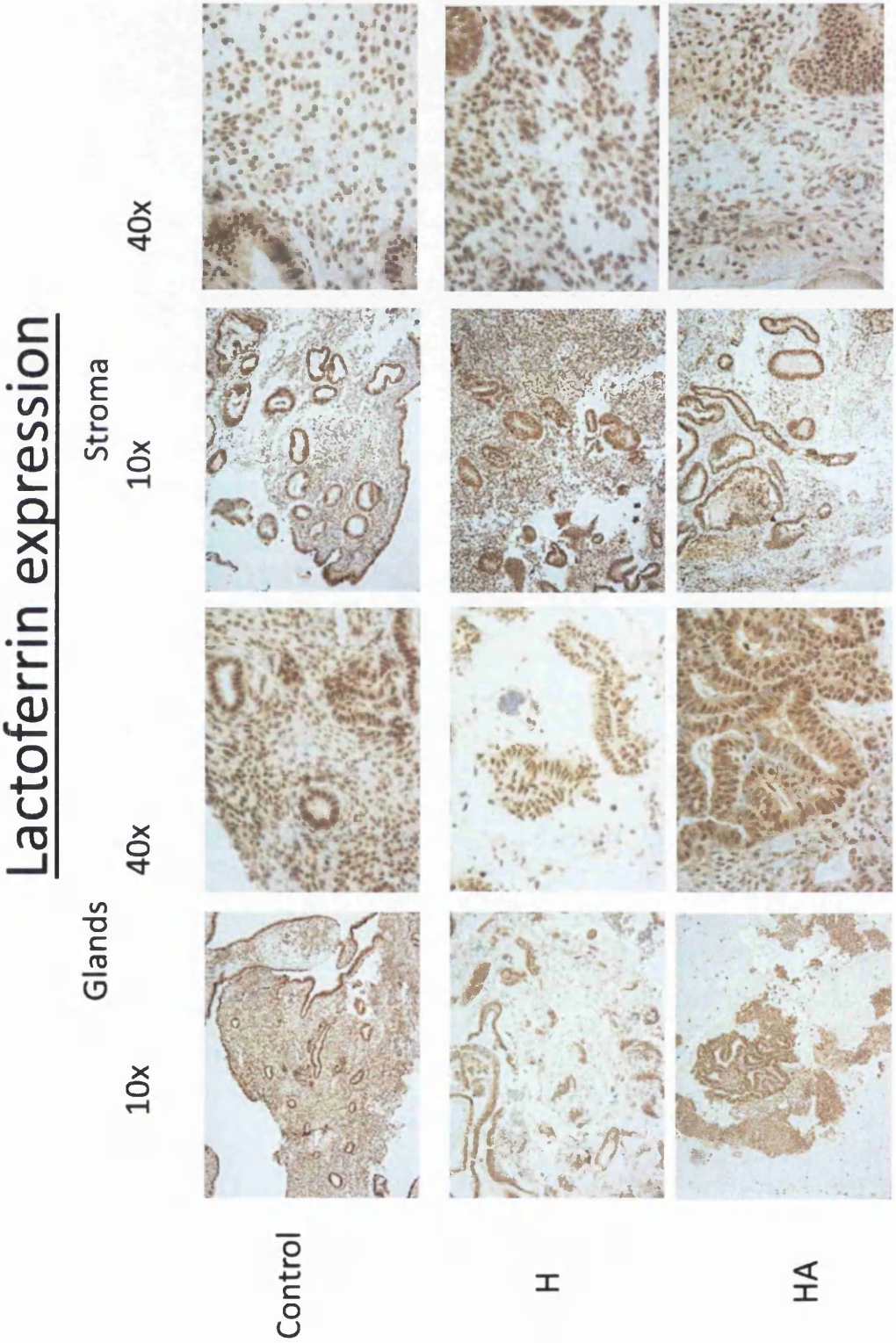
Appendix E: FFPE samples obtained from healthy (controls), patients diagnosed with hyperplasia without atypia (H) and hyperplasia with atypia (HA) were stained with a mouse anti-FOXO1 monoclonal antibody. Expression of FOXO1 protein was identified in glands and stroma. Representative images (10x and 40x magnification) per group are shown.



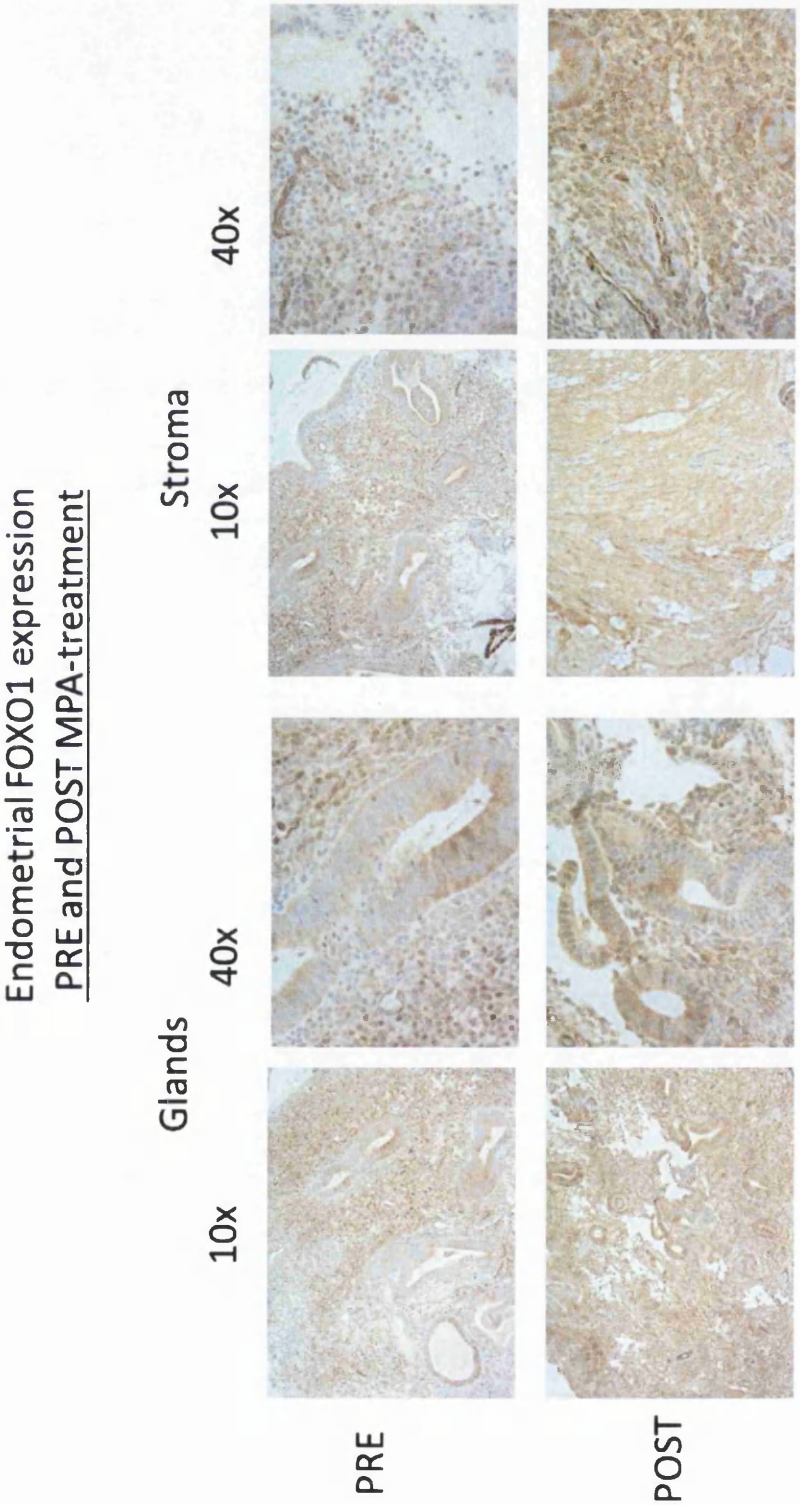
Appendix F: FFPE samples obtained from healthy (controls), patients diagnosed with hyperplasia without atypia (H) and hyperplasia with atypia (HA) were stained with a mouse anti-amphiregulin monoclonal antibody. Expression of Amphiregulin protein was identified in glands and stroma. Representative images (10x and 40x magnification) per group are shown.



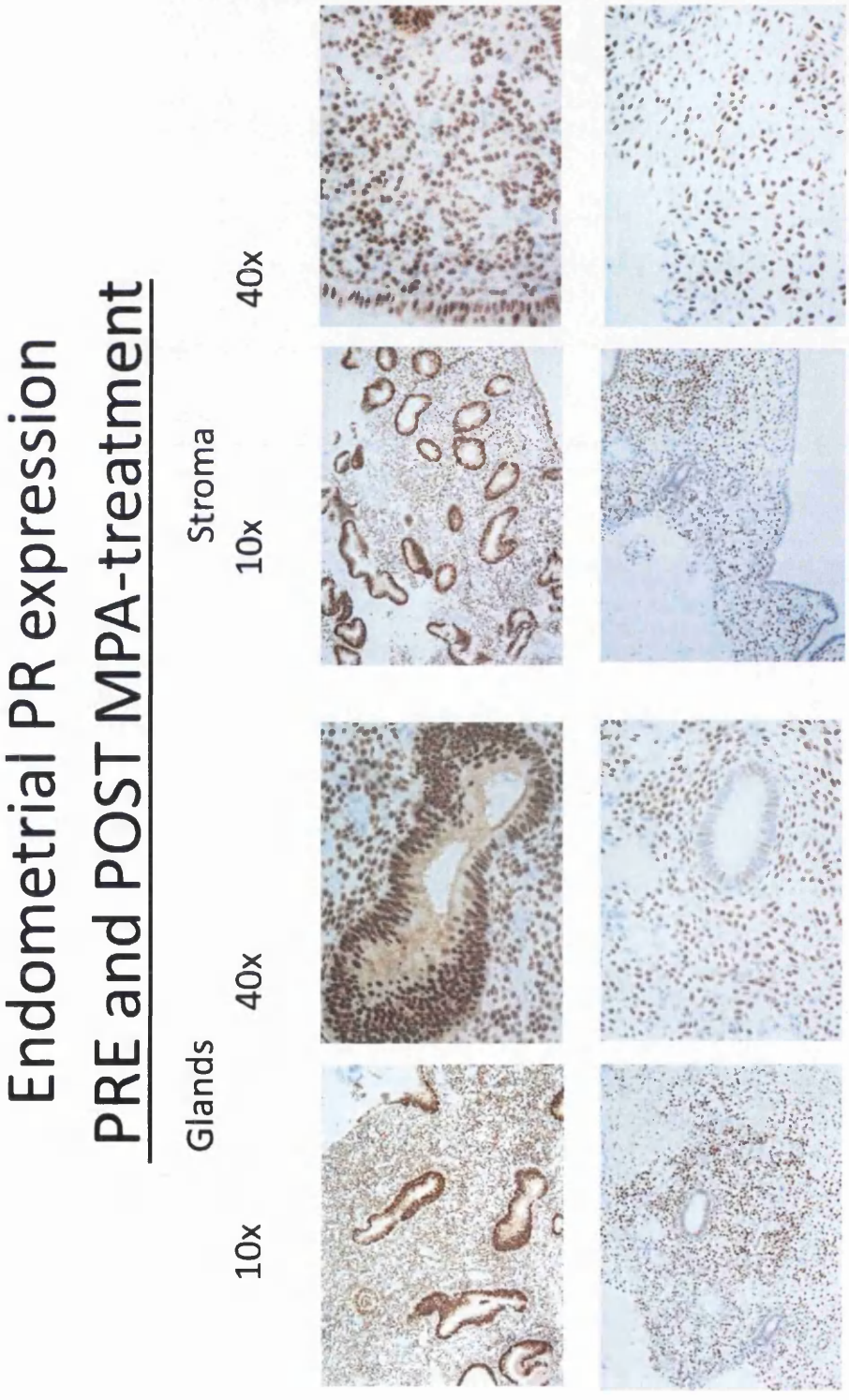
Appendix G: FFPE samples obtained from healthy (controls), patients diagnosed with hyperplasia without atypia (H) and hyperplasia with atypia (HA) were stained with a mouse anti-lactoferrin monoclonal antibody. Expression of Lactoferrin protein was identified in glands and stroma. Representative images (10x and 40x magnification) per group are shown.



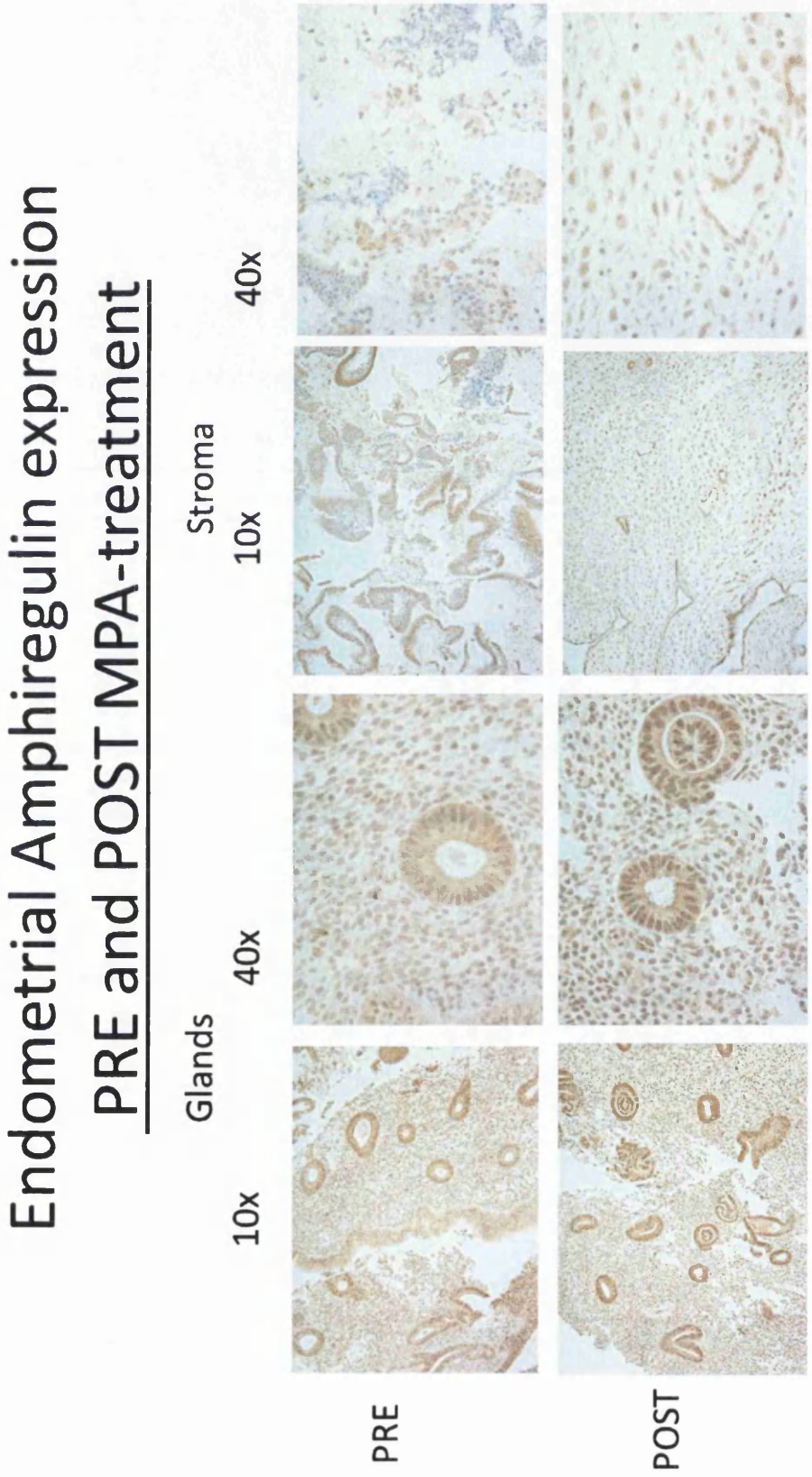
Appendix H. Effect of MPA on endometrial expression of FOXO1 in vivo. FFPE samples from patients diagnosed with Hyperplasia without atypia was interrogated for expression of FOXO1 by IHC. Samples were collected from patients before and after MPA treatment (3 months). FOXO1 expression was identified in glands and stroma using a specific anti-FOXO1 antibody. Representative images (10x and 40x magnification) per group are shown



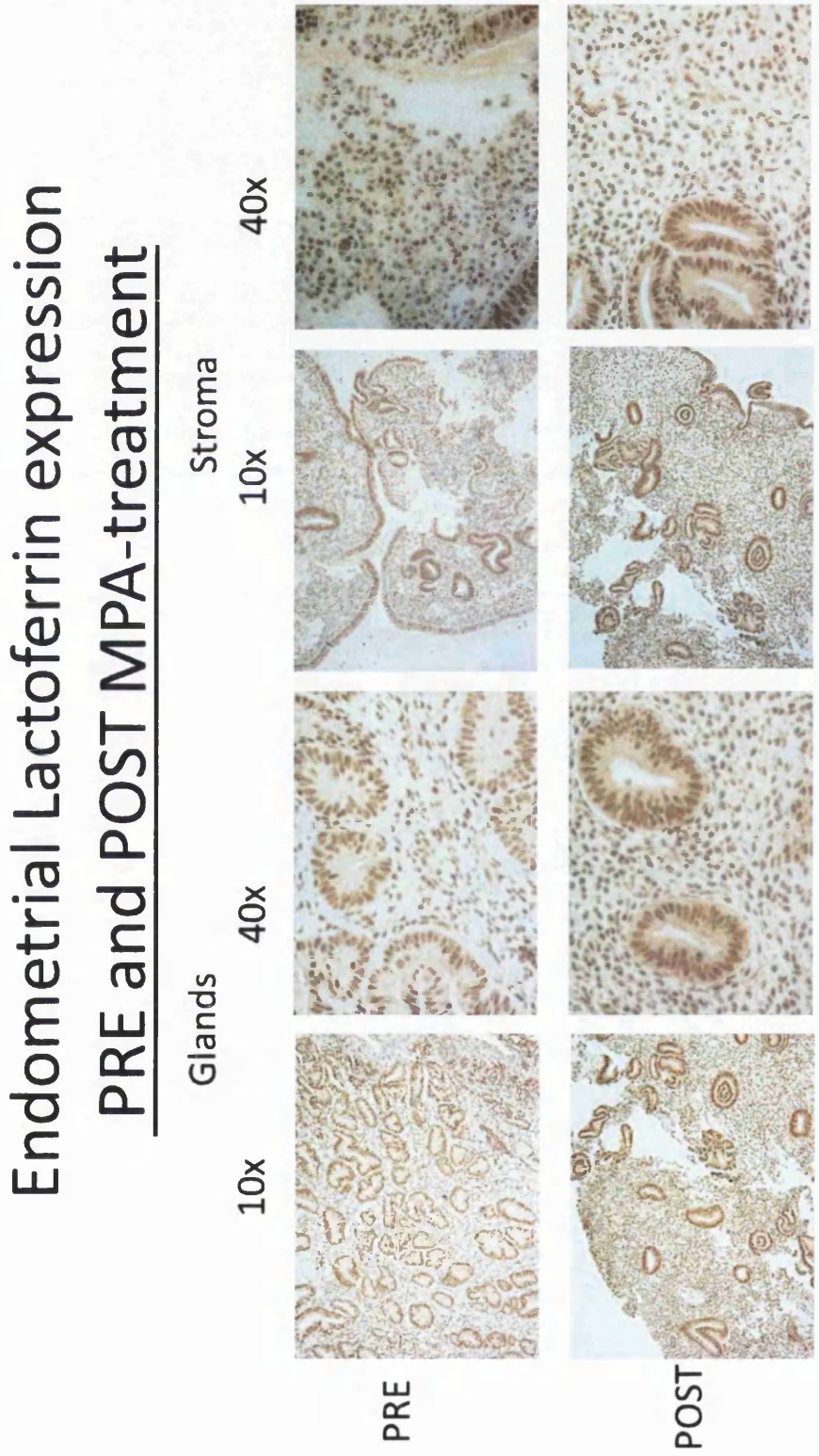
Appendix I. Effect of MPA on endometrial expression of PR in vivo. FFPE samples from patients diagnosed with Hyperplasia without atypia was interrogated for expression of PR by IHC. Samples were collected from patients before and after MPA treatment (3 months) . Progesterone Receptor expression was identified in glands and stroma using a specific anti-PR antibody. Representative images (10x and 40x magnification) per group are shown.



Appendix J. Effect of MPA on endometrial expression of Amphiregulin *in vivo*. FFPE samples from patients diagnosed with Hyperplasia without atypia was interrogated for expression of amphiregulin by IHC. Samples were collected from patient before and after MPA treatment (3 months). Amphiregulin expression was identified in glands and stroma using a specific antibody. Representative images (10x and 40x magnification) are shown.



Appendix K. Effect of MPA on endometrial expression of Lactoferrin *in vivo*. FFPE samples from patients diagnosed with Hyperplasia without atypia was interrogated for expression of Lactoferrin by IHC. Samples were collected from patients before and after MPA treatment (3 months) . Lactoferrin expression was identified in glands and stroma using a specific antibody. Representative images (10x and 40x magnification) per group are shown



L: Fluorescent images showing formaldehyde fixed cells with DAPI-stained cell nucleus (blue), Texas-Red stained GREB1 protein (red) and fused DAPI/Texas Red image at 10x optical zoom. Images obtained using the InCell Analyser