THE ROLES OF PROSTAGLANDIN E_2, PROSTAGLANDIN $F_{2\alpha}$ AND ALDO-KETO REDUCTASE 1C ISOENZYMES IN ENDOMETRIOSIS AND BREAST CANCER

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A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abstract

University of Manchester

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Doctor of Philosophy (PhD)

Thesis title: The roles of prostaglandin E_2, prostaglandin $F_{2\alpha}$ and aldo-keto reductase 1C isoenzymes in endometriosis and breast cancer

February 2017

Endometriosis and breast cancer are sex hormone dependent diseases characterised by the local production of high levels of 17β-oestradiol. The relationship between prostaglandins and sex steroid hormones is one of the focal questions in endometriosis, breast cancer and other sex steroid hormone related disorders.

Therefore, the main hypothesis was that the aldo-keto reductase (AKR) 1C isoenzymes are responsible for controlling the availability of 17β-oestradiol, progesterone and prostaglandins in the microenvironment of the endometrium, and surrounding adipose tissues of endometriotic lesions and breast tumours. This was investigated using quantitative real-time polymerase chain reaction (PCR) for measuring the gene expression of AKR1C1-3 enzymes, and prostaglandin E $_{(1\text{-}4)}$ and F receptors in the endometrium, and surrounding adipose tissues of endometriotic lesions and breast tumours. This was then followed by investigating the role of one of the AKR1C enzymes - AKR1C3 - by inhibiting its catalysis using bimatoprost, followed by using PGE_2 as one of the main candidates acting as a transcription factor for upregulating the expression of AKR1C3 which in turn upregulates the production of the local 17β-oestradiol.

The gene expression of AKR1C1 was significantly higher in endometriotic lesions compared to eutopic endometrium of endometriosis patients. However, there was no significant difference in the gene expression of AKR1C (1-3) enzymes in the surrounding adipose tissues of endometriotic lesions between patients with or without endometriosis. Also, there was no significant difference in the gene expression of AKR1C (1-3) enzymes in the breast adipose tissues of patients with breast tumours, regardless of the oestrogen or progesterone receptor status.

The gene expression of prostaglandin E (EP) receptor subtype 3 was significantly higher in the endometriotic lesions compared to eutopic endometrium of endometriosis patients. In the omental adipose tissue, there was no significant difference in the gene expression of $EP_{1\text{-}4}$ and FP receptors between endometriosis and non-endometriosis patients. In the breast adipose tissue, there was also no significant difference in the gene expression of $EP_{1\text{-}4}$ and FP receptors in patients with breast cancer regardless of the oestrogen or progesterone receptor status.

The inhibitory constant (Ki) of bimatoprost was determined using oestrone as a substrate: Ki = 2.9µM and αKi = 0.7µM. Bimatoprost also significantly inhibited the

production of 17β-oestradiol and inhibited the production of 9α,11β PGF_2 in a dose dependent manner in the human endometrial cells.

The effect of PGE_2 on the expression of AKR1C1 and AKR1C3 was assessed in the human endometrial cells. The EP_4 receptor agonist, L-902688, increased the gene expression of AKR1C1 and AKR1C3. Despite gene expression elevation, L-902688 did not increase the production of 17β-oestradiol.

In conclusion, the results were contradictory and highlighted the need for further investigation into the relationship between prostaglandins and sex steroid hormones in the microenvironment of the endometrium, and surrounding adipose tissues of endometriotic lesions and breast tumours.

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List of abbreviations

2-deoxyglucose (2-DG)

15-hydroxyprostaglandin dehydrogenase (HPGD)

17β-oestradiol (E2)

17-hydroxylase/17-20-lyase (P450c17)

Acetyl coenzyme A (acetyl-CoA)

Aldo-keto reductase (AKR)

Aromatase (P450arom or CYP19A1)

BCL2-antagonist/killer (BAK)

cAMP-dependent protein kinase (PKA)

cAMP response element binding protein (CREB)

Carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP)

Carbonyl reductase (CBR)

Cell adhesion molecules (CAMs)

Cholesterol side-chain cleavage enzyme (P450scc)

Coefficients of variation (%CV)

Connective tissue growth factor (CTGF)

Cycle threshold (Ct)

Cyclic adenosine monophosphate (cAMP)

Cyclin-dependent kinase (CDK)

Cyclooxygenase (COX)

Cysteine-rich angiogenic protein 61 (Cyr61)

Cytochrome P450 17A1 (CYP17A1)

Cytosolic PGE synthase (cPGES)

Diacylglycerol (DAG)

Dimethyl sulphoxide (DMSO)

Ductal carcinoma in situ (DCIS)

Dulbecco's Phosphate Buffered Saline (DPBS)

Enzyme-linked Immunosorbent Assay (ELISA)

Epidermal growth factor receptor (EGFR)

European Medicines Agency (EMA)

Extracellular acidification rate (ECAR)

Extracellular flux (XF)

Extracellular membranes/matrix (ECM)

Fibroblast growth factor (FGF)

Foetal Bovine Serum (FBS)

Follicle-stimulating hormone (FSH)

Gonadotrophins releasing hormone analogues (GnRHa)

Human genome project (HUGO)

Hydroxysteroid dehydrogenase (HSD)

Hypoxia-inducible factor (HIF)

Inhibitory constant (Ki)

Inositol trisphosphate (IP3)

Interferon (INF)

Interleukins (IL)

Invasive ductal carcinoma (IDC)

Invasive lobular carcinoma (ILC)

Invasive mammary carcinoma (IMC)

Luteinising hormone (LH)

Luteinising hormone releasing hormone (LHRH or GnRH)

Macrophage colony stimulating factor (MCSF)

Matrix metalloproteinases (MMPs)

Maximum binding (B_0)

Maximum velocity (Vmax)

Membrane-bound PGE synthase (mPGES)

Michaelis constant (K_M)

Mitogen-activated protein kinases (MAPK)

Monocyte chemotactic protein (MCP)

Natural killer (NK)

Nicotinamide adenine dinucleotide phosphate (NADP)

Non essential Amino Acids (NEAA)

Non-specific binding (NSB)

Nottingham Prognostic Index (NPI)

Oestrogen receptors (ER)

Oestrone (E1)

One-way analysis of variance (ANOVA)

Oxygen consumption rate (OCR)

Penicillin-streptomycin solution (Pen-Strep)

Phospholipase (PLC)

Progesterone receptor (PR)

Proliferating cell nuclear antigen (PCNA)

Prostacyclin (PGI_2)

Prostaglandin (PG)

Prostaglandin E receptor (EP)

Prostaglandin F receptor (FP)

Prostaglandin D synthase (PGDS)

Prostaglandin E synthase (PGES)

Prostaglandin F synthase (PGFS)

Prostaglandin transporter (PGT)

Prostamide F/prostaglandin F synthase (PM/PGFS)

Protein kinase (PK)

Pyruvate dehydrogenase kinase (PDK)

Real time quantitative polymerase chain reaction (qRT PCR)

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH)

Regulated on activation normal T cell expressed and secreted (RANTES)

Selective oestrogen receptor modulator (SERMs)

Steroidogenic acute regulatory protein (StAR)

Steroidogenic factor (SF)

Sulforhodamine B (SRB)

The American Society of Reproductive Medicine (ASRM)

Thiazolyl Blue Tetrazolium Bromide (MTT)

Thromboxane A_2 (TXA_2)

Tissue inhibitor of metalloproteinase (TIMP)

Tissue type plasminogen activator (tPA)

Total activity (TA)

Transforming growth factor (TGF)

Tris-acetate-EDTA (TAE)

Tumour necrosis factor (TNF)

Turnover number (K_{cat})

Urokinase-type plasminogen activator (uPA)

Vanilloid receptor 1 (VR1)

Vascular endothelial growth factor (VEGF)

1. Introduction

1.1. The human uterus

The uterus is the female reproductive sex organ responsible for the development of the embryo and foetus during pregnancy. It is a dynamic organ influenced by sex steroid hormones and physical stimuli, such as the implantation of a blastocyst (Yoshinaga, 1988).

The uterus undergoes physical changes during the menstrual cycle, pregnancy and parturition. The inherent dynamic nature of uterine tissues renders it susceptible to a number of gynaecological diseases, such as excessive blood loss (menorrhagia), excessive myometrial contractility (dysmenorrhoea) and endometriosis.

The uterus is a hollow fibromuscular organ that sits in the pelvic cavity. The narrow inferior region of the uterus, known as the cervix, connects the uterus to the vagina (Ludmir and Sehdev, 2000). The body of the uterus is the wider region of the uterus superior to the cervix. Superior to the body is the dome-shaped region called the fundus. The fallopian tubes extend laterally from the corners of the fundus (Fig 1.1.1).

The uterus is composed of three distinct layers: The outer serosa that forms a protective layer, followed by the myometrium, a thick muscular layer, and the endometrium which is the inner mucosal layer of the uterus. There is also a less distinct region between the myometrium and endometrium called the junctional zone (Brosens et al., 1995).

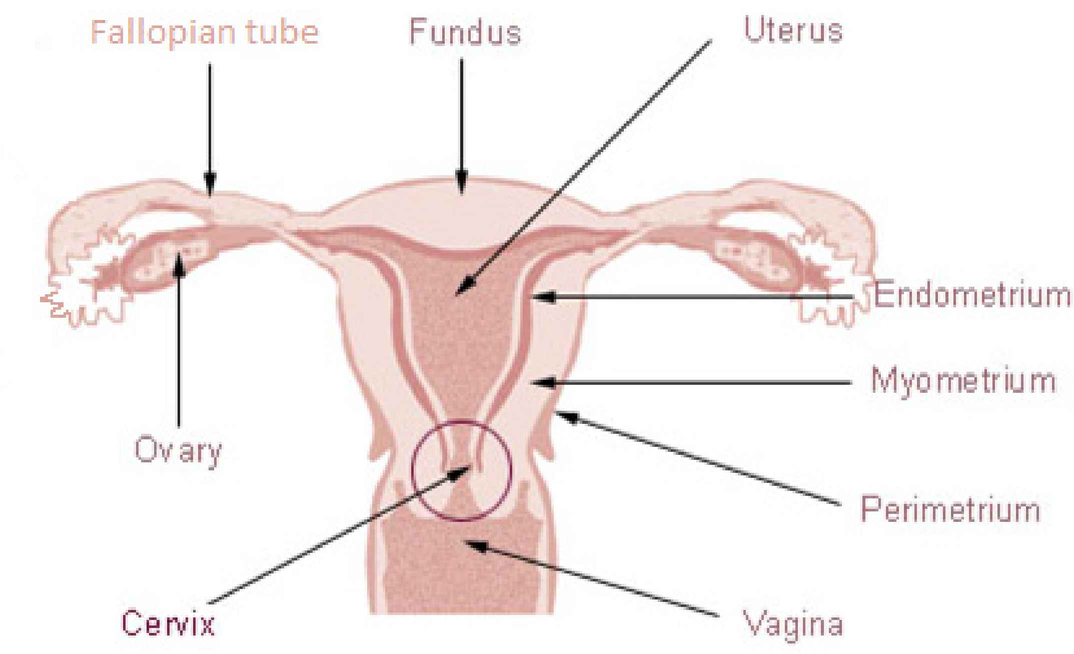


Fig 1.1.1: Anterior view showing the anatomy of the uterus. (adapted from https://commons.wikimedia.org)

1.1.1. The menstrual cycle

The menstrual cycle is influenced by periodic changes in ovarian sex steroid hormones which prepare the uterus to receive a fertilised ovum. In healthy women of reproductive age, the menstrual cycle spans approximately 28 days.

Menses marks the first 4-5 days of the cycle, during which the superficial endometrial layer is sloughed together with blood. After menstruation, circulatory 17β-oestradiol gradually increases during the proliferative phase of the menstrual cycle. During this phase, the endometrial layer starts to rebuild. The systemic level of 17β-oestradiol level reaches its maximum approximately a day earlier before a surge in luteinising hormone (LH) which triggers the release of an ovum (Farage et al., 2009).

The subsequent phase is dominated by progesterone secretion from the corpus luteum (Erden et al., 2005). During this phase, progesterone suppresses myometrial

activity and thickens the endometrial lining. Spiral arteries also fully develop in preparation for pregnancy. However, if fertilisation does not occur, the corpus luteum regresses and progesterone and oestrogen secretion declines. As a result, uterine spiral arteries constrict and the superficial endometrial layer starts to slough off to prepare for the next cycle (Farage et al., 2009) (Fig 1.1.2).

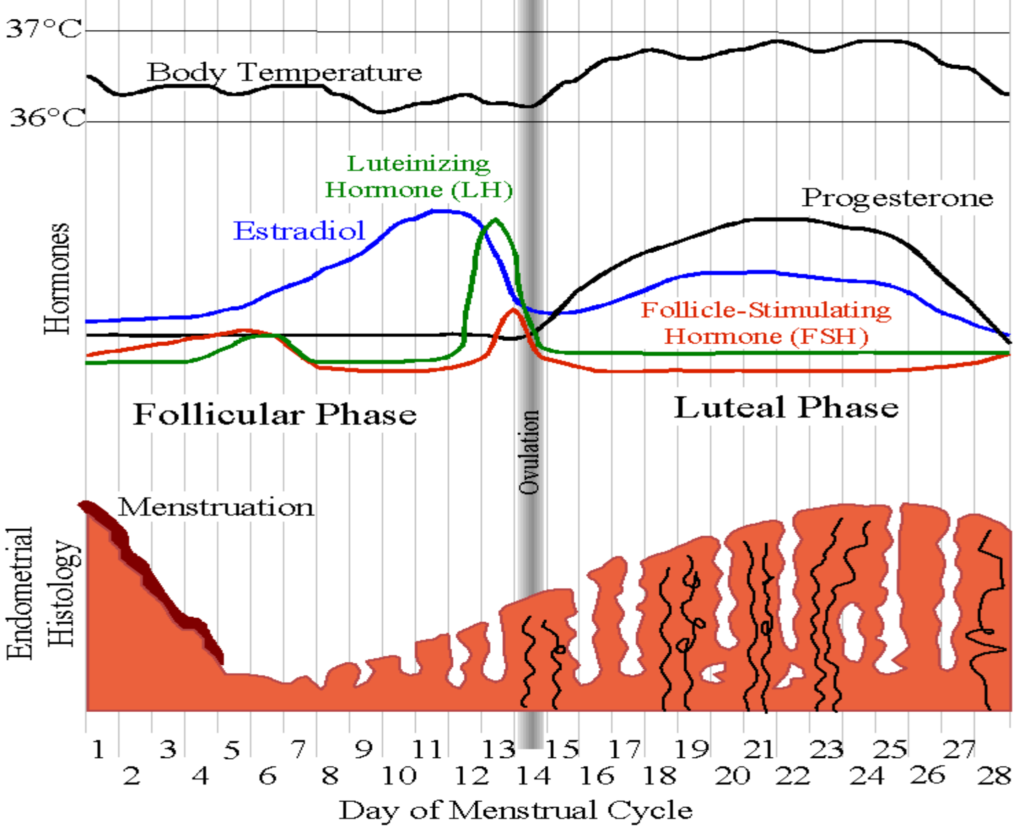


Fig 1.1.2: A typical menstrual cycle starts with the shedding of superficial endometrial layer (menses). Systemic levels of 17β-oestradiol gradually increase during the proliferative phase. Also, luteinising hormone (LH) stimulates the production of androgens by theca cells in the ovary, providing a substrate for oestrogen synthesis by ovarian granulosa cells. Follicle-stimulating hormone (FSH) facilitates follicle maturation and oestrogen-dependent proliferation of endometrial and myometrial cells. Approximately at day 14, LH triggers ovulation, followed by a drop in FSH and LH release. The ruptured follicle transforms into corpus luteum to secrete progesterone, causing endometrium to thicken. If pregnancy is not established, menstruation results from endometrial shedding secondary to the rapid decline in oestrogen and progesterone synthesis as a result of corpus luteum regression (adapted from https://commons.wikimedia.org)

1.2. An overview of endometriosis

1.2.1. Background

Endometriosis is a chronic inflammatory condition that affects women of reproductive age. It is characterised by the presence of endometriotic lesions in extra-uterine areas (Fig 1.2.1). It is estimated that 6-10% of the general population are diagnosed with endometriosis (Giudice and Kao, 2004). Endometriosis occurs in 15% of women who complain of chronic pelvic pain and also occurs in 25% who undergo hysterectomy (Mahmood and Templeton, 1991). These figures might be underestimated since no robust diagnostic method currently exists for endometriosis without involving the visualisation of endometriotic lesions (Brown and Farquhar, 2014).

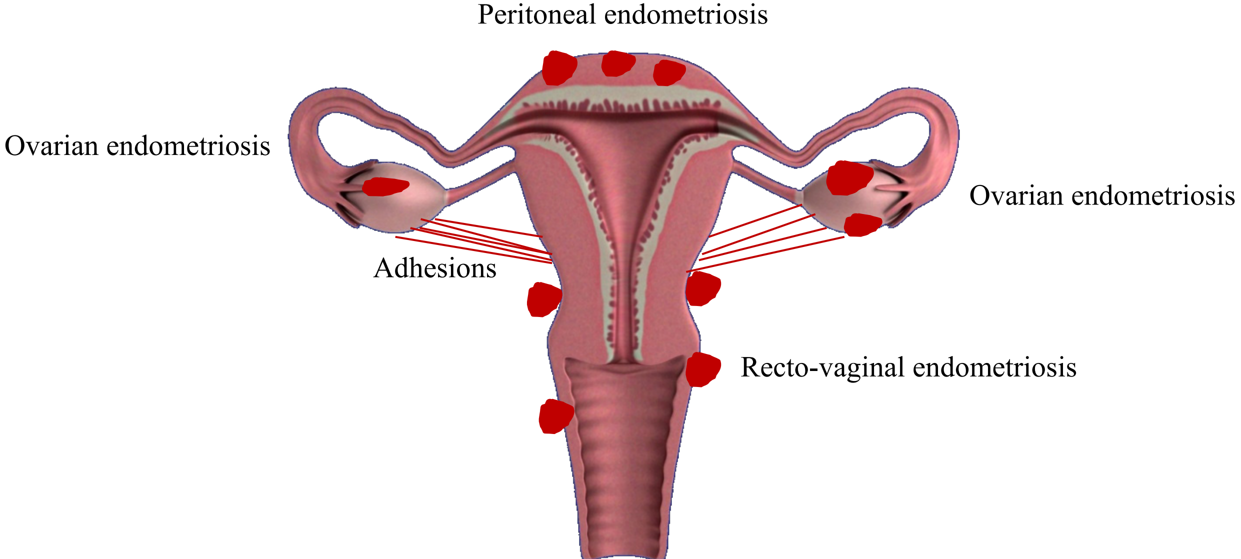


Fig 1.2.1: A diagram to show potential sites for the endometriotic lesion development outside the uterus: peritoneum, ovaries and rectovaginal septum. In severe cases, adhesions can occur between different organs within the pelvic area. Adapted from (https://pixabay.com/en/uterus-apparatus-ovaries-1089344/)

1.2.2. Symptoms of endometriosis

The symptoms of endometriosis overlap with many gynaecological diseases, making it difficult for the clinician to diagnose the condition (Table 1.2.1).

Symptoms of endometriosis	Other potential gynaecological conditions
1) Painful sex (deep dyspareunia)	Psychosexual problems, vaginal dryness
2) Chronic painful periods	Adenomyosis
3) Painful defecation (dyschezia)	Constipation, anal fissure
4) Painful micturition	Cystitis
5) Adnexal masses	Benign and malignant ovarian cysts, hydrosalpinges
6) Chronic lower back pain	Musculoskeletal strain
7) Infertility	Other causes of infertility
8) Chronic lower abdominal pain	Irritable bowel syndrome, neuropathic pain, adhesions

Table 1.2.1: The symptoms of endometriosis which are commonly manifested in other gynaecological diseases (Hickey et al., 2014)

1.2.3. Diagnosis of endometriosis

The gold standard diagnostic test for endometriosis is laparoscopy, even though it is an expensive and invasive procedure. This diagnostic procedure requires a high level of surgical expertise for successful diagnosis (Wykes et al., 2004). A meta-analysis showed that laparoscopic examination increases the chance of detecting positive diagnosis to 32%, whilst it decreases the chance of detecting false diagnosis to 0.7% (Wykes et al., 2004).

The American Fertility Society (1979) had initially created a classification system to determine the stage of disease so as to improve clinical outcome and prognosis. Since then, the classification system had gone through several changes until 1996, when The American Society of Reproductive Medicine (ASRM) had come up with an updated classification system (American Society for Reproductive Medicine,

1997) (Table 1.2.2). In this system, the extent of endometriosis is evaluated during laparoscopy and classified according to a score that describes the extent of endometriosis, adhesions and endometrioma cyst in the ovary.

Stage	Description (points)	Total Points
Stage I (Minimal)	**Peritoneum**: Superficial Endo 1-3 cm (2) **R. Ovary**: Superficial Endo <1cm (1) & filmy adhesions 1/3 (1)	4
Stage II (Mild)	**Peritoneum**: Deep Endo >3cm (6) **R. Ovary:** Superficial Endo <1cm (1) & Filmy adhesions <1/3 (1) **L. Ovary**: Superficial Endo <1cm (1)	9
Stage III (Moderate)	**Peritoneum**: Deep Endo >3cm (6) **Cul –de-sac**: Partial obliteration (4) **L. Ovary**: Deep Endo 1-3cm (16)	26
Stage III (Moderate)	**Peritoneum**: Superficial Endo >3cm (4) **R. Tube**: Filmy adhesions <1/3 (1) **R. Ovary**: Filmy adhesions <1/3 (1) **L. Tube**: Dense adhesions <1/3 (16) **L. Ovary**: Deep Endo <1cm (4) & Dense adhesions <1/3 (4)	30
Stage IV (Severe)	**Peritoneum:** Deep Endo >3cm (6) **Cul –de-sac**: Complete obliteration (40) **R. Ovary**: Deep Endo 1-3cm (16) & Dense adhesions <1/3 (4) **L. Tube**: Dense adhesions >2/3 (16) **L. Ovary**: Deep Endo 1-3cm (16) & Dense adhesions >2/3 (16)	114
Stage IV (Severe)	**Peritoneum**: Superficial Endo >3cm (4) **L. Ovary**: Deep Endo <1cm (32) & Dense adhesions <1/3 (8) **L. Tube**: Dense adhesions <1/3 (8)	52

Table 1.2.2: The American Fertility Society for reproductive medicine (ASRM) endometriosis classification system

1.2.4. Treatment of endometriosis

Treatment of endometriosis is based mainly on managing the symptoms. Empirical treatment of pelvic pain usually consists of non-steroidal anti-inflammatory drugs, such as mefenamic acid, in unconfirmed cases (Dunselman et al., 2014). If endometriosis is confirmed, hormonal therapies are mainly used for controlling endometriosis-associated pain. For instance, combined oral contraceptives, progestagens, anti-progestagens, danazol and gonadotrophins releasing hormone analogues (GnRHa) are used according to patient’s preference and side effect profile

(Dunselman et al., 2014). Aromatase inhibitors can also be used in endometriosis refractory to other medical or surgical treatment (Dunselman et al., 2014). However, the adverse effects, especially suppression of ovulation, have negative impact on the quality of life and therefore could limit their use. If the pharmacological approach fails or pregnancy is desired, laparoscopy is an alternative option to ablate ectopic endometrial tissues (Sutton et al., 1994). However, 54% of patients might have more than one laparoscopic procedure within a 10 year period (Cheong et al., 2008).

1.2.5. The aetiology of endometriosis

The aetiology of endometriosis is not fully understood. Different theories have been hypothesised, which are not entirely proven and do not fully explain all the mechanisms associated with the initiation and development of endometriosis. The most widely accepted theory is by Sampson (1927) which suggests that retrograde menstruation carries endometrial tissue outside the uterus, through the fallopian tubes and towards the pelvis, causing the endometrial cells to attach at extra-uterine sites. The endometriotic lesions act in the same manner as normal eutopic endometrial tissues, meaning that they have the ability to respond to sex steroid hormones and produce debris during menses.

Alternatively, the “theory of müllerianosis” postulates that endometriosis occurs due to the disruption in organogenesis during foetal development where primitive endometrial tissues are dislocated outside the uterine cavity (Signorile et al., 2009). Also, Meyer’s theory postulates that the formation of endometriomas is caused by metaplasia or transformation of the coelomic epithelium to different types of endometrial cells (Nisolle and Donnez, 1997).

The different theories about the aetiology of endometriosis have arisen due to the non-uniformity of location, morphology and progression of the disease in different patients. This consequently contributed to dividing endometriosis into mainly three different entities: peritoneal endometriosis, ovarian endometriosis and rectovaginal endometriosis (Nisolle and Donnez, 1997).

Peritoneal endometriosis can be explained by the aforementioned theory by Sampson (1927). The ectopic endometrial tissue initially appears red and is morphologically similar to eutopic endometrial tissues. After consecutive menstrual shedding, the lesion becomes encapsulated and turns black, and ultimately, turns into white or yellow-brown lesion due to fibrosis (Nisolle and Donnez, 1997). Hughesdon (1957) attempted to explain the pathogenesis of ovarian endometriosis and suggested that it occurs due to the accumulation of menstrual debris from the bleeding endometriotic implants on the surface of ovaries, causing invagination of menstrual debris into the ovarian cortex, which progressively leads to the formation of "chocolate cysts" (Brosens et al., 1994). However, later on, Donnez et al (1996) disagreed with Hughesdon's theory and suggested that ovarian endometriosis is rather caused by the metaplasia of invaginated coelomic epithelium, which turns into typical endometrial epithelial and stromal cells.

Donnez et al (1996) also disagreed with Sampson's theory on deep infiltrating endometriosis of the rectovaginal septum. They suggested that it occurs by extensive adhesion and infiltration of endometrial adenoma into the rectovaginal septum, and in fact, discarded the idea of mere implantation of regurgitated endometrial cells, arguing that the morphology of endometriotic nodule of rectovaginal septum is

similar to that of adenomyoma and the histopathogenesis of this type is related to the metaplasia of Müllerian rests located in the rectovaginal septum.

1.2.6. Pathophysiology of endometriosis

I. Adhesion:

Cell adhesion molecules (CAMs) are transmembrane receptors that facilitate adhesion of one cell to another or to the extracellular membranes (ECM). There are five different types of CAMs, namely integrins, cadherin, selectin, mucins and immunoglobulin superfamily.

In eutopic endometrial cells, integrins are hormonally regulated in a cyclical pattern. Ectopic endometrial cells, however, are able to express different integrins independently of the hormonal phase. The integrin, $\alpha_v\beta_3$ receptor, is normally expressed during luteal phase when the endometrium is highly receptive for egg implantation, but in endometriosis this receptor may be absent, suggesting that endometrial cells of patients with endometriosis lack differentiation and as a result they are more motile (Lessey et al., 1994).

Cadherins are calcium dependent transmembrane proteins that have a crucial role in cell-cell adhesion. There are four types of cadherins: E-cadherin, N-cadherin, P-cadherin and M-cadherin. E-cadherin is expressed in differentiated cells and acts as a suppressor of metastasis and tumour progression, whereas N-cadherin is found in mesenchymal or undifferentiated cells and promotes metastasis and invasion. E-cadherin is downregulated in ectopic endometrial epithelial cells (Gaetje et al., 1997), whereas N-cadherin is upregulated in ectopic endometrial epithelial cells

(Zeitvogel et al., 2001). Collectively, this suggests that ectopic endometrial cells are not properly differentiated and have enhanced invasive properties.

II. Implantation:

Following successful adhesion, proteolytic digestion of the extracellular matrix (ECM) is the next crucial step for establishing an endometriotic lesion at extra-uterine sites. ECM degradation is mediated by enzymes such as matrix metalloproteinases (MMPs), cysteine proteinases, aspartic proteinases, and serine proteinases.

Matrix metalloproteinases (MMPs) are zinc-dependent of endopeptidases. Deregulations of MMPs synthesis in endometriosis can cause local destruction of ECM which enhances the ability of ectopic endometrial cells to invade the ECM and consequently establish endometriotic lesions. This was shown by upregulation of MMP-2 and downregulation of tissue inhibitor of metalloproteinase-2 (TIMP-2) in women with endometriosis (Chung et al., 2002). Additionally, plasminogen, a protein secreted by the liver, is activated to a highly potent protease, plasmin, by two activators: (i) tissue type plasminogen activator (tPA) which is important for fibrinolysis and (ii) urokinase-type plasminogen activator (uPA), which is responsible for localised proteolysis. In endometriosis, the level of soluble uPA receptor (uPAR) is significantly higher compared to endometriosis-free women (Sillem et al., 1998).

III. Angiogenesis:

Using laparoscopy, endometriotic lesions, especially in peritoneal and deep-infiltrating endometriosis, are easily recognised as having an abundant blood supply. This demonstrates the importance of angiogenesis in establishing and maintaining endometriosis (Groothuis et al., 2005). At the start of angiogenesis, the basal membrane of ectopic sites is degraded by proteases released from vascular endothelial cells which is then followed by endothelial cell migration, proliferation and subsequently capillary tube formation (Folkmann and Haudenschild, 1980). The key player in angiogenesis is the vascular endothelial growth factor (VEGF). It promotes angiogenesis during embryogenesis, skeletal growth and reproductive functions, but also has a pathologic role in many diseases such as cancer and endometriosis (Ferrara et al., 2003). It induces endothelial cell activation and increases the permeability of blood vessels in the peritoneal wall which causes extravasation of fibrinogen and fibrin. This extravasation leads to the generation of vascularised connective tissues (Nagy et al., 1995).

Consequently, the level of VEGF in the peritoneal fluid of endometriosis patients is higher, compared to healthy subjects (McLaren et al., 1996). Furthermore, McLaren et al (1996) have shown that peritoneal macrophages are the principal source of VEGF, along with prostaglandin (PG) E_2, which also modulates the expression of VEGF.

IV. Aberrations in the immune system of the pelvis:

Halme et al (1984) postulated that peritoneal macrophages have central role in the pathogenesis of endometriosis. In endometriosis, peritoneal macrophages,

lymphocytes and ectopic endometrial cells release different cytokines. The cytokines detected in the peritoneal fluid of endometriosis patients are: interleukins (IL-1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13), interferon (INF)-γ, tumour necrosis factor (TNF)-α, regulated on activation normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), macrophage colony stimulating factor (MCSF) and transforming growth factor (TGF)-β (Harada et al., 2001) (Table 1.2.3).

Halme et al (1987) have shown that peritoneal macrophages are elevated in patients with endometriosis, indicating a role in the development of endometriosis. Despite being highly elevated in endometriosis patients, peritoneal macrophages are quite dysfunctional. They cannot release MMP-9 to facilitate phagocytosis of ectopic endometrial cells (Wu et al., 2005); furthermore, the macrophages have dysfunctional scavenger receptor (CD36) which compromises their phagocytic ability (Chuang et al., 2009). Similarly, natural killer (NK) cells are also found to be higher in the patients with endometriosis but they show dysfunctional traits which also limit their phagocytic activities (Eisenberg et al., 2012).

Cytokines	Role in endometriosis	reference
IL-1β	- ↑ soluble intercellular adhesion molecules (sICAMs) allowing ectopic endometrial cells to escape immunosurveillance - ↑ VEGF to promote angiogenesis	(Viganò et al., 1998)
IL-8	- Elevated in patients with endometriosis - Acts as an autocrine growth factor which promotes the growth of endometriotic stromal cells	(Calhaz-Jorge et al., 2003)
TGF-β	- Elevated in patients with endomertriosis - ↓T & B lymphocyte and NK cell activities and enhances the invasiveness of epithelial endometrial cells	(Liu et al., 2009)
TNF-α	- Elevated in patients with endometriosis - Activates proinflammatory cytokines (IL-1, IL-6, TNF-α) and promotes the growth of endometriotic stromal cells - ↑MMP expression	(Harada et al., 2001)

Table 1.2.3: The roles of different cytokines in the development of endometriosis. For full review, refer to (Herington et al., 2011).

1.3. The aldo-keto reductase (AKR) Superfamily

Aldo-keto reductase (AKR) enzymes are quaternary proteins composed of 140 enzymes and divided into 15 families. AKRs are widely found in vertebrates, invertebrates, plants, protozoa, fungi, eubacteria, and archaebacteria (Jez et al., 1997). Within species, AKRs are virtually found in all tissues, however, they are found in high concentrations in the kidneys and liver of mammals, birds, reptiles and fish (Barski et al., 2008).

They have $(\beta/\alpha)_8$ structure motif, which consists of 8 inner strands of β sheets and 8 outer α helices (Jez et al., 1997). The inner β sheets are constrained and have same arrangements throughout the superfamily, whereas the outer α helices differ in arrangement from one AKR enzyme to another. Additionally, neighbouring domains interrupt the β/α motif structure, adding more conformational diversity as well as diverse biochemical activities (Barski et al., 2008).

The active site of AKRs can bind to a vast range of substrates, which denotes the significance of these enzymes, since they have a role in the detoxification of many endogenous and exogenous carbonyl-containing molecules in the body, especially drugs such as daunorubicin, doxorubicin, oracin, ketotife and oxonortriptyline (Bachur, 1976; Barski et al., 2008). This important feature of the active site is attributed to the catalytic tetrad – four amino acid residues at the active site (aspartate, tyrosine, lysine and histidine) – which facilitates the recognition of carbonyl groups (C=O) at certain positions within the compound (Kilunga et al., 2005).

The enzyme is dependent on reduced nicotinamide-adenine dinucleotide (NADH) or reduced nicotinamide-adenine dinucleotide phosphate (NADPH) cofactors to facilitate reduction reactions. During the reduction reactions, AKR enzymes reduce aldehydes and ketones to their correspondent alcohols. The cofactor binds to the AKR enzyme at the cofactor binding site and thereafter releases hydride (H^-) to attack the substrate carbonyl group at the active site. Tyrosine residues act as acid and facilitate the addition of a proton (H^+) to the negatively charged oxygen atom, forming a hydroxyl group (-OH) (Mindnich and Penning, 2009) (Fig 1.3.1).

The rate limiting factor for the majority of AKR enzymes, which have long cofactor binding loop, is mainly the rate of NADPH release; however AKR1C isoenzymes have shorter loops and are therefore limited by the product release (Bennett et al., 1996; Barski et al., 2008).

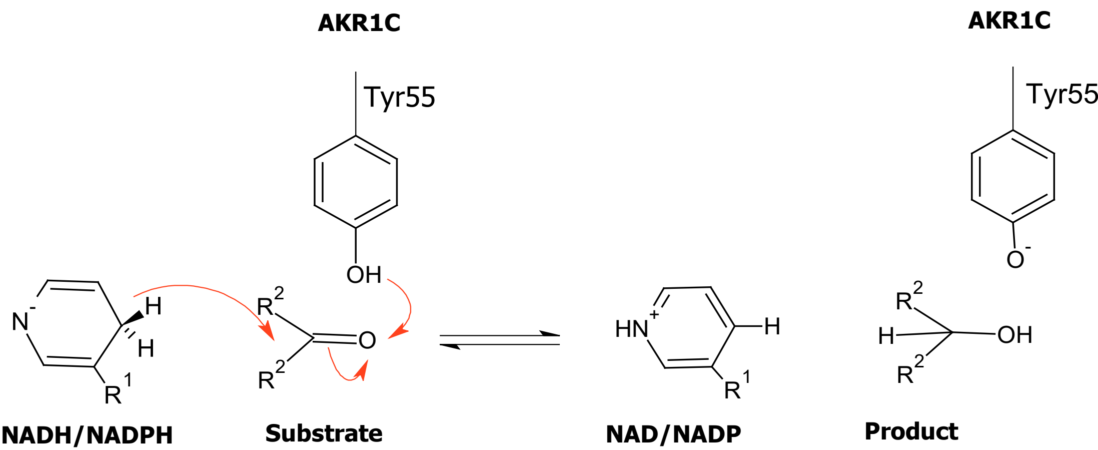


Fig 1.3.1: The reaction mechanism of carbonyl reduction catalysed by AKR1C enzymes

1.3.1. The role of AKR1C isoenzymes in humans

Thirteen AKR enzymes have been identified in humans by the human genome project (HUGO). These are AKR1A1, AKR1B1 and AKR1B10; AKR1C1, AKR1C2, AKR1C3 and AKR1C4; AKR1D1; AKR6A3, AKR6A5 and AKR6A9; and AKR7A2 and AKR7A3 (Barski et al., 2008).

The AKR1C1-4 enzymes are only found in humans which makes it challenging to use rodent animal model since there is no uniformity in homologous AKR1C enzymes found in rodents. For example, the murine AKR1C18 shares 73.4% of amino acid sequence to the human AKR1C3, but based on substrate specificity, AKR1C18 is homologous to the human AKR1C1 (Barski et al., 2008).

The AKR1C isoenzymes are one of the key groups of enzymes in the reproductive system, except AKR1C4, which is predominantly present in the liver. They play a diverse role in the formation and/or inactivation of hormones involved in the reproductive tract, thus regulate the access of such hormones to their corresponding receptors. *In vivo,* the AKR1C subfamily predominantly acts as 3-, 17- and 20-ketoreductases. For example, AKR1C1 (20α-hydroxysteroid dehydrogenase (20α-HSD)) converts progesterone to 20α-hydroxyprogesterone (Piekorz et al., 2005). Also, AKR1C3 (type 5 17β-HSD and type 2 3α-HSD) catalyses the conversion of oestrone to 17β-oestradiol, and catalyses the conversion of 4-androsterone-3,17-dione to testosterone which can be aromatised to 17β-oestradiol by aromatase (Penning et al., 2000; Lin et al., 2004). AKR1C3 also has 20α-hydroxysteroid dehydrogenase activity and hence converts progesterone to 20α-hydroxyprogesterone (Sharma et al., 2006). Furthermore, AKR1C3 catalyses the

reduction of peroxide groups on PGH_2 and carbonyl groups on PGD_2 to hydroxide groups on $PGF_{2\alpha}$ and 9α,11β-$PGF_{2\alpha}$, respectively (Byrns et al., 2010) (Table 1.3.1).

AKR1 Subfamily	Species	Enzyme	Substrate	Product	K_M (µM)	Kcat (min^{-1})	Reference
AKR1C	**Human**	AKR1C1	Progesterone	20α OH-progesterone	1.9	0.57	(Beranič et al., 2011)
			PGD_2	9α, 11β PGF_2	140	0.015	(Nishizawa et al., 2000)
			PGE_2	$PGF_{2\alpha}$	1400	0.48	(Nishizawa et al., 2000)
		AKR1C2	Progesterone	20α OH-progesterone	7.7	0.21	(Beranič et al., 2012)
			PGD_2	9α, 11β PGF_2	120	0.051	(Nishizawa et al., 2000)
			PGE_2	$PGF_{2\alpha}$	98	0.082	(Nishizawa et al., 2000)
		AKR1C3	Progesterone	20α OH-progesterone	5.6	0.17	(Beranič et al., 2011)
			Oestrone	17β oestradiol	9	0.068	(Byrns et al., 2010)
			PGD_2	9α, 11β PGF_2	1.7	9.5	(Koda et al., 2004)
			PGH_2	$PGF_{2\alpha}$	10	3.7	(Koda et al., 2004)

Table 1.3.1: The enzymatic activities of AKR1C (1-3) enzymes for sex steroid hormones and prostaglandin substrates. **K_M**: Michaelis constant and **Kcat**: Turn over number.

1.3.2. The role of AKR1C isoenzymes and prostaglandin biosynthesis

Prostaglandins (PG) are synthesised ubiquitously from arachidonic acid, which is converted to PGG_2 by the constitutive cyclooxygenase (COX)-1 and the inducible COX-2 enzymes. PGG_2 is then metabolized by the peroxidase activity intrinsic to both COX-1 and -2 to the unstable PGH_2 (precursor prostaglandin). PGH_2 is converted to PGD_2, PGE_2, PGF_2, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) by prostaglandin D synthase, prostaglandin E synthases, prostaglandin F synthases, prostacyclin synthase and thromboxane A synthase, respectively (Catalano et al., 2011).

PGE_2 and $PGF_{2\alpha}$ are the main prostanoids synthesised in the human endometrium and in the menstrual fluid (Smith and Kelly, 1988). PGE_2 and $PGF_{2\alpha}$ act as mitogens during the proliferative phase of the menstrual cycle, both of which are aided by coincident elevation in prostaglandin E receptor subtype 4 (EP_4) and prostaglandin F receptor (FP) expression (Milne and Jabbour, 2003). Additionally, PGE_2 acts on the hypothalamus to stimulate the release of luteinising hormone releasing hormone (LHRH or GnRH) which induces the release of FSH and LH from the pituitary gland (Ojeda and Campbell, 1982). $PGF_{2\alpha}$, on the other hand, plays a role in luteolysing the corpus luteum in the late secretory phase (Anderson et al., 2001). Furthermore, prostaglandins regulate the local vasculature in the endometrium, whereby PGE_2 and PGI_2 cause vasodilation, and PGF_2 and TXA_2 cause vasoconstriction (Sales and Jabbour, 2003).

PGE_2 is synthesised by a number of distinct forms of PGE synthase: membrane-bound type 1 (mPGES-1), polymorphic type 2 (mPGES-2) and cytosolic (cPGES) prostaglandin E synthases (Kudo and Murakami, 2005). $PGF_{2\alpha}$ is synthesised by a number of PGF synthases which belong to the AKR superfamily (AKR1C3 and AKR1B1) and carbonyl reductases (CBR1) (Catalano et al., 2011). AKR1C3 and AKR1B1 catalyse the reduction of PGH_2 to $PGF_{2\alpha}$ (Bresson et al., 2011). Additionally, AKR1C3 reduces PGD_2 to 9α11β-PGF_2, which is a stereoisomer of $PGF_{2\alpha}$ and has the same biological action as $PGF_{2\alpha}$ (Watanabe, 2011). Recently, another enzyme has been characterised, prostamide F/prostaglandin F synthase (PM/PGFS), which is capable of converting prostamide H_2 to prostamide $F_{2\alpha}$ as well as PGH_2 to $PGF_{2\alpha}$ (Moriuchi et al., 2008). Moreover, CBR1 can convert PGE_2 to $PGF_{2\alpha}$ (Schieber et al., 1992).

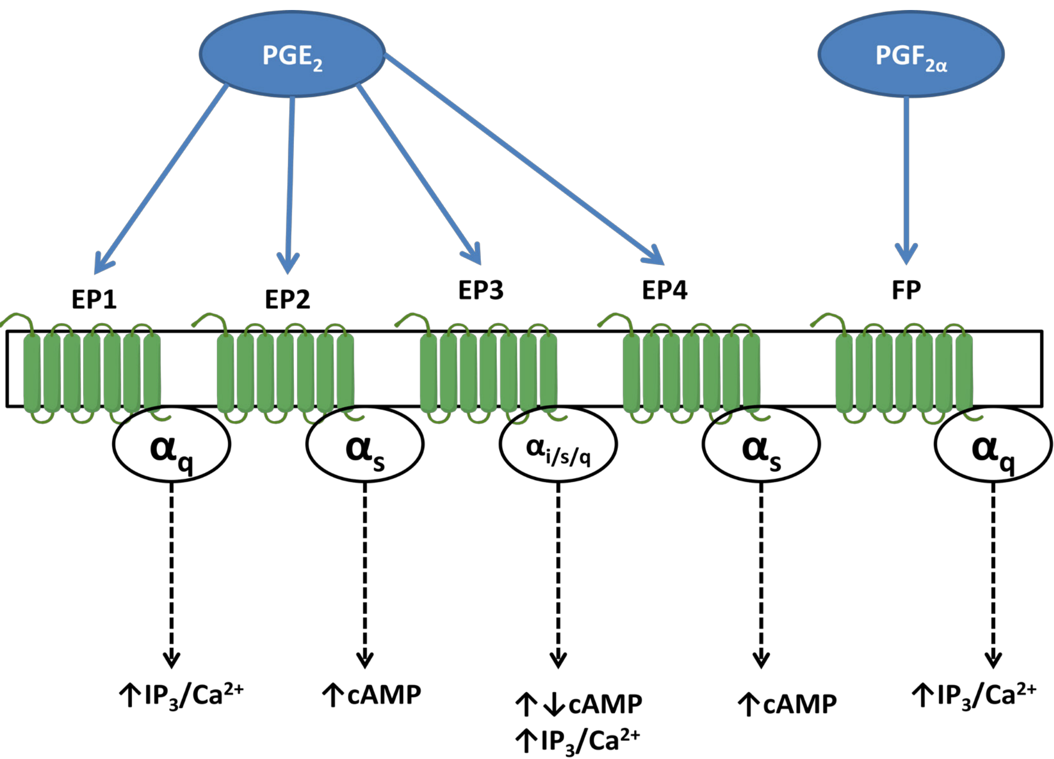


Fig 1.3.2: A schematic diagram of the signal transduction for the G-protein coupled receptors: prostaglandin E receptors ($EP_{1\text{-}4}$) and prostaglandin F (FP) receptor.

I. PGE_2: The key element in the inflammatory process

PGE_2 is heavily involved in the initiation and progression of endometriosis; in fact it is believed that PGE_2 is the key mediator in directing the inflammatory process. The COX-2 enzyme is abundantly present in endometriotic cells, whereas it is virtually undetected in normal endometrial cells. The concentration of PGE_2 in endometriotic tissues is significantly higher than in normal endometrial tissues, indicating that it is the COX-2 enzyme that predominantly synthesises PGE_2 in ectopic endometrial cells (Banu et al., 2008).

PGE_2 mediates its effect through prostaglandin E (EP) receptors, namely EP_1, EP_2, EP_3 and EP_4. EP_1 receptor activation mobilises intracellular calcium and inositol trisphosphate (IP3) via Gαq. Activation of the EP_2 and EP_4 receptors results in an increase in cyclic adenosine monophosphate (cAMP) accumulation via Gαs. However, EP_3 activation results in different responses, depending on the splice variant and cell type. It either increases or decreases cAMP, or increases intracellular calcium mobilisation and accumulation of IP_3 (Sales and Jabbour, 2003) (Fig 1.3.2).

Angiogenesis and proliferation are mainly mediated via PGE_2 in endometriotic tissues. The expression of fibroblast growth factor (FGF)-9 was induced in endometriotic stromal cells after the addition of PGE_2 (Chuang et al., 2006). Additionally, PGE_2 is directly involved in enhancing the expression of VEGF mRNA in rat gastric microvascular endothelial cells, indicating that PGE_2 is capable of enhancing angiogenesis in endometriosis (Pai et al., 2001).

PGE_2 also contributes to the local immunological dysfunctions in endometriosis, as it has the ability to downregulate the expression of scavenger receptor, CD36, on peritoneal macrophages in endometriosis patients (Chuang et al., 2010).

II. The mitogenic effect of $PGF_{2\alpha}$

$PGF_{2\alpha}$ is synthesised by a different array of enzymes as mentioned previously. It acts via the prostaglandin F (FP) receptor, a G-protein coupled receptor subtype ($G\alpha_q$). The signalling transduction through FP receptors causes the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) to facilitate the mobilisation of calcium (Ca^{+2}) from the endoplasmic reticulum (Narumiya and FitzGerald, 2001) (Fig 1.3.2).

The FP receptor has an important role in parturition which was demonstrated by Sugimoto et al (1997) who reported that FP knockout mice failed to deliver normal foetuses at term, despite oxytocin treatment. This is because in normal conditions, $PGF_{2\alpha}$ causes the levels of progesterone to drop and as a result oxytocin receptor levels increase (Sugimoto et al., 1997).

Indeed, $PGF_{2\alpha}$ is an inflammatory mediator and has been investigated in endometriosis by Karck et al (1996) who reported that macrophages in the peritoneal fluid of endometriosis patients release more PGE_2 and $PGF_{2\alpha}$ than healthy patients. Furthermore, some cytokines such as TNF-α and IL-1α induce the release of $PGF_{2\alpha}$ and PGE_2 in a dose dependent fashion (Chen et al., 1995).

$PGF_{2\alpha}$ has also been linked to other painful gynaecological disease, such as dysmenorrhoea. Higher levels of $PGF_{2\alpha}$ cause myometrial contractions, leading to uterine contractility and ischaemia (Senior et al., 1993). Also, the effect of $PGF_{2\alpha}$ on tubal and uterine contractility has led to the postulation that it may be interfering with fertility in endometriosis patients (Karck et al., 1996).

$PGF_{2\alpha}$-FP receptor interaction comprises a mitogenic effect through cross communication with epidermal growth factor receptor (EGFR), which switches on the mitogen activated protein kinase (MAPK) signalling pathway. Although this was

investigated in Ishikawa cells, $PGF_{2\alpha}$ might have a similar proliferative role in endometriosis (Sales, Milne, et al., 2004).

1.3.3. AKR1C isoenzymes contribute to aberrations of oestrogen biosynthesis

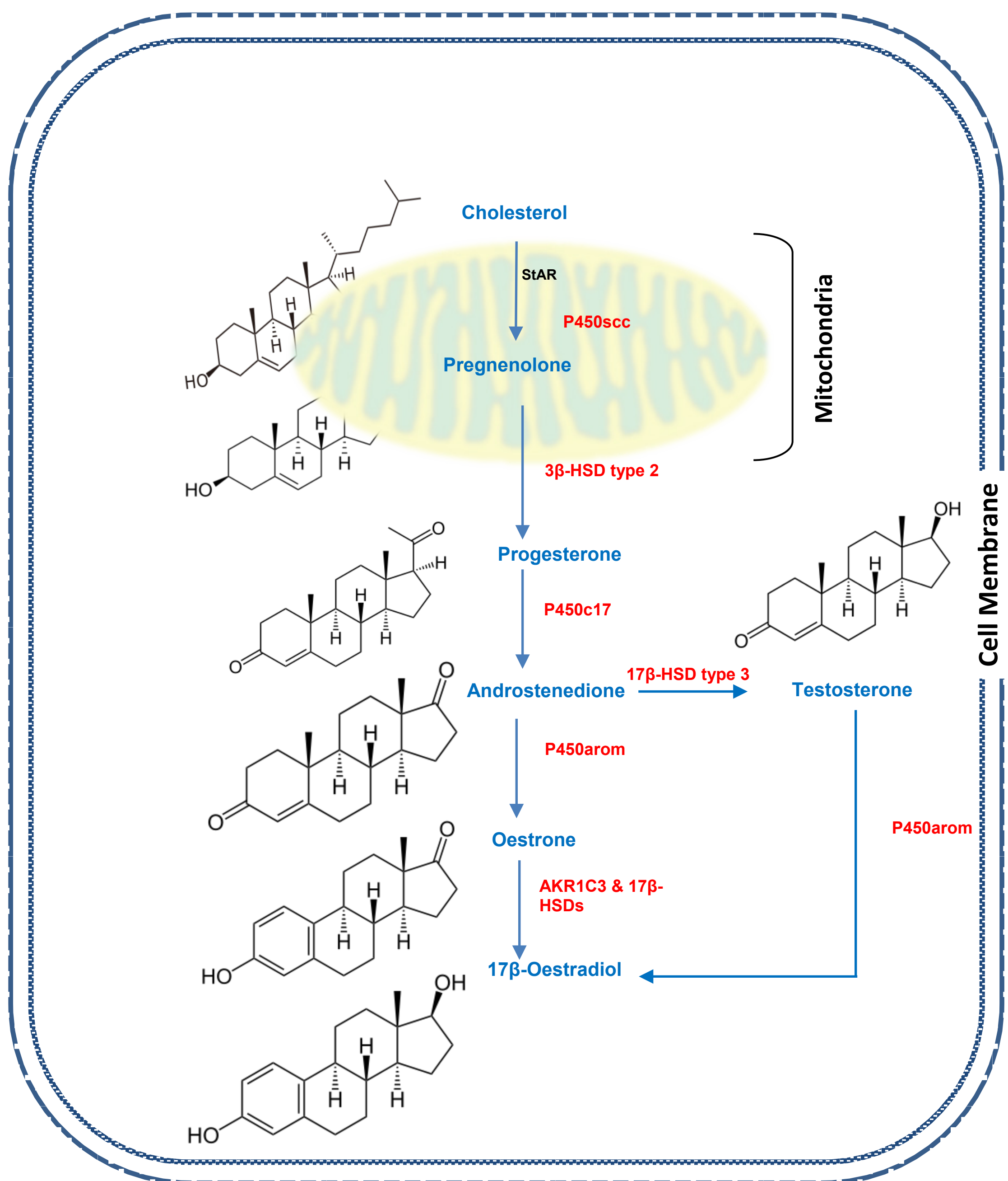


Fig 1.3.3: A schematic model showing oestrogen and progesterone biosynthesis in a cell. Cholesterol is carried from the mitochondrial outer membrane to the mitochondrial inner membrane by steroidogenic acute regulatory protein (StAR). It is then converted to pregnenolone by the mitochondrial cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone is converted to progesterone by 3β-hydroxysteroid type 2 (3β-HSD type 2) and then converted to androstenedione via 17-hydroxylase/17-20-lyase (P450c17). Aromatase (P450arom) converts androstenedione to oestrone, which is further converted to 17β-oestradiol by the enzyme AKR1C3. Androstenedione is reduced to testosterone by 17β-HSD type 3 which can be converted to 17β-oestradiol by aromatase

Oestrogens are essential sex steroid hormones, synthesised mainly by the ovaries. They are synthesised from cholesterol which is carried from the mitochondrial outer membrane to the mitochondrial inner membrane by steroidogenic acute regulatory protein (StAR). The transformed cholesterol gets converted to pregnenolone by the mitochondrial cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone is converted to progesterone by 3β-hydroxysteroid type 2 (3β-HSD type 2) and then converted to androstenedione via 17-hydroxylase/17-20-lyase (P450c17). Aromatase or P450arom is responsible for the conversion of androstenedione to oestrone, which is further converted to 17β-oestradiol by the enzyme AKR1C3 (Attar et al., 2009) (Fig 1.3.3). Androstenedione is mainly reduced to testosterone by 17β-HSD type 3 (Moghrabi et al., 1998). Aromatase can convert testosterone directly to 17β-oestradiol (Stocco, 2012). In addition, some 17β-hydroxysteroid dehydrogenases (17β-HSDs) are responsible for the interconversion of oestrone and 17β-oestradiol (Marchais-Oberwinkler et al., 2011).

Aromatase is expressed in the ovaries of sexually mature animals, specifically in the differentiated pre-ovulatory granulosa cells and luteal cells. It is expressed in a timely manner to regulate the 17β-oestradiol levels according to the phase of the menstrual cycle. Additionally, aromatase is expressed in extragonadal areas in the body, such as the bone, breast, adipose tissue and brain (Stocco, 2012). On the other hand, aromatase is not expressed in normal endometrium due to the absence of the stimulatory transcription factor, SF-1, which binds to aromatase promoter II to induce aromatase expression. But, in endometriotic lesions, aromatase is found to be elevated, due to the aberrant production of SF-1 (Bulun et al., 2001; Smuc et al.,

2009). Also, surprisingly, eutopic endometrial tissues express aromatase in women with severe endometriosis (Maia et al., 2012).

Additionally, the work of Hevir et al. (2011) has shown that AKR1C isoenzymes are upregulated in ovarian endometriosis. This was demonstrated by significant increase in mRNA expression of AKR1C1 and AKR1C2, but a borderline significant increase with AKR1C3. Similarly, Smuc et al. (2009) reported elevated levels of AKR1C3 in ovarian endometrioma in contrast with control group. However, the control subjects were women with fibroids, making the findings questionable because there is a possibility of hormonal imbalance. It is also noteworthy that elevation of 17β-oestradiol levels leads to the upregulation COX-2 mRNA expression and protein levels in the human uterine microvascular endothelial cells (HUMECs). This consequently upregulates the synthesis of PGE_2, which also elevates aromatase synthesis via EP_2 receptors, creating a positive feedback loop whereby more oestrogens and PGE_2 are perpetually produced (Tamura et al., 2004).

Besides the aromatase pathway, there is also a complimentary pathway by which *de novo* biosynthesis of oestrogen can occur. The sulphatase pathway can convert oestrogen sulphate, produced by the adrenal gland, to oestrone which is further converted to 17β-oestradiol by AKR1C3 and other 17β-HSDs. This pathway seems to be upregulated in endometriosis since sulphatase mRNA were found to be significantly higher in ectopic endometrial tissues (Smuc et al., 2007).

As a result of elevated levels of 17β-oestradiol, the secretion of VEGF and FGF9 in ectopic endometrial tissues was found to be elevated (Tsai et al., 2002; Lin and Gu, 2005). Furthermore, Pellegrini et al. (2012) found that mRNA expression of oestrogen receptors (ER) α and β in ectopic endometriomas was upregulated, despite

ERβ only being expressed in 40% of the samples. In contrast, Bulun et al. (2012) found that mRNA expression of ERβ is markedly higher than that of ERα in ectopic endometrial cells which had led them to suggest that overexpression of ERβ inhibits the expression of ERα.

Taken all together, ectopic endometrial tissues have the ability to carry out *de novo* biosynthesis of oestrogen, regardless of the phase of menstrual cycle and thus provide consistent supply of oestrogen to maintain their proliferation and survival (Wu et al., 2010).

1.3.4. AKR1C isoenzymes contribute to the disruption of progesterone metabolism and functions

Progesterone is a female sex hormone synthesised mainly in the ovaries and adrenal glands. The progesterone level is elevated during the luteal or secretory phase, after the release of an ovum. The biosynthetic pathway of progesterone is initiated by transferring cholesterol to the inner membrane of mitochondria, which is converted to pregnenolone by P450scc (Sugawara et al., 2004). Pregnenolone is then converted to progesterone by 3β HSD type 1 & 2 (Sinreih et al., 2013) (Fig 1.3.3). Conversely, the metabolism of progesterone is catalysed predominantly by AKR1C1 and AKR1C3 to 20α-hydroxyprogesterone, which has lower affinity for progesterone receptors (Sharma et al., 2006).

Progesterone acts on the two nuclear progesterone receptors, progesterone receptor A and B (PRA & PRB). The two isoforms arise from the same mRNA, though the initiation of protein translation is different (Conneely et al., 2002). PRA has a dual

role in repressing uterine cell proliferation mediated by PRB and oestrogen receptors (Kraus et al., 1995). At the mRNA level, a significant downregulation in the expression of progesterone receptor A and B (PRA/B) was reported in the ovarian endometriosis specimens in comparison to control specimens (Hevir et al., 2011). This was explained by Bulun et al. (2012) who proposed that lower ERα:ERβ ratio (i.e. low levels of ERα) causes a decline in progesterone receptor expression because the induction of PR expression is only mediated via ERα.

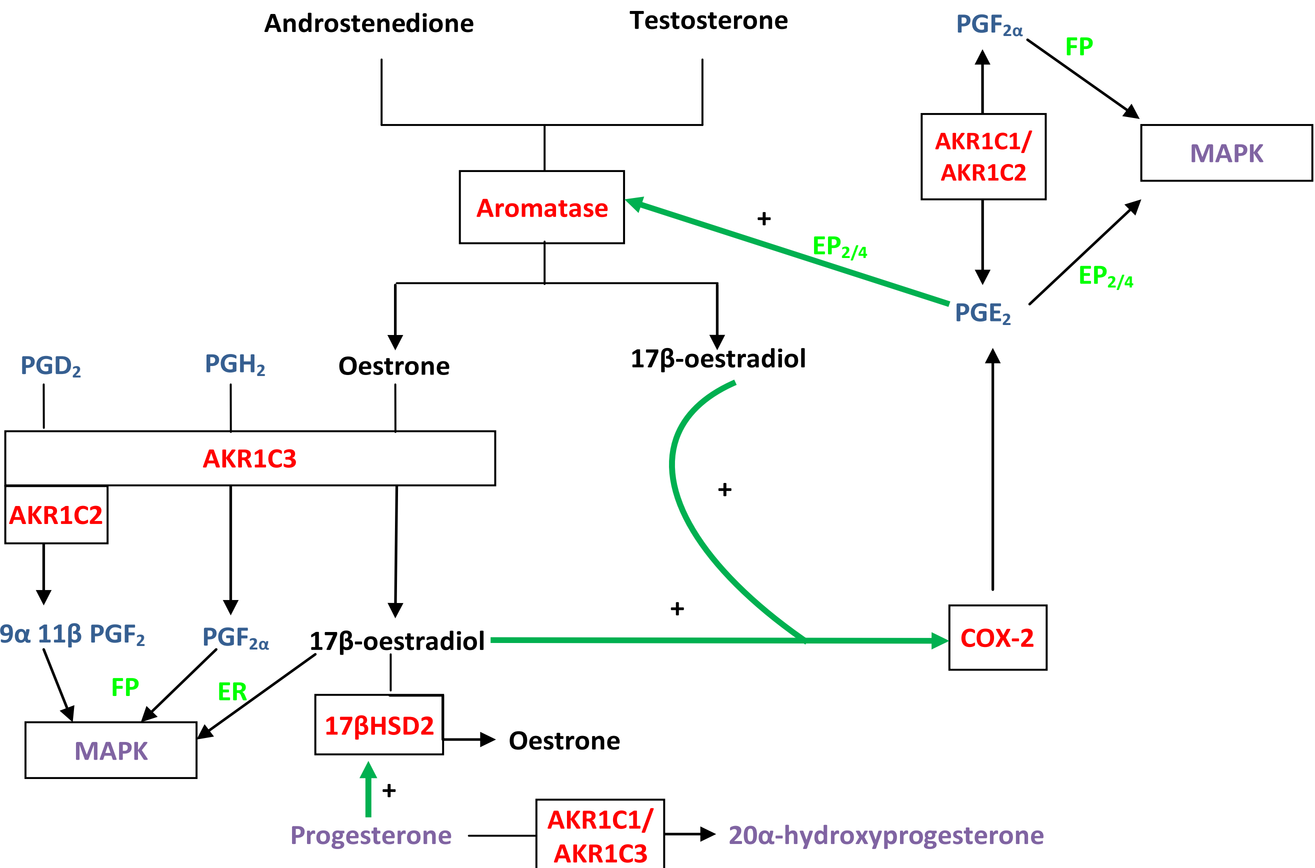


Fig 1.3.4: A schematic diagram showing the interrelationship between sex steroid hormones - 17β-oestradiol and progesterone - and PGE_2 and $PGF_{2\alpha}$ in the human reproductive system. Adapted from:(Casey et al., 1994; Hara et al., 1996; Bulun et al., 2001; Dozier et al., 2008; Penning and Byrns, 2009).
Abbreviations: **MAPK**: mitogen-activated protein kinases; **17βHSDs**: 17β hydroxysteroid dehydrogenase; **AKR**: Aldo keto reductase; **COX-2**: cyclooxygenase 2; **EP**: prostaglandin E receptor; **FP**: prostaglandin F receptor

1.3.5. The role of sex steroid hormones, prostaglandins and AKR1C isoenzymes in breast cancer

Mainly, oestrogen and progesterone receptors have a significant role in breast cancer. *In vitro* administration of 17β-oestradiol causes the proliferation of MCF-7 breast cancer cell line. This effect is attenuated by the administration of the selective oestrogen receptor modulator, tamoxifen, indicating it is oestrogen receptor mediated effect (Aitken and Lippman, 1983). Moreover, 17β-oestradiol regulates the induction of mitogens, such as epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and Transforming growth factor α (TGF-α) in MCF-7 breast cancer cell lines and other hormone responsive (Kasid and Lippman, 1987).

On the other hand, the role of progesterone is poorly defined, compared to that of oestrogen. However, progesterone receptor status is important for the prognosis of the disease, as progesterone receptor negative (PR-) breast tumours are more resistant to treatment with tamoxifen, even when the tumour is oestrogen receptor positive (ER+) (Arpino et al., 2005; Purdie et al., 2014).

The importance of AKR1C isoenzymes in breast cancer still remains to be clarified. However, AKR1C3 converts androstenedione to testosterone, which is followed by aromatisation to 17β-oestradiol. Also, 0.1μM oestrone was converted via AKR1C3 to 17β-oestradiol in MCF-7 breast cancer cell line, indicating that 17β-oestradiol can be produced through different pathways in the breast tumour and the surrounding environment (Byrns et al., 2010).

Elevated COX-2 protein levels have been detected in breast tumours (Howe, 2007). The overexpression of COX-2 positively correlates with overexpression of human

epidermal growth factor receptor 2 (HER-2) which is a characteristic of aggressive form of breast cancer. The relationship between oestrogen and prostaglandins are essentially characterised by the induction of aromatase enzyme by PGE_2 in the breast adipose tissue and breast tumour (Zhao et al., 1996). This effect was mediated by the EP_2 and EP_4 receptor stimulation(Subbaramaiah et al., 2008). Furthermore, PGE_2 has a role in angiogenesis through the stimulation of EP_3 in HEK-293 cells which induced the mRNA expression of VEGF and VEGF -1 receptor (Taniguchi et al., 2008).

Alternatively, PGE_2 has the ability to attenuate the immune system which prohibits cell apoptosis in the breast tumour and allows its progression. Attenuation of the immune occurs due to the reduced cytotoxic activity of natural killer (NK) and lymphokine-activated killer (LAK) cells in the breast tumour (Baxevanis et al., 1993). Additionally, PGE_2 attenuates the activity of macrophages through inhibition of IL-12 and TNF_{α}, which is mediated via EP_4 receptor (Ikegami et al., 2001).

Overall, the relationship between oestrogen and prostaglandins seems to be the driving force in the progression of hormone responsive breast tumours. However, there is a paucity of studies investigating the control 17β-oestradiol synthesis via AKR1C isoenzymes in adipose tissues surrounding breast tumours.

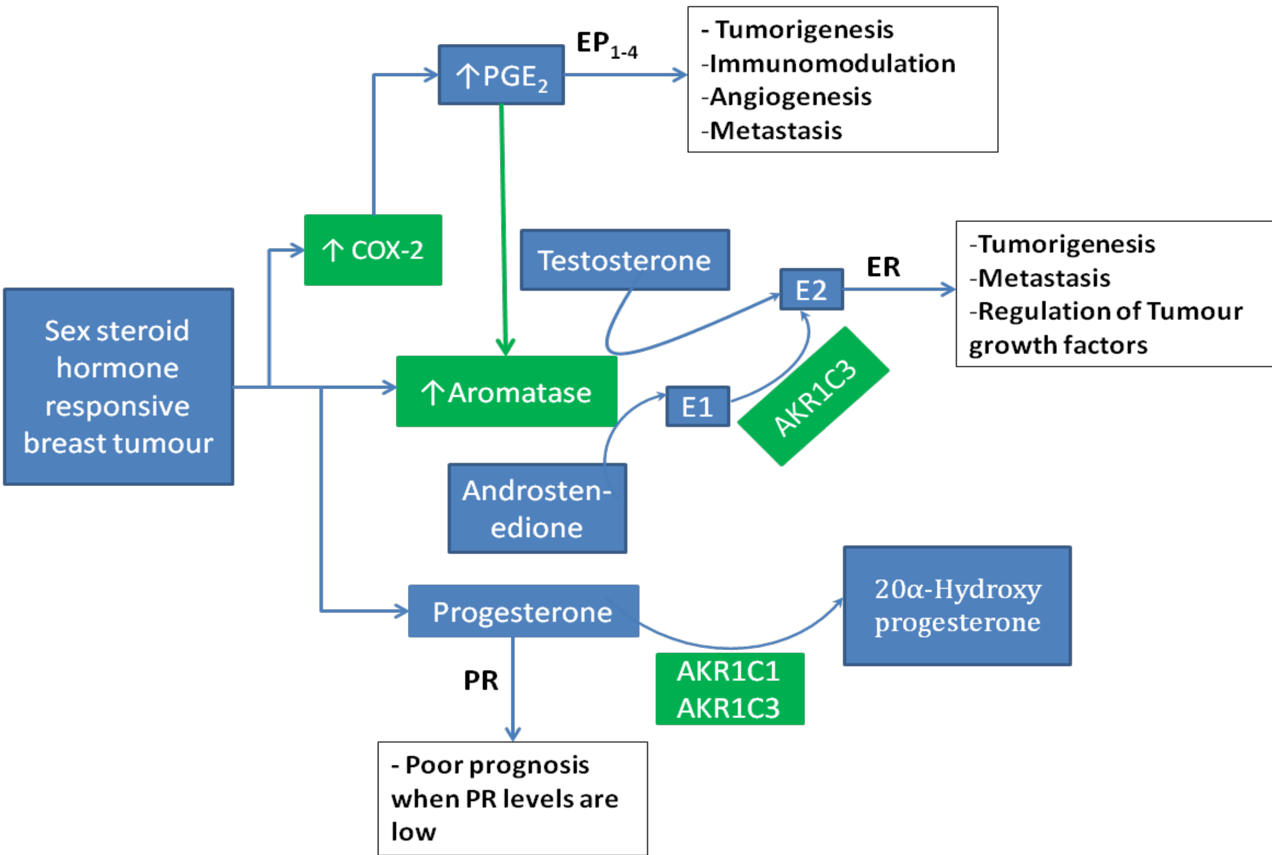


Fig 1.3.5: A summary of the role of oestrogen, progesterone and prostaglandin E_2 in the progression of sex steroid hormone responsive breast tumour.

1.4. The role of adipose tissue in hormone dependent diseases: endometriosis and breast cancer

Adipose tissues were considered to be inert organs that are merely used for storing fat as a source of energy. However, since the discovery of leptin in the mid-1990s, adipose tissues have been considered as endocrine tissues responsible for the secretion of several hormones (Considine et al., 1996). Adipose tissues play important roles in energy homeostasis, lipid metabolism, immune response and reproduction (Coelho et al., 2013).

Adipose tissues are considered to be the major tissue for synthesising oestrogen in postmenopausal females, as mRNA expression of aromatase is increased in subcutaneous fat and omental fat with advancing age (Bulun and Simpson, 1994). Moreover the omental fat cell size is significantly larger in postmenopausal females (Tchernof et al., 2004).

Oestrogen plays a part in distributing fatty tissues in the body, as fat generally accumulates in the gluteofemoral area in premenopausal females and then redistributes to the intra-abdominal area (omental adipose tissue) after menopause (Toth et al., 2000).

There is contradictory evidence for the expression of oestrogen and progesterone receptors in adipose tissues (Broonnegard et al., 1994; Pedersen, Hansen, et al., 1996). However, recent studies have reported the presence of low levels of oestrogen and progesterone receptors (Pedersen, Fuglsig, et al., 1996; O'Brien et al., 1998).

The effect of oestrogen seems to reduce fat density in females, as observed by the ERα subtype knockout female mouse model, which showed a marked increase in adipose tissue density (Heine et al., 2000). On the other hand, progesterone, but not oestrogen, has the ability to induce differentiation of preadipocytes to mature adipocytes. The effect of progesterone was further corroborated using a rat *in vivo* model treated with progesterone which demonstrated higher total body fat content and increased basal lipogenesis (Shirling et al., 1981).

I. Omental adipose tissue and its role in endometriosis

Omental adipose tissue is located in the intra-abdominal area of the body. The omental fat is associated with increased risk of several metabolic diseases, such as diabetes and cardiovascular diseases (Kannel et al., 1991). The omental fat demonstrated higher number of mitochondria and elevated oxidative phosphorylation activity in comparison to subcutaneous fat, suggesting that it is more metabolically active than subcutaneous adipose tissues (Kraunsøe et al., 2010).

The omental adipose tissue has the potential to have a supportive role in the development and maintenance of endometriotic lesions in the peritoneal cavity. In addition to being more metabolically active, omental fat has higher 17β-hydroxysteroid dehydrogenase (17β-HSD) activity compared to subcutaneous adipose tissues (Deslypere et al., 1985). This suggests that if oestrone and 17β-oestradiol ratios are dysregulated in the omental adipose tissue, resulting in higher 17β-oestradiol concentrations, it can support endometriotic lesion survival. Moreover, oestrogen receptors behave differently at different adipose sites, as

oestrogen treatment increased ERα protein expression in omental adipocytes but inhibited it in subcutaneous adipocytes (Anwar et al., 2001).

On the other hand, the effects of progesterone on adipose tissue and adipose cell regulation have been investigated in several studies. Progesterone can promote lipogenesis in the murine 3T3-L1 cell line by stimulating adipocyte differentiation (Rondinone et al., 1992). This effect is not mediated through the progesterone receptors, as it had no effect, but through antagonising the glucocorticoid receptor (Xu et al., 1990).

In the omental fat, AKR1C1-3 enzymes were all detectable in the omental fat (Blanchette et al., 2005). AKR1C1, which catalyses the conversion of progesterone to 20α-hydroxyprogesterone, has a positive correlation between visceral adipose tissue area and its enzymatic activity in the omental fat (Blanchette et al., 2005).

Although aromatase and 17β-HSD activities are significantly reduced in adipose tissues of postmenopausal females compared to premenopausal counterparts, the combination of adipose tissues and skin are capable of inducing postmenopausal endometriosis, especially in obese female patients (Deslypere et al., 1985; Bendon and Becker, 2012).

As with prostaglandins, Affymetrix cDNA microarray data showed that omental adipose tissue had higher gene expression of COX-1 and PGDS, compared to subcutaneous adipose tissue; however AKR1C3 was higher in subcutaneous fat (Quinkler et al., 2006). Also, PGE synthases were detected in human adipocytes (Hétu and Riendeau, 2007).

In summary, omental adipose tissue is sufficiently active to supply 17β-oestradiol and prostaglandins, especially PGE_2 and $PGF_{2\alpha}$. This potentially allows the two entities to interplay and support the survival and development of endometriotic lesions.

II. Breast fat tissues and its role in breast cancer

Breast cancer is the most common cancer in women in England. In 2010, 41,259 new cases of breast cancer were diagnosed in England. Breast cancer is the second most common cause of cancer death in women, affecting 1 in 8 women at some point in their lifetime (Office for National Statistics, 2012).

The association of breast adipose tissue with breast cancer still remains controversial. Some studies have reported that breast non-dense areas (i.e. fatty tissues) have a protective effect on reducing the risk of developing breast cancer (Torres-Mejía et al., 2005; Pettersson et al., 2011). Alternatively, there is another study which showed a positive correlation between breast fatty tissues and breast cancer risk (Lokate et al., 2011). These contradictory reports are probably due to the multi-factorial nature of breast cancer.

Furthermore, there is a positive association with dietary fat intake and breast cancer risk which suggests that fat has indirect supportive role in breast cancer through its ability to synthesise oestrogen (Lee and Lin, 2000).

The progesterone receptors were detected in the breast tissues, indicating that they may have a role in the regulation and distribution of breast tissue (O'Brien et al., 1998). However, with the exception of progesterone's role in lipogenesis, there are

limited data on the role of progesterone receptors in breast fat tissue and how they affect breast cancer.

Furthermore, PGE_2 is a stimulator of aromatase in breast adipose stromal cells. Also, the release of TNF_{α} in breast adipose stromal cells has a dual stimulatory effect on aromatase, as well as PGE_2 via the upregulation of COX-2 and PGE_2 (Karuppu et al., 2002). The convergence of these mechanisms leads to the stimulation of oestrogen in the local environment of breast.

Adipose tissues enhanced tumour growth in an *in vivo* mouse model when they were co-injected with F442A adipocytes and breast cancer cell line (MCF-7) (Liu et al., 2013). Furthermore, leptin, a polypeptide cytokine, induced the expression of aromatase in F442A preadipocytes and mature adipocytes (Liu et al., 2013). Also, breast tumours express more leptin and leptin receptors compared to normal mammary gland, suggesting that breast tumours are capable of expressing aromatase which is the limiting step for synthesising oestrone and 17β-oestradiol (Ishikawa et al., 2004).

In conclusion, breast adipose tissue and breast tumours form a dynamic partnership in which breast adipose tissue maintains the survival of breast tumours. Therefore, there is a need to investigate the role of enzymes, such as AKR1C isoenzymes, which are involved in sex steroid hormone and prostaglandin synthesis in breast fat tissues and how they affect the development and progression of breast tumours.

1.5. Aim

It has been proposed that there is a synergistic relationship between prostaglandins and sex steroid hormones in endometriosis and breast cancer, which is partly characterised by the AKR1C isoenzymes, as they utilise sex steroid hormones and a number of prostaglandins as substrates. As a result, the gene expression of AKR1C isoenzymes, EP receptor subtypes (1-4) and FP receptor were compared in the endometrium, endometriotic lesions, and the adipose tissues surrounding endometriotic lesions and breast tumours to investigate whether they are implicated in endometriosis and breast cancer. This is the first comparison of gene expression of AKR1C isoenzymes, EP receptor subtypes (1-4) and FP receptor in adipose tissues surrounding endometriotic lesions and breast cancer (Chapter 3).

Moreover, the physiological effect of one of the AKR1C isoenzymes (AKR1C3) was further investigated using a potential AKR1C3 inhibitor, bimatoprost, to determine whether the levels of 17β-oestradiol and prostaglandins (such as 9α, 11β PGF_2) were affected in human endometrial cells (Chapter 4).

Another characteristic of the synergistic relationship between prostaglandins and sex steroid hormones is the ability of PGE_2 to influence the levels of 17β-oestradiol. Therefore, the effect of PGE_2 on the expression of AKR1C isoenzymes was investigated in human endometrial cells (Chapter 5).

Finally, ectopic endometrial cells have the ability to migrate, invade surrounding tissues and develop blood vessels. As a result, mitochondrial respiration and glycolytic functions of ectopic endometrial cells were investigated in endometriosis to determine whether they undergo any metabolic changes. Since ectopic cells are

hormone dependent, certain potential modifiers, namely oestrone and 17β-oestradiol, were used to investigate their effects on mitochondrial and glycolytic functions of endometrial cells (Chapter 5).

2. General Methodology

2.1. Human endometrial cell culture

2.1.1. Materials

a) Enzymatic Solution

	Catalogue Number	Company
DMEM/F12 Glutamax-I with phenol red	A1515501	Gibco
Collagenase Type IA	C2674	Sigma-Aldrich

b) Basic Cell Culture Media

	Catalogue Number	Company
DMEM/F12 Glutamax-I with phenol red	A1515501	Gibco
Foetal Bovine Serum (FBS)	10270-106	Gibco
Non essential Amino Acids (NEAA)	M7145	Sigma-Aldrich
Penicillin-streptomycin solution (Pen-Strep)	P4333	Sigma-Aldrich

c) Cell Dissociation reagent

	Catalogue Number	Company
TrypLE Express Solution (1X)	12605-036	Gibco
Dulbecco's Phosphate Buffered Saline (DPBS)	D8537	Sigma-Aldrich

d) Cell viability assay

	Catalogue Number	Company
Trypan Blue 60% Solution	302643	Sigma-Aldrich
DMEM/F12 Glutamax-I with phenol red	A1515501	Gibco

e) MTT assay

	Catalogue Number	Company
Thiazolyl Blue Tetrazolium Bromide (MTT)	M2128	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline	D8537	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	D8418	Sigma-Aldrich

f) Conditioned Media

	Catalogue Number	Company
DMEM/F12 without phenol red	21063-029	Gibco
Heat Inactivated, Charcoal Stripped Foetal Bovine Serum	12676-029	Gibco

g) Equipment

Equipment	Company
Cell Culture Flask (25 and 75 cm^2)	Greiner Bio-one (GBO)
Petri dishes 60mm diameter	Greiner Bio-one (GBO)
Centrifuge tubes (15ml and 50ml)	Greiner Bio-one (GBO)
Centrifuge	Eppendorf
5% CO2 Incubator	NuAire
Inverted phase contrast microscope	Olympus Optical Co. Ltd
Orbital incubator	Stuart scientific

2.1.2. Methods

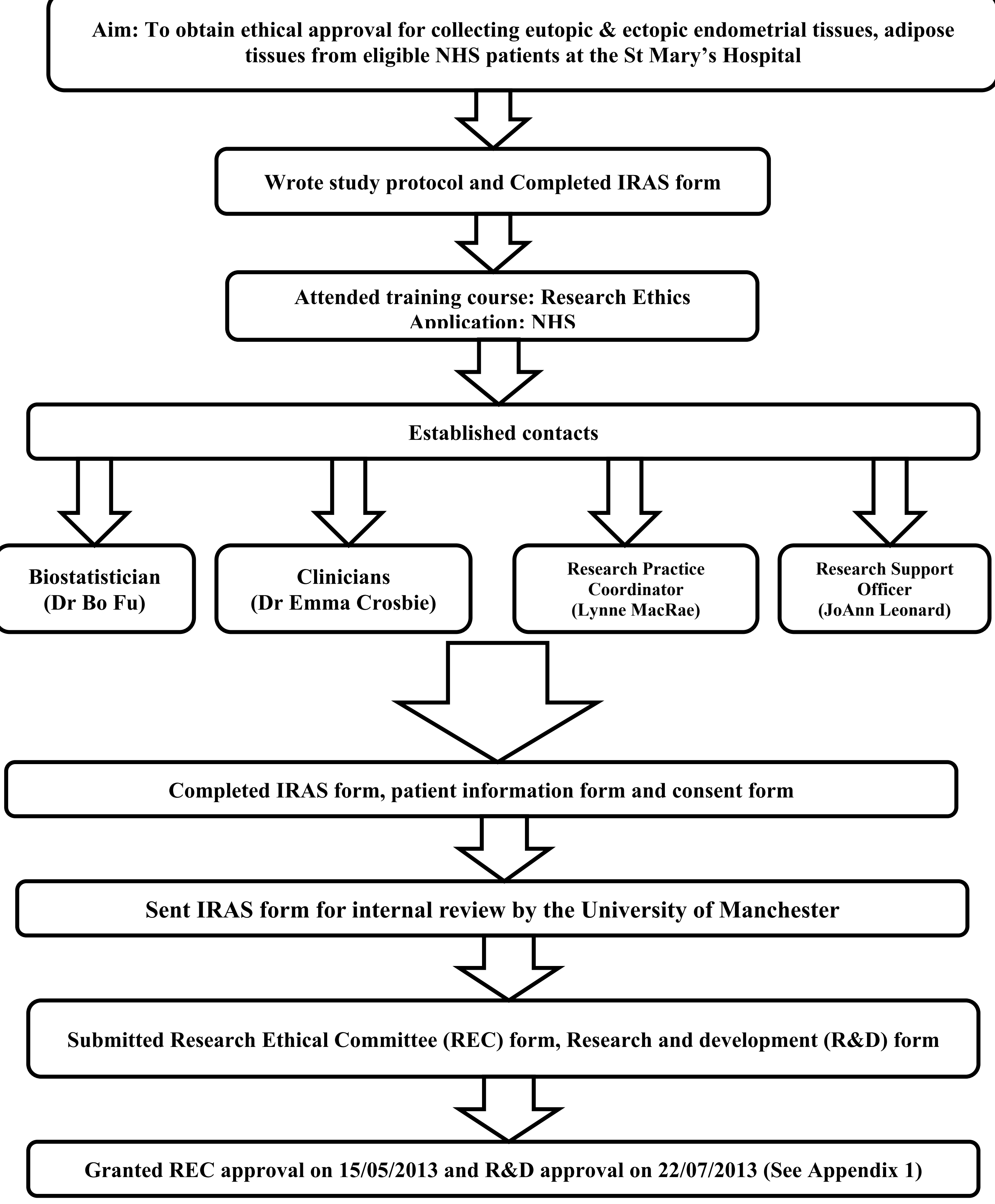
I. Endometrial and omental adipose tissue biopsy donation

Human endometrial and omental adipose biopsies were obtained from consenting female donors who were undergoing laparoscopy or hysterectomy at the Central Manchester University Hospitals. Potential donors were approached by a clinician and were asked to sign a consent form and give non-identifiable clinical information prior to the procedure. This Study was approved by the Bristol Research Ethics Committee, **REC reference number: 13/SW/0123**, and by the Research and Ethics Office at the Central Manchester University Hospitals (see flowchart below).

Human eutopic endometrial tissues were obtained during a pre-operative clinical assessment in the morning of laparoscopy or hysterectomy operation. The ectopic endometrial tissues and omental adipose tissues were obtained during the laparoscopy or hysterectomy procedure.

Over the period of two years, a total of 41 eutopic endometrial samples, 11 endometriotic lesions and 32 omental adipose tissue samples.

The breast adipose tissues surrounding breast tumours, which were redundant, were obtained at the Institute for Cancer Sciences, the University of Manchester. The majority of breast adipose tissues were processed at the Institute for Cancer Sciences and were taken as ribonucleic acid (RNA). However, due to limited space and time, the remaining breast fat tissues were processed in our laboratory at Stopford building, the University of Manchester.


Aim: To obtain ethical approval for collecting eutopic & ectopic endometrial tissues, adipose tissues from eligible NHS patients at the St Mary's Hospital
Wrote study protocol and Completed IRAS form
Attended training course: Research Ethics Application: NHS
Established contacts
Biostatistician (Dr Bo Fu)
Clinicians (Dr Emma Crosbie)
Research Practice Coordinator (Lynne MacRae)
Research Support Officer (JoAnn Leonard)
Completed IRAS form, patient information form and consent form
Sent IRAS form for internal review by the University of Manchester
Submitted Research Ethical Committee (REC) form, Research and development (R&D) form
Granted REC approval on 15/05/2013 and R&D approval on 22/07/2013 (See Appendix 1)

II. Human endometrial cell isolation

Endometrial biopsies were placed in a tight seal container containing DMEM F12-Glutamx-I (Gibco, USA). The biopsies were transported in an Ethical Tissue Transport Box (ETTB) to the laboratory.

The endometrial tissue (0.2 – 0.6g) was washed 3 times in DPBS (Sigma, Germany) and finely minced with a scalpel or scissors. It was incubated in DMEM/F12 media plus Glutamax-I (Gibco, USA), containing 400 units/ml collagenase type 1A (Sigma, Germany), for 3 hours in an orbital incubator (Stuart Scientific, UK) at 37°C. The use of an orbital incubator shortened the incubation period specified on the previous laboratory protocol which stated that digestion period should be 8 hours in a conventional 5% CO_2 incubator.

The enzymatic mixture was then passed through a 100μm cell strainer (BD falcon, USA) to exclude tissue debris. The filtrate was centrifuged at 500x*g* for 10 minutes at room temperature, followed by discarding the supernatant. The cell pellet was suspended in 1ml of fresh media and placed on a 25cm^2 cell culture flask (GBO, Germany) containing 5ml DMEM/F12-Glutamax-I media (Gibco, USA), supplemented with 10% v/v FBS (Gibco, USA), 1% v/v pen-strep (Sigma, Germany) and 1%v/v NEAA (Sigma, Germany).

It is worth noting that endometrial cells were mixed population of epithelial and stromal cells, as cell separation by cell size was not effective in obtaining a homogenous population and prolonged the length of time needed to become confluent. Also, the mixed population of epithelial and stromal endometrial cells represents what happens naturally in the human endometrium (Fig 2.1.1).

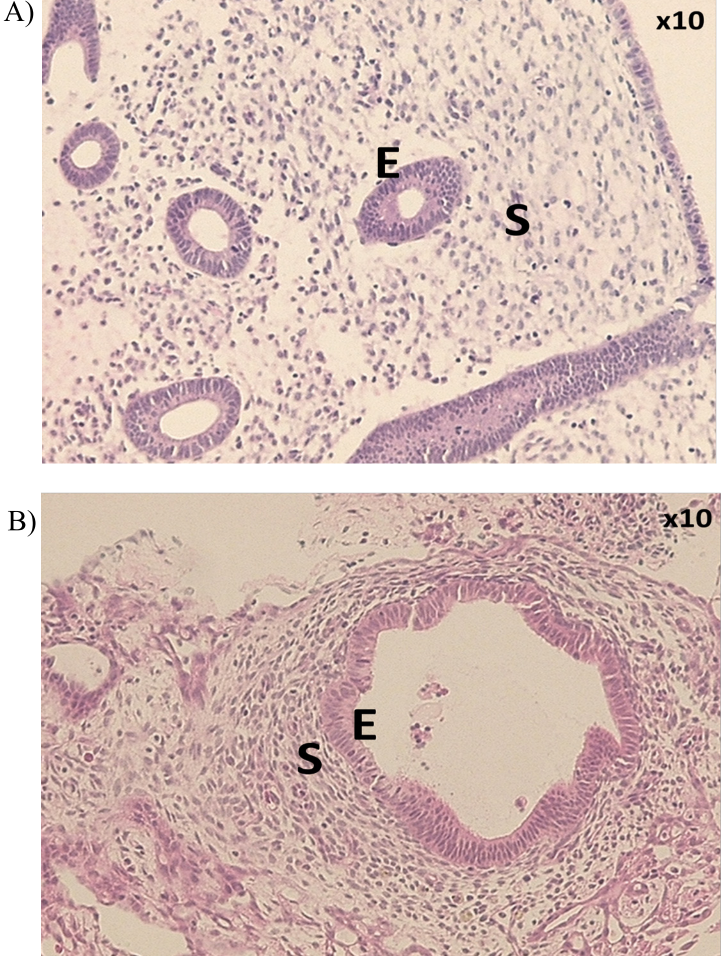


Fig 2.1.1: Haematoxylin and eosin staining of human eutopic endometrial tissues (A) and human ectopic endometrial tissues (B), showing epithelial (E) and stromal (S) layers at 10x magnification.

III. Subculture of human endometrial cells

The endometrial cells usually took 1-2 weeks to become 80-90% confluent. Once it reached that level of confluence, the cells were subcultured enzymatically using trypsin.

Firstly, the medium was discarded and the cells were washed with DPBS (Sigma, Germany) 3 times. Approximately 4 ml of TrypLE Express solution, which is recombinant human trypsin, (Gibco, USA) was added and followed by 5-10 minute

incubation at 37°C. The cells were monitored regularly under the microscope to assess cell dissociation. Once the majority of cells had lifted off, the suspension was collected in a 15ml centrifuge tube and centrifuged at 500x*g* for 10 minutes. The supernatant was discarded, by decanting initially, and then by pipetting to remove the remainder of supernatant. The cell pellet was resuspended in 1 ml of fresh DMEM/F12 Glutamax-I media. A small volume (10µl) was taken for quantifying cells, whilst the rest was divided between two 75cm^2 flasks (GBO, Germany) containing 10ml DMEM/F12 Glutamax-I media (Gibco, USA), supplemented with 10%v/v FBS (Gibco, USA), 1%v/v Pen-Strep (Sigma, Germany) and 1%v/v NEAA (Sigma, Germany).

IV. Cell counting:

Once the cells were resuspended in 1ml media, 10µl was added to trypan blue solution (sigma, Germany) at a dilution factor of 4. The trypan blue stains dead cells blue, whereas live cells appear clear or colourless (Altman et al., 1993). The counting of cells is performed on a haemocytometer (Bright-line, Cambridge Instruments, USA), which is divided into 9 large squares, under an inverted microscope (Olympus Optical Co. Ltd, Japan). At 10x magnification, the total number of cells in each of the 5 squares was counted to determine the number of cells in 1ml suspension (Fig 2.1.2).

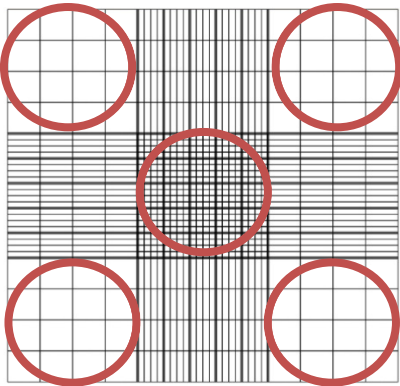


Fig 2.1.2: Cells (live and dead) are counted on the squares circled in red to estimate the total number of cells in 1ml suspension. The area of each square is $1mm^2$ and the depth of the chamber is 0.1mm.

$$\textbf{\% Viability} = \frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100$$

Number of cells per 1 millilitre (cells/ml) = Average number of live cells per one square x dilution factor x *correction factor

* Correction factor = 10000; to convert from $0.1mm^3$ to $1cm^3$

V. **MTT assay**

a) **Procedure**

Cell proliferation can be indirectly measured by the ability of living cells to metabolise tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl bromide (MTT) to insoluble formazan insoluble crystals. This enzymatic reaction is dependent on NAD(P)H (Liu et al., 1997). Thiazolyl Blue Tetrazolium Bromide or MTT (Sigma, Germany), a yellow crystal powder, was diluted in DPBS at a concentration of 5mg/ml. The solution was sterile filtered and aliquoted in 1ml stock vials.

At the end of an experiment, the conditioned media was aspirated, followed by the addition of 100µl of 0.5mg/ml MTT to each well. The plate was incubated for 3 hours at 37°C with 5%CO_2. The MTT was then aspirated and 100µl of dimethyl

sulphoxide or DMSO (Sigma, Germany) was added to solubilise the formazan crystals. The plate was stirred gently to enhance dissolution of formazan and form a homogenous purple solution. The plate was read at 570nm using BioTek ELx 800 microplate reader (BioTek, USA) (Riss et al., 2013).

b) Determining the cell density for 96-well plate

The cell density per well was opitimised using different cell densities which were incubated in DMEM/F12 phenol red free media for 48 hours. There is a correlation between cell density and intensity of absorabance, as demonstrated below. A cell density of $0.15x10^6$ cells/ml was used in the subsequent experiments, as it was more economical and hence would allow high throughput experimentations. Also it lies within a region of the plot where sensitivity to changes induced by experimental parameters could be observed (Fig 2.1.3).

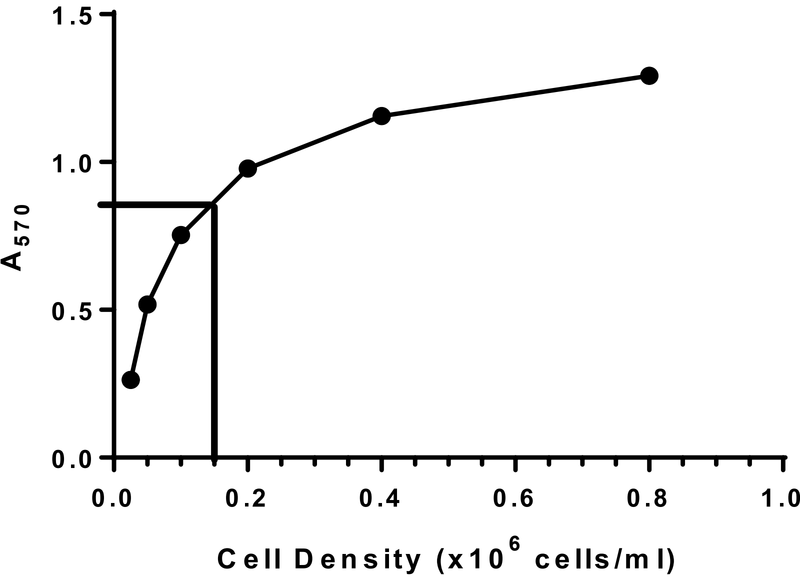


Fig 2.1.3: Human endometrial cells plated on 96-well plate at different cell densities and incubated for 48 hours. 100µl of MTT (0.5mg/ml) was added and followed by 3 hour incubation. MTT was removed and DMSO was added to the cells to dissolve the formazan crystals. The colour absorbance was measured using a plate reader at 570nm.

2.2. Explant Culture

2.2.1. Materials

I. Explant culture Media

	Catalogue Number	Company
Dulbecco's Phosphate Buffered Saline (DPBS)	D8537	Sigma-Aldrich
DMEM/F12 without phenol red	21063-029	Gibco
Heat Inactivated, Charcoal Stripped Foetal Bovine Serum	12676-029	Gibco

II. Equipment

Equipment	Company
24 Multi-well plate	Corning Inc
0.4μm well inserts	Greiner Bio-one
5% CO_2 incubator	Nuaire

2.2.2. Method

Endometrial biopsies were washed 3 times with DPBS (Sigma, UK) and weighed into 10-15mg portions. The tissues were cut using a scalpel into approximately 1mm^2 in DMEM/F12 media without phenol red (Gibco, USA), supplemented with 1% v/v charcoal-stripped and heat inactivated foetal calf serum (Gibco, USA). The tissues were then transferred into 0.4µm well inserts (Greiner Bio-one, Germany) and placed in duplicates in a 24 well plate (Corning, USA) containing 1 ml of DMEM/F12 Glutamax-I media (Gibco, USA), supplemented with 10% foetal calf serum (Gibco, USA) and 1% Pen-Strep (Sigma, Germany).

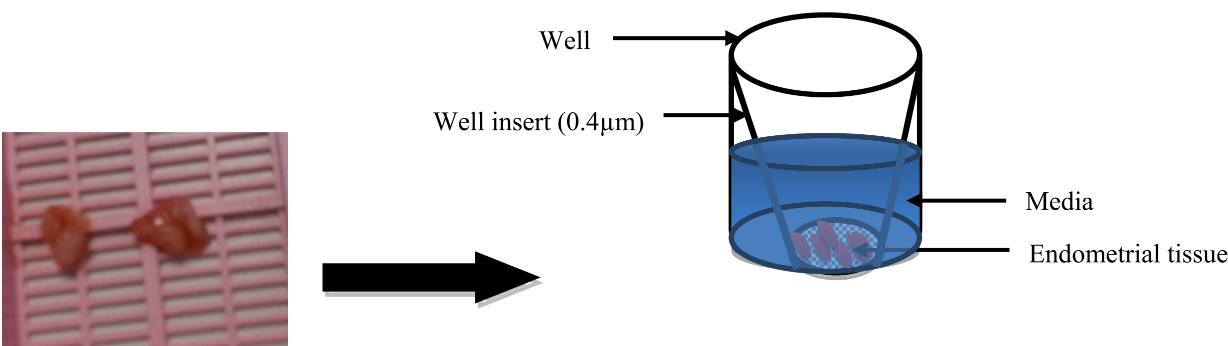


Fig 2.2.1: Schematic diagram of explant culture experiment

The explants were incubated at 37°C for 24 hours prior to the start of treatment to allow the tissue to settle in the media. They were treated for 48 hours with different treatments diluted in DMEM/F12 without phenol red (Gibco, USA), supplemented with 1%v/v charcoal stripped and heat inactivated foetal calf serum (Gibco, USA). The treatments were replaced at 24-hour interval and aliquots of the conditioned media were collected and stored at -80°C for further analysis.

2.3. Enzyme-linked Immunosorbent Assay (ELISA)

2.3.1. Materials

ELISA kit	*Catalogue Number*	*Manufacturer*
PGE_2 ELISA kit	ADI-900-001	Enzo Life Sciences Ltd
17β-oestradiol ELISA kit	582251	Cayman Chemical
$PGF_{2\alpha}$	ADI-900-069	Enzo Life Sciences Ltd
9α, 11β PGF_2 ELISA kit	516521	Cayman Chemical

2.3.2. Methods

I. Preparation of standards:

i. ***PGE_2***: the sensitivity of the kit ranged from 39.1 to 2500 pg/ml. 50 ng/ml PGE_2 standard solution was serially diluted using culture media to make up 7 standards with the following concentrations: 2500, 1250, 625, 313, 156, 78.1 and 39.1 pg/ml.

ii. ***17β-oestradiol:*** the sensitivity of the kit ranged from 6.6 to 4000 pg/ml. 400ng/ml of 17β-oestradiol standard solution was serially diluted using culture media to make up 8 standards with the following concentrations: 4000, 1600, 1875, 640, 256, 102.4, 41, 16.4 and 6.6pg/ml.

iii. ***9α, 11β PGF_2:*** the sensitivity of the kit ranged from 1.6 to 1000 pg/ml. 100ng/ml of 9α,11β PGF_2 was serially diluted using culture media to make up 8 standards: 1000, 400, 160, 64, 25.6, 10.2, 4.1 and 1.6pg/ml.

iv. ***$PGF_{2α}$:*** the sensitivity of the kit ranged from 3.05 - 50000 pg/ml. 100ng/ml of $PGF_{2α}$ was serially diluted using culture media to make up 8 standards: 50000, 12500, 3125, 781.25, 195.31, 48.83, 12.2 and 3.05 pg/ml.

II. Assay procedure:

a) ENZO ELISA kit

The media samples were first thawed in ice, whereas ENZO ELISA kit components were thawed at room temperature for 30 minutes.

According to manufacturer's protocol, 100µl of tissue culture media was added into the nonspecific binding (NSB) and maximum binding (B_0) wells, whilst 100µl of standards and samples were added to their designated wells. This was followed by adding 50µl of alkaline phosphatase covalently attached to PGE_2 (blue solution) to standards, samples, NSB and B_0 wells. Subsequently, 50µl of the primary monoclonal PGE_2 antibody (yellow solution) was added to standards, samples and B_0 wells. The plate was then incubated at room temperature on a plate shaker for 2 hours at ≈500rpm.

The content of wells was emptied and all wells were washed 3 times with 400µl of 1Xwash solution. After aspirating the wells and removing any remaining wash buffer, 5ul of alkaline phosphatase conjugated with PGE_2 (blue solution) was added to the total activity (TA) well to determine the percentage bound by tracer. This was followed by the addition of 200µl of p-nitrophenyl phosphate (pNpp) substrate solution to all wells. The plate was sealed with aluminium foil and incubated for 45 minutes at room temperature. This would allow alkaline phosphatase to convert

pNPP to p-nitrophenol (pNp) which has a yellow colour. For the termination of enzymatic reaction, 50µl of stop solution was added to all wells and the plate was read immediately at 405nm using Biotek absorbance reader (Biotek, USA).

b) Cayman ELISA kit

The media samples were first thawed in ice, whereas Cayman ELISA kit components were thawed at room temperature for 30 minutes.

According to the manufacturers protocol, 50µl of culture media was added into the nonspecific binding (NSB) and maximum binding (B_0) wells. Also, 50µl of standards and samples were added to their designated wells. This was followed by the addition of 50µl of the tracer - containing acetylcholinesterase covalently attached to 17β-oestradiol or 9α, 11β PGF_2 - to standards, samples, NSB and B_0 wells. Subsequently, 50µl of the primary antibody for 17β-oestradiol or 9α, 11β PGF_2 was added to standards, samples and B_0 wells. 17β-oestradiol ELISA plate was incubated at room temperature on a plate shaker for 1 hour at ≈500rpm, whereas 9α, 11β PGF_2 ELISA plate was incubated for 18 hours at room temperature.

The content of wells was emptied and washed 5 times with 400µl 1X wash buffer. 5ul of the tracer was added to the total activity (TA) well to determine the percentage bound by tracer. Ellman's reagent was reconstituted with 20ml of UltraPure water (Cayman, USA) and 200µl were added to all the wells.

Ellman's reagent contains acetylcholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylcholine by acetylcholinesterase produces thoicholine, which can

react non-enzymatically with 5,5'-dithio-bis-(2-nitrobenzoic acid). The resulting product (5-thio-2-nitrobenzoic acid) has a yellow colour and has a strong absorbance at 405nm. After 60-minute incubation in the dark on a plate shaker, the plate was measured using Biotek absorbance reader (Biotek, USA).

III. Data analysis:

a) Determining sample concentration

The averaged absorbance values of standards and samples were subtracted from averaged blank and non-specific binding (NSB) wells. The binding of standards and samples was calculated as a percentage of the maximum binding (B_0) using the following equation:

$$\%\text{Bound } (\%B/B_0) = \frac{\text{Net absorbance of standards or samples}}{\text{Net absorbance of } B_0} \times 100$$

b) Four-parameter logistic non-linear regression model

The 4-parameter logistic non-linear regression model was used to produce the standard curve. The following equation was incorporated into GraphPad Prism software (version 6.04) and used for interpolating the concentration of analytes (Herman et al., 2008):

Y= ((Bottom-Top)/ (1+ ((X/Inflection) ^Slope))) +Top

X = concentration of analyte

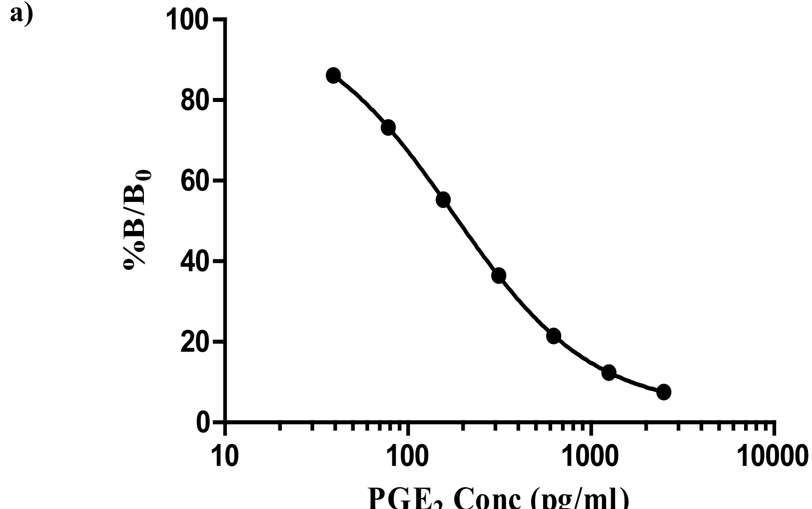
Y = Response; (%B/B_0)

Top and Bottom = minimum and maximum values of %B/B_0

Inflection point = the concentration (X) at which the curve changes direction

Slope = the steepness of the curve

a)



b)

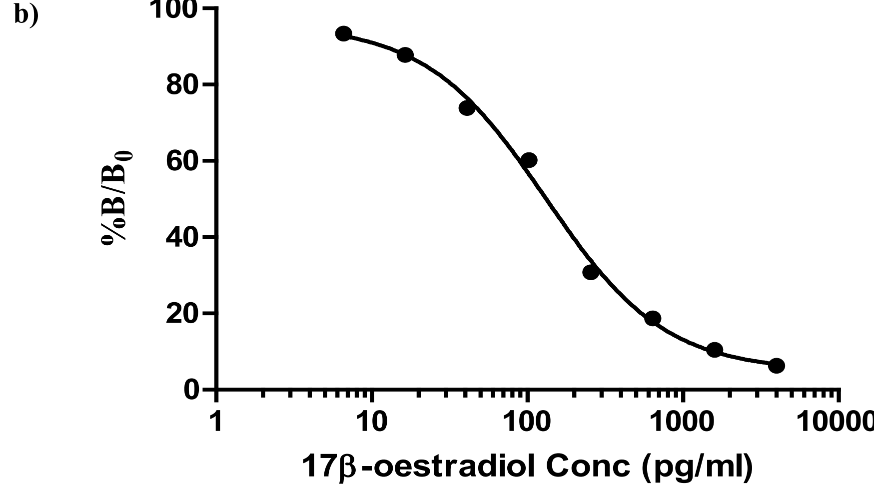


Fig 2.3.1: Typical standard curves of (a) PGE_2 and (b) 17β-oestradiol

c) Intra-assay and inter-assay coefficients of variation (%CV)

The intra- and inter-assay coefficients of variation were calculated using concentrations determined by a standard curve. Intra-assay coefficient of variation was calculated to determine the accuracy of sample replicates within each plate, whereas inter-assay coefficient of variation was calculated to give an indication of the reproducibility of results between different plates.

Analyte	Intra-assay %CV	Inter-assay %CV
PGE_2	6.9	18.5
17β-oestradiol	15.5	32.6
$PGF_{2\alpha}$	8	ND
9α, 11β PGF_2	8.5	19.2

Table 2.3.1: The intra- and inter-assay coefficients of variation (%CV) for the following analytes: PGE_2, 17βoestradiol, $PGF_{2\alpha}$ and 9α, 11β PGF_2.

2.4. Real time quantitative polymerase chain reaction (qRT PCR)

2.4.1. RNA isolation

a) Tissues:

Prior to conducting any RNA isolation, the area must be free of RNase contamination. RNaseZap (Ambion, USA) was used to denature any RNase on the bench surface, glassware and other laboratory equipment used for the procedure.

Human endometrial or adipose tissue samples (≈20-90mg) were stabilised in aliquots of RNAlater (Ambion, USA) and kept at -80ºC for later use. Tissues were thawed and placed in 1 ml of TRIzol (Sigma, Germany) and disrupted using a rotor-stator homogeniser (The Scientific Instrument Centre, Germany) for 30 – 60 seconds. The homogenate was passed through a QIAshredder (Qiagen, Germany) and centrifuged at 12000xg for 2 minutes. The homogenate was incubated for 5-10 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes.

b) Cells grown in monolayer

Human endometrial cells cultured as a monolayer were lysed by adding 1 ml TRIzol (Sigma, Germany) and incubated at room temperature for 2-3 minutes. The cell lysate was passed through a pipette a few times. It was then transferred into QIAshredder (Qiagen, USA) and centrifuged at 12000xg for 2 minutes. The homogenate was incubated for 5-10 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes.

2.4.2. Purification of RNA

a) Purification of RNA using phenol-chloroform extraction

This method was first developed by Chomczynski and Sacchi in 1987. Initially, 200µl of chloroform was added to lysate or homogenate and vortexed for 15 seconds. The samples were incubated at room temperature for 2-3 minutes and centrifuged at 12000xg for 15 minutes at 2-8°C. The mixture separated into three layers:

a) Chloroform layer: The upper layer; contains RNA
b) Interphase: The middle layer; contains DNA
c) Phenol layer: The lower layer; contains proteins

The upper layer containing RNA was carefully removed and transferred into a fresh tube. To precipitate RNA, 500µl of isopropyl alcohol (Fisher, UK) was added and mixed gently with the clear solution containing RNA. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12000xg for 10 minutes at 2-8°C. After removing the supernatant, RNA pellet was washed by adding 1ml of 75%v/v ethanol (Sigma, Germany) and mixed together by vortexing. The mixture was centrifuged at 7500xg for 5 minutes at 2-8°C. This washing step was repeated once. Ethanol was removed and the pellet was air-dried for 5 minutes. RNA was dissolved in 25-30µl of DPEC treated water (Invitrogen, USA).

b) Purification of RNA using RNEasy kit

RNeasy Mini Kit (Qiagen, Germany) was used for the purification of total RNA. As per manufacturer's protocol, 350 - 600µl of 70% ethanol (Sigma, Germany) was added to the supernatant and mixed properly by pipetting up and down. The mixture was then transferred into an RNeasy spin column, fitted with a 2 ml collection tube, and centrifuged at 8000xg for 15 seconds. After discarding the flow through, 700µl of RW1 buffer (Qiagen, Germany) was added to the spin column and centrifuged at 8000xg for 15 seconds. The flow through was discarded and 500µl of RPE buffer (Qiagen, Germany) was added to the spin column and centrifuged at 8000xg for 15 seconds. RPE buffer (Qiagen, Germany) was added again and centrifuged at 8000xg for 2 minutes. After discarding the flow through, the spin column was centrifuged at full speed for 1 minute to remove any carryover of RPE buffer. The column was placed in a new collection tube, followed by adding 25-30µl of DPEC treated water (Qiagen, Germany) to the spin column and centrifuging at 8000xg for 1 minute to elute RNA. To elute more RNA, the eluent was added back again into the spin column and centrifuged at 8000xg for 1 minute.

2.4.3. Quantification of RNA

After purification of RNA, 2µl of RNA was added to a special 96 well plate, DropPlate (Integrated Sciences, Australia). The plate was inserted into the DropSense 96 (Integrated Sciences, Australia), which is a UV and Visible light spectrophotometer. DropSense 96 measured the optical density at 230nm (A_{230}), 260nm (A_{260}) and 280nm (A_{280}). The analytical software, cDrop (Integrated

Sciences, Australia), calculated the concentration of RNA (ng/µl), as well as A_{260}/ A_{280} ratio for determination of RNA purity. Generally, A_{260}/ A_{280} ratio value of 1.8 and above was indicative of low DNA contamination.

2.4.4. Checking RNA integrity

According to the manufacturer's protocol, 2-5µg of total RNA was suspended in nuclease free water (Ambion, USA). NorthernMax formaldehyde load dye (Ambion, USA) was added in a ratio of 1:3 v/v (1 volume RNA and 3 volumes dye). The mixture was incubated for 15 minutes in a 65°C water bath. The mixture was centrifuged briefly (≈ 7500xg for 5-10 seconds). NorthernMax denaturing gel buffer (10X) (Ambion, USA) was diluted 10x with nuclease free water (Ambion, USA) and added to agarose powder (Invitrogen, USA) to prepare 1% agarose gel. The powder was dissolved by heating in the microwave for 30-60 seconds. Subsequently, 10mg/ml ethidium bromide (Sigma, Germany) was added at a concentration of 0.1µl/1ml of agarose gel. The gel was poured onto the gel tray with comb mounted. After 30 minutes, the gel solidified and the comb was carefully removed from the gel, creating wells in which the samples would be added. The Loading dye containing RNA was added to each well and the tray was then placed in the buffer tank containing 1X MOPS buffer (Santa Cruz, USA). A voltage of 120V was applied for 75 minutes. At the end of electrophoresis, the gel was visualised under UV light. Intact total RNA run on a denaturing gel will have clear 28S and 18S rRNA bands.

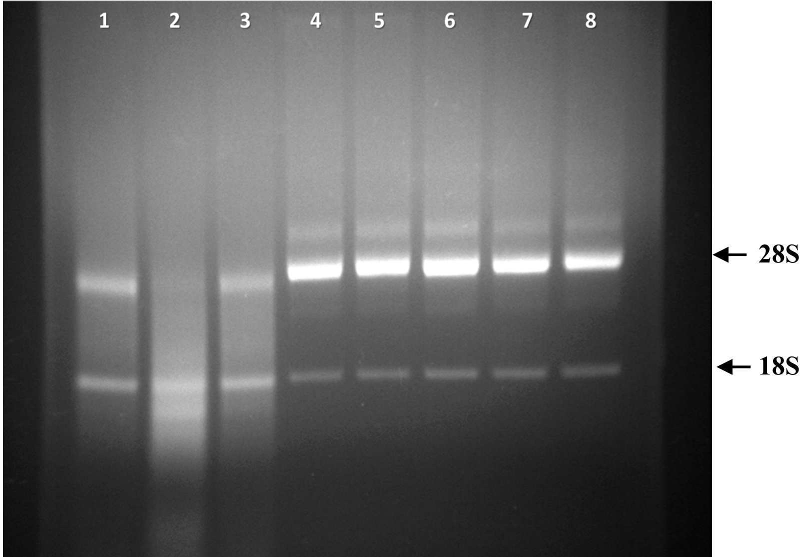


Fig 2.4.1: Two µg of total RNA samples prepared in formaldehyde load dye were run on a 1%w/v agarose gel using 1x MOPS buffer. Lanes 1 to 3 are breast fat tissues and lanes 4 to 8 are human eutopic endometrial cells

2.4.5. cDNA synthesis using QuantiTect Reverse Transcription kit

For cDNA synthesis, QuantiTect Reverse Transcription kit (Qiagen, Germany) was used and the manufacturer's protocol was followed accordingly. Genomic DNA was eliminated by adding 2µl of gDNA wipeout buffer to a total volume of 14µl containing 1µg RNA. The mixture was incubated for 5 minutes at 42°C.

For reverse transcription, Quantiscript RT buffer, RT primer mix, Reverse-transcription master mix and template RNA were mixed in a 4:1:1:14 v/v/v/v ratio to a total volume of 20µl. The mixture was incubated for 20 minutes at 42°C for cDNA synthesis and the reaction was terminated by denaturing Quantiscript reverse transcriptase at 95°C for 3 minutes. cDNA samples were stored at -20°C.

2.4.6. Primer design

A web-based primer designing tool “Primer-BLAST” was used for designing primers [http://www.ncbi.nlm.nih.gov/tools/primer-blast/]. The accession number of the gene of interest was entered into the “PCR template” box. The PCR product size was chosen to be 70 to 200 base pairs, whilst the primer melting temperature (Tm) was chosen to be 55 to 59°C. To circumvent the issue of amplifying genomic DNA, primers were designed such that they spanned an exon-exon junction.

Design Criteria for qRT PCR primers were adapted from (Dorak et al., 2006):

1. Primer length between 18-24 nucleotides
2. Amplicon length should not exceed 250 base pairs
3. The difference between melting temperature of sense and antisense primers should be no more than 2°C
4. Primer melting temperature (Tm) is between 50 to 60°C (optimal 55-59°C)
5. No runs of more than three consecutive “G” nucleotides
6. No consecutive runs of the same nucleotide more than six times
7. % GC content of primer should be approximately 50% (35% <GC<65%)
8. No 3’ CG clamp on primers
9. $\leq$ 2 GC in the last 5 nucleotides of the 3’end of the primer

PCR Template
Reset page Save search parameters Retrieve recent results
Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear
NM_003739.5
Range
From To
Forward primer Clear
Reverse primer
Or, upload FASTA file Choose File No file chosen

Primer Parameters
Use my own forward primer (5'->3' on plus strand) Clear
Use my own reverse primer (5'->3' on minus strand) Clear
PCR product size Min 70 Max 200
\# of primers to return 10
Primer melting temperatures (Tm) Min 55.0 Opt 59.0 Max 60.0 Max Tm difference 2

Exon/intron selection
A refseq mRNA sequence as PCR template input is required for options in the section
Exon junction span Primer must span an exon-exon junction
Exon junction match Exon at 5' side 7 Exon at 3' side 4
Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction
Intron inclusion Primer pair must be separated by at least one intron on the corresponding genomic DNA
Intron length range Min 1000 Max 1000000

Note: Parameter values that differ from the default are highlighted in yellow

Primer Pair Specificity Checking Parameters
Specificity check Enable search for primer pairs specific to the intended PCR template
Search mode Automatic
Database Refseq mRNA
Organism Homo sapiens
Enter an organism name, taxonomy id or select from the suggestion list as you type.
Add more organisms
Exclusion (optional) Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences
Entrez query (optional)
Primer specificity stringency Primer must have at least 2 total mismatches to unintended targets, including
at least 2 mismatches within the last 5 bps at the 3' end.
Ignore targets that have 6 or more mismatches to the primer.
Misprimed product size deviation 4000
Splice variant handling Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)

Primer Pair Specificity Checking Parameters
Max number of Blast target sequences 50000
Blast expect (E) value 30000
Blast word size 7
Max primer pairs to screen 500
Max targets to show (for designing new primers) 20
Max targets to show (for pre-designed primers) 1000
Max targets per sequence 100

Primer Parameters
PCR Product Tm Min Opt Max
Primer Size Min 18 Opt 20 Max 24
Primer GC content (%) Min 35.0 Max 65.0
GC clamp 0
Max Poly-X 5
Max 3' Stability 9
Max GC in primer 3' end 2
Secondary Structure Alignment Methods Use Thermodynamic Oligo Alignment Use Thermodynamic Template Alignment (warning: search may be very slow with this option on)
TH: Max Template Mispriming Primer 40.00 Pair 70.00 (For thermodynamic alignment model only)
TH: Max Self Complementarity Any 45.0 3' 35.0 (For thermodynamic alignment model only)
TH: Max Pair Complementarity Any 45.0 3' 35.0 (For thermodynamic alignment model only)
TH: Max Primer Hairpin 24.0 (For thermodynamic alignment model only)
Max Template Mispriming Primer 12.00 Pair 24.00 (For old secondary structure alignment model only)
Max Self Complementarity Any 8.00 3' 3.00 (For old secondary structure alignment model only)
Max Pair Complementarity Any 8.00 3' 3.00 (For old secondary structure alignment model only)
Excluded regions
Overlap junctions
5' side overlaps 7 3' side overlaps 4
Minimal number of nucleotides that the left or the right primer must have at the 5' or 3' side of the junctions

Fig 2.4.2: A screenshot of the web-based primer design tool "Primer-BLAST".

Primer	Sequence	Melting Temp (°C)	CG% content	Amplicon length	Accession No.
β-actin (F)	5’-GTACCACTGGCATCGTGATGGAC-3’	63.37	56.52	403	NM_001101
β-actin (R)	5’-GAGTTGAAGGTAGTTTCGTGGATG-3’	59.61	45.83		
GAPDH (F)	5’-TGTTCGTCATGGGTGTGAAC-3’	58.41	50.00	143	NM_002046.5
GAPDH (R)	5’-TGTGGTCATGAGTCCTTCCA-3’	58.27	50.00		
AKR1C1 (F)	5’-ATTTGCCAGCCAGGCTAGTG-3’	60.68	55.00	178	NM_001353
AKR1C1 (R)	5’-GAATCAATATGGCGGAAGCCAG-3’	59.77	50.00		
AKR1C2 (F)	5'-AACTTCTTTTCCACAGGTAAGA-3'	54.70	36.40	75	NM_001354.5
AKR1C2 (R)	5'-ACAGAGGCAGTCTTACACAA-3'	55.30	45.00		
AKR1C2 (F)	5’-CACAGGTAAGAAACGGTTGAACT-3’	59.12	43.48	184	NM_001354.5
AKR1C2 (R)	5’-TTCTGTCACTGGCCTGGTTA-3’	58.57	50.00		
AKR1C3 (F)	5'-TCTGGGATCTCAACGAGACAA-3'	58.47	47.62	207	NM_003739
AKR1C3 (R)	5'-TGGAACTCAAAAACCTGCACG-3'	59.60	47.62		
Aromatase (F)	5’-ACGTCGCGACTCTAAATTGC-3’	59.01	50.00	174	NM_000103
Aromatase (R)	5’-GCCCTCATAATTCCACACCAA-3’	58.27	47.62		
Aromatase (F)	5’-GGCAAGCTCTCCTCATCAAAC-3’	59.26	52.38	199	NM_000103
Aromatase (R)	5’-TCACCACGTTTCTCTGCTAAA-3’	57.54	42.86		
Aromatase (F)	5’-TTGGAAATCCCTGTGGACTCT-3’	58.65	47.62	105	NM_031226
Aromatase (R)	5’-GCACGATGCTGGTGATGTTAT-3’	59.06	47.62		
EP_1 (F)	5’-GTGTACATCCTACTGCGCCA-3’	59.83	55.00	177	NM_000955.2
EP_1 (R)	5,-GGCCTCTGGTTGTGCTTAGA-3’	59.68	55.00		

EP$_2$ (F)	5’-GAGACGGACCACCTCATTCTC-3’	59.86	57.14	76	NM_000956.3
EP$_2$ (R)	5’-CAAAAATCGTGAAAGGCAAGGA-3’	58.02	40.91		
EP$_3$ (F)	5’-CGCCTCAACCACTCCTACAC-3’	60.39	60.00	107	NM_001126044.1
EP$_3$ (R)	5’-GACACCGATCCGCAATCCTC-3’	60.88	60.00		
EP$_4$ (F)	5’-ACTGAGACCGGCTTTGAGAA-3’	58.95	50.00	157	NM_000958.2
EP$_4$ (R)	5’-GTGCAAGGCTGGGTCTGTA-3’	59.63	57.89		
FP (F)	5’-CTGGGATCGGTGGAACTTGA-3’	59.39	55.00	122	NM_001039585.1
FP (R)	5’-GCAGTCCAGACATCTTGTCA-3’	57.54	50.00		

Table 2.4.1: A list of primers designed using Primer-BLAST.

Preparation of primers:

The primers were synthesised by Eurofins Genetic Services Limited. The primers were then reconstituted in Tris-acetate-EDTA (TAE) buffer at pH 8 to 100µM stock solution and stored at -20°C.

2.4.7. Quantitative Real-Time PCR

For quantitative real-time PCR, QuantiTect SYBR Green PCR kit (Qiagen, Germany) was used. The QuantiTect SYBR Green master mix contained HotStarTaq DNA polymerase, SYBR Green PCR buffer, Deoxyribonucleotide triphosphate (dNTP) mix, ROX passive reference dye and 5mM $MgCl_2$. cDNA, primers (forward and reverse) and RNase free water were added to QuantiTect SYBR Green master mix in 384 well plates (Table 2.4.2). The reference gene used was (β-actin and/or GAPDH). Negative controls included in the experiment were no template control (NTC), where all the components where added except cDNA, and no reverse transcription control (-RT), where RNA was added instead of cDNA template.

Components	**Concentration**	**Volume**	**Final concentration**
QuantiTect SYBR Green master mix	2X	5µl	1X
Primer (forward)	15µM	0.5µl	0.3µM
Primer (reverse)	15µM	0.5µl	0.3µM
cDNA	5-50ng/µl	1µl	0.05-0.5ng/µl
RNase free water		3.5µl	
Total Volume		**10µl**	

Table 2.4.2: Components of the qRT-PCR reaction added to a 384 well plate

The 384-well plate was sealed and centrifuged at 1000xg for 30 seconds. The plate was inserted into the Quantstudio 12K flex real-time PCR system (Applied Biosystems, USA). The reaction setup was followed according to the manufacturer's protocol for 45 cycles:

Step	Time	Temperature (°C)	No of cycles
Activation of DNA polymerase	15 min	95	1 cycle
Denaturation	15 sec	94	45 cycles
Annealing	30 sec	60	
Extension	15 sec	72	

Table 2.4.3: qRT- PCR reaction setup according to the manufacturer's protocol (Qiagen, Germany)

2.4.8. Determining the efficiency of different primers

a) Serial dilution method:

Initially, the efficiency of PCR reaction for each different set of primers was determined as it was crucial for data analysis. cDNA of endometrial cells was serially diluted by a factor of 10 over 5-log range. They were added to PCR reaction components along with the primer of interest (Table 2.4.3). At the end of reaction, the cycle threshold (Ct) values - the cycle number at which the curve intersects the threshold value - were plotted against the "log cDNA dilution". The linear regression value (r^2) and the slope of standard curve were determined from the graph. For calculating the efficiency of each primer, the (r^2) value must be ≥ 0.99.

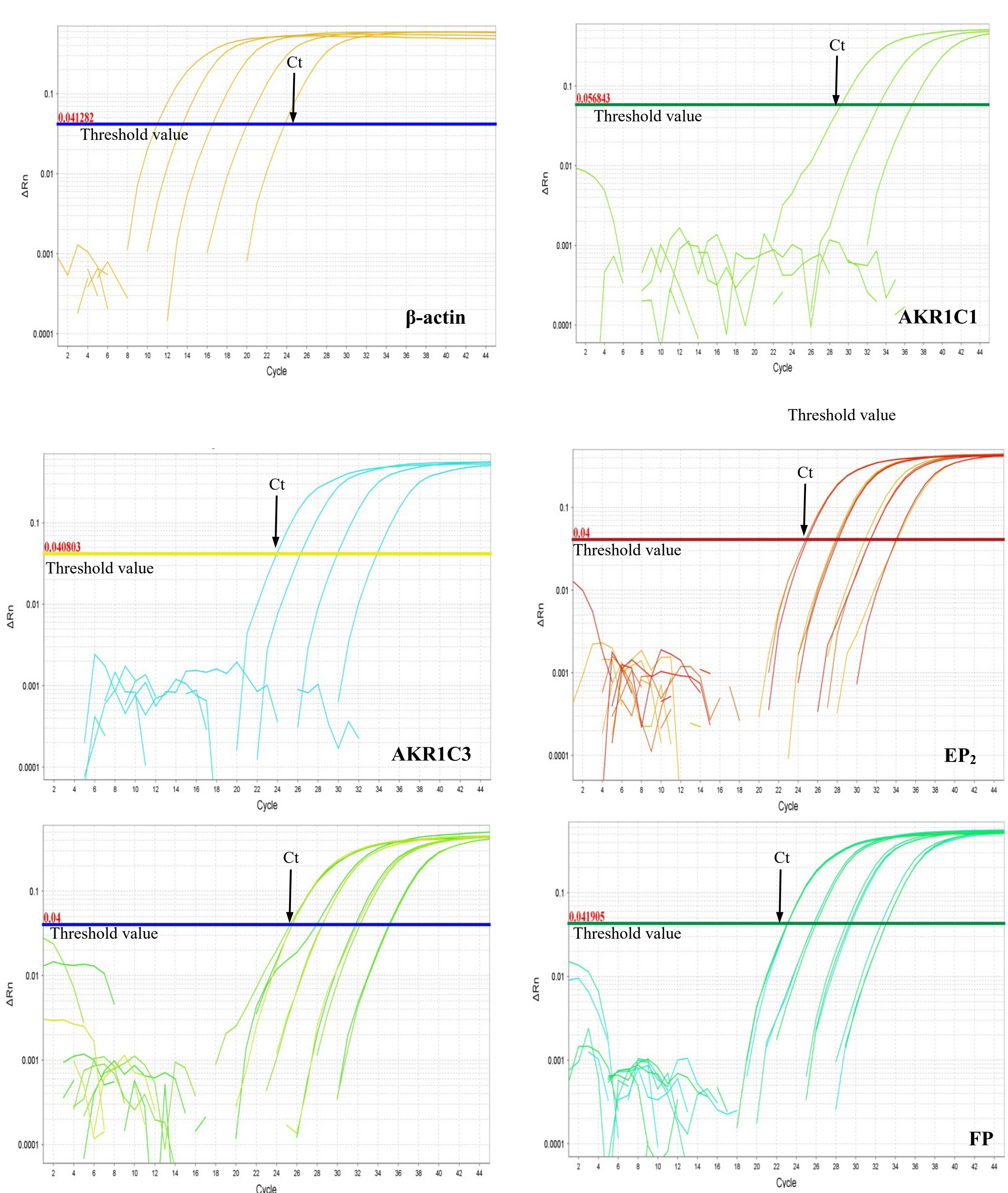


Fig 2.4.3: The amplification curves of serially diluted cDNA over 5-log dilutions by different primers (β-actin, AKR1C1, AKR1C3, EP_2 and EP_4. As shown, the cycle threshold (Ct) value increased as cDNA was diluted.

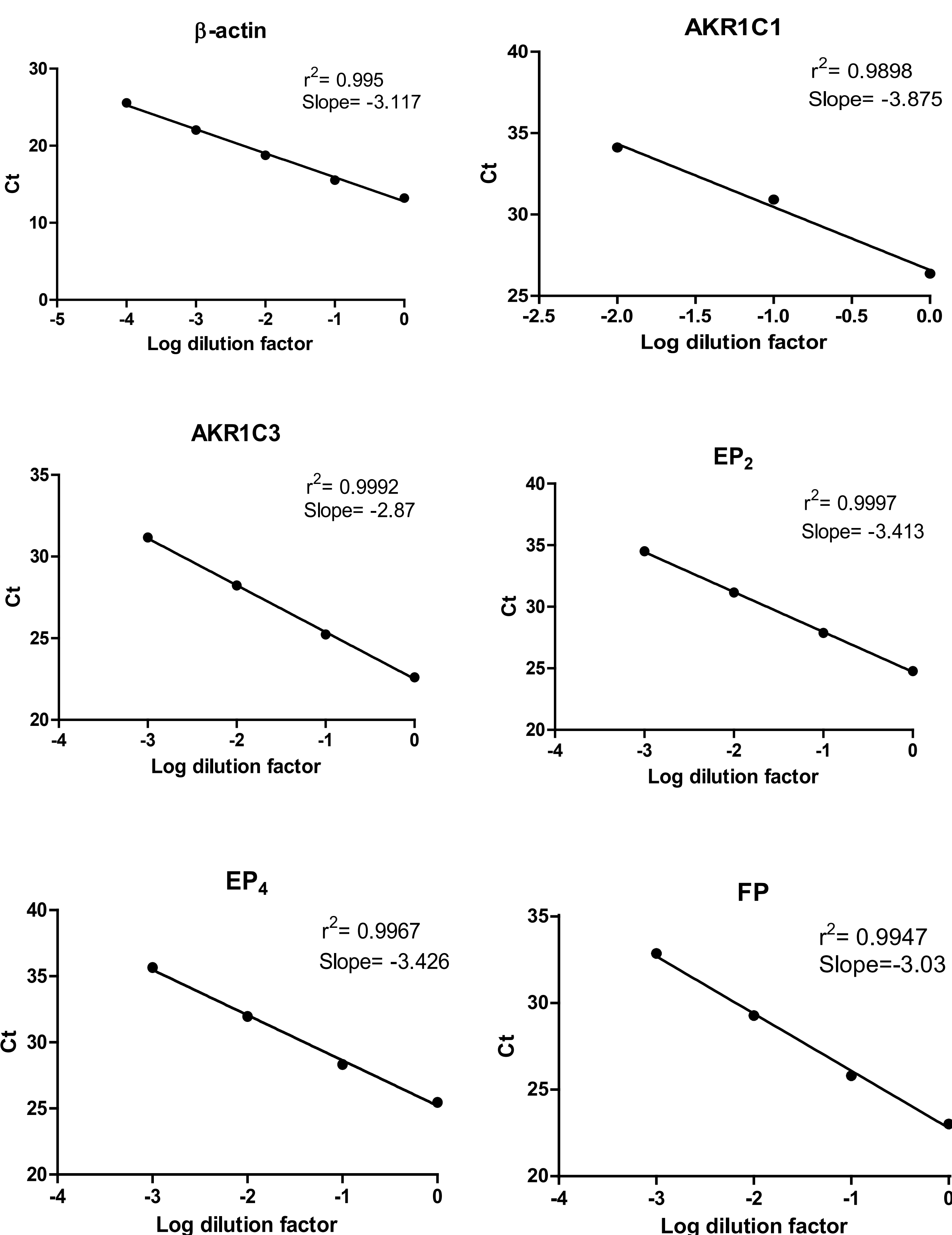


Fig 2.4.4: Standard curves of serially diluted cDNA for the following primers: β-actin, AKR1C1, AKR1C3 and EP_2 and EP_4.

$$\text{Primer efficiency} = 10^{(-1/\text{slope})}$$

Primer	Efficiency
β-actin	2.0
AKR1C1	1.8
AKR1C3	2.2
EP_2	2.0
EP_4	2.0
FP	2.0

Table 2.4.4: Primer efficiencies determined by the cDNA serial dilution method. C_t values of serially diluted cDNA were plotted against log dilution factor. The slope of standard curve was used to calculate primer efficiency.

b) Deriving primer efficiency using amplification curve

Liu and Saint had developed a new method whereby efficiency of a primer can be derived from the amplification curve (Liu and Saint, 2002). This method determines primer efficiency for each sample. This was useful for cDNA obtained from endometrial and adipose tissues, as it was very expensive and time-consuming to determine primer efficiencies for different tissue types using serial dilutions.

To calculate primer efficiency, C_t values were determined at two ΔRn values along the exponential phase of the amplification curve (Fig 2.4.5). The following equation was used to determine primer efficiency:

$$E = \left(\frac{\Delta Rn\ A}{\Delta Rn\ B}\right)^{\left(\frac{1}{Ct_A - Ct_B}\right)}$$

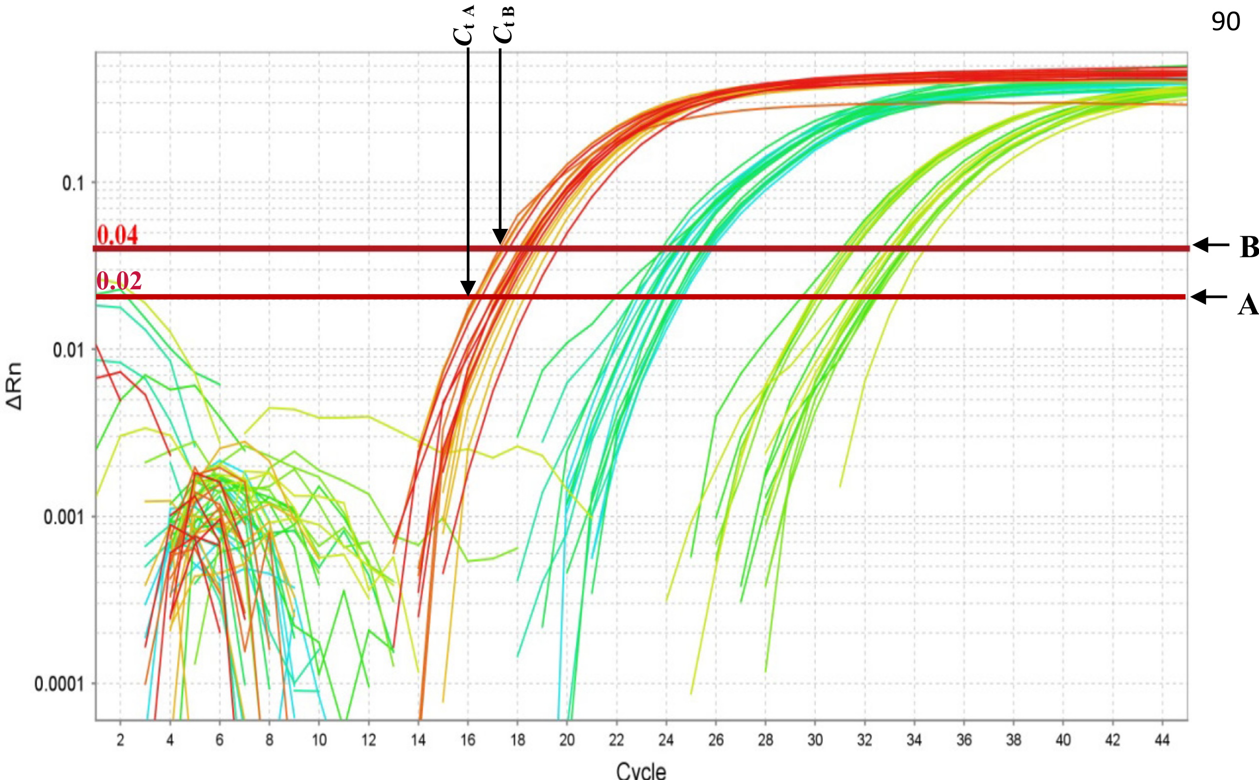


Fig 2.4.5: Calculation of primer efficiency using the amplification curve. C_t values at two different ΔRn values along the exponential phase of curve were used to calculate the primer efficiency values.

2.4.9. Checking product specificity

At the end of PCR reaction, amplicon specificity was confirmed using either of the following techniques:

a) ***Melting curve analysis:*** This analysis is built into the software of real-time cyclers. At the end of PCR reaction, fluorescence is measured continuously as the temperature is gradually increased from 65°C to 95°C. At low temperatures the amplicon is double stranded and SYBR Green dye is intercalated in between, hence fluorescence is high. Conversely, at high temperatures fluorescence decreases rapidly as the amplicon becomes denatured. The first derivative (-dF/dT) is plotted against temperature (T) which results in curves with peaks at the respective melting point (Tm).

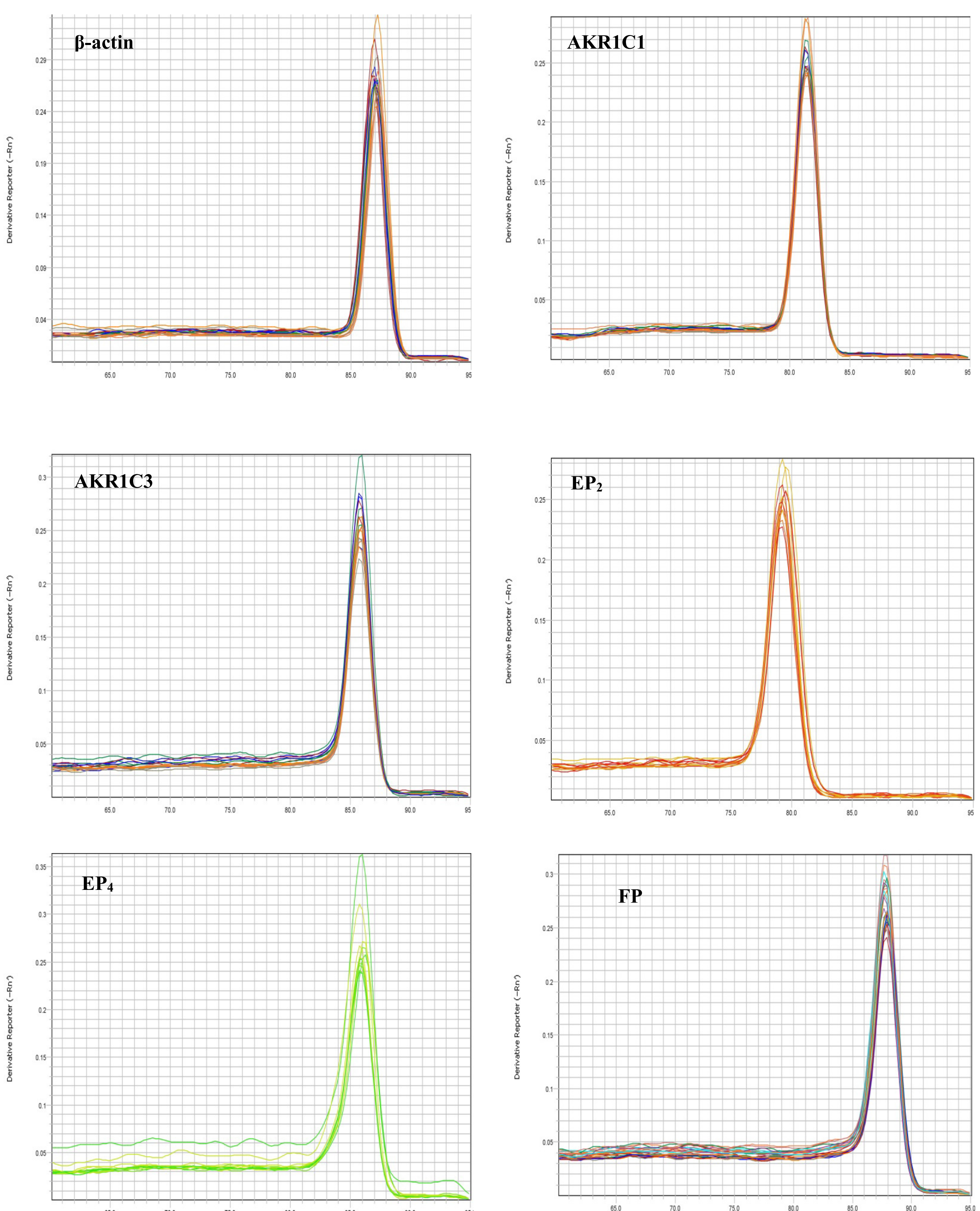


Fig 2.4.6: The melt curve analysis of PCR products formed using the following primers: β-actin, AKR1C1, AKR1C2, EP_2 and EP_4. The appearance of one single peak indicates the specificity of PCR product that had been measured.

b) ***Agarose gel electrophoresis:*** This method was first developed by Aaij and Borst (Aaij and Borst, 1972). 1%w/v Agarose gel (Invitrogen, USA) was prepared using 0.5X Tri-borate-EDTA (TBE) buffer (Ambion, USA). The agarose gel was dissolved by heating in the microwave for 30-60 seconds. Subsequently, ethidium bromide 10mg/ml (Sigma, Germany) at a concentration of 0.1µl/1ml of agarose gel was added. The gel was poured onto the gel tray with comb mounted. After 30 minutes, the gel solidified and the comb was carefully removed from the gel, creating wells in which the samples would be added.

Four parts of the DNA samples were added to one part of BlueJuice loading buffer (Invitrogen, USA) (Table 2.4.5). Also, four parts of 1Kb plus DNA ladder (Invitrogen, USA) was added to one part of the loading buffer.

DNA samples were added in their respective wells and the tray was then placed in the buffer tank containing 0.5X TBE buffer (Ambion, USA). Since DNA carries negative charge, the wells were placed next to the anode electrode. A voltage of 120V was applied for 75 minutes. At the end of electrophoresis, the gel was visualised under UV light.

Component	**Concentration**
Sucrose	65% w/v
Tris-HCl	10mM
EDTA	10mM
Bromophenol Blue	0.3% w/v

Table 2.4.5: Composition of BlueJuice (Invitrogen, USA) gel loading buffer

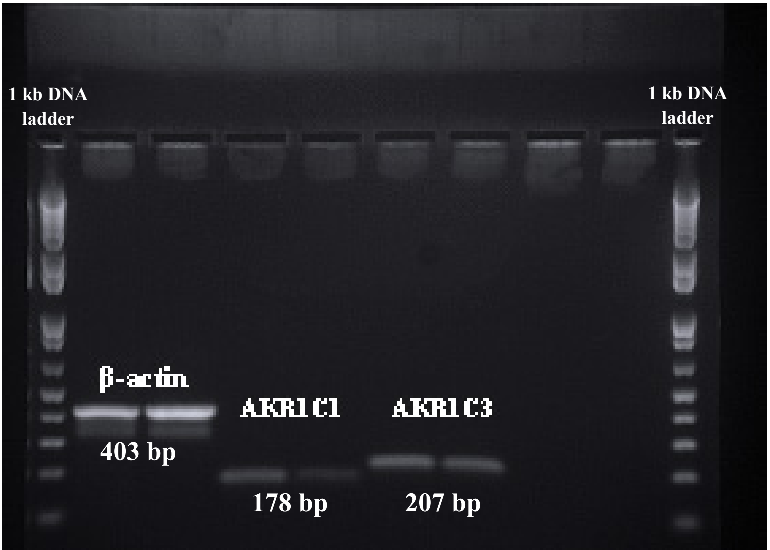


Fig 2.4.7: Gel electrophoresis: PCR products from two different endometrial cells were mixed with loading buffer and added to agarose gel containing ethidium bromide, which binds to DNA molecules and fluoresce under UV light.

2.4.10. Data analysis of relative mRNA expression

The C_t values which were less than 5 cycles of the negative template control (or less than 40 cycles) were accepted and used in the data analysis. Relative mRNA quantification of target genes was normalised against housekeeping genes, provided that no change was observed between the different biological groupings. All relative mRNA quantification was performed using the Pfaffl mathematical model (Pfaffl, 2001), as this method accounts for the different efficiencies of different genes, as shown in table 2.4.4. The data was expressed as a ratio where control is calibrated at 1.

The following equation was used:

$$\textbf{Relative Expression Ratio } (\Delta\Delta C_t) = \frac{(E_{Target})^{(C_t \text{ control} - C_t \text{ treated})}}{(E_{Reference})^{(C_t \text{ control} - C_t \text{ treated})}}$$

Where,

E = efficiency of primer

C_t = the threshold value

Target = the gene of interest

Reference= the reference gene

Alternatively, in the case of comparing control and case, the equation above was modified so as to avoid calibrating control:

$$\Delta C_t = \frac{(\mathbf{E}_{\mathbf{Reference}})^{(C_t)}}{(\mathbf{E}_{\mathbf{Target}})^{(C_t)}}$$

2.5. The Aldo-keto reductase (AKR) 1C3 kinetics

2.5.1. Materials

I. Chemicals:

	Catalogue Number	Company
Monobasic dihydrogen phosphate	10458153	Fisher Scientific
Dibasic monohydrogen phosphate	10468153	Fisher Scientific
β-Nicotinamide adenine dinucleotide phosphate (β-NADP)	N5755	Sigma-Aldrich
Glucose-6-phosphate	446980010	Acros Organics
Glucose-6-phosphate dehydrogenase	G8529	Sigma-Aldrich
Ethyl acetate	10080130	Fisher Scientific
Chloroform	10102190	Fisher Scientific
anhydrous sodium sulphate	424410010	Acros Organics
Methanol	10674922	Fisher Scientific
Sulphuric acid	10294300	Fisher Scientific
Opti-Fluor	6013199	PerkinElmer
Oestrone	10006485	Cayman
17β-oestradiol	E8875	Sigma-Aldrich
[^{3}H] oestrone	NET319250UC	PerkinElmer

II. Equipment

	Company
Shaking Water bath	VWR UK
Thin layer chromatography (TLC) plate	Merk Millipore
Tricarb 2100TR scintillation counter	PerkinElmer

2.5.2. Method:

The standard assay mixture for the purified AKR1C3 was adopted from professor Kikuko Watanabe's lab (Koda et al., 2004). The standard assay mixture contained 0.1M potassium phosphate buffer (KPB, pH=6.5) (Sigma, Germany), 0.5mM β-NADP$^+$ (Sigma, Germany), 5mM glucose-6-phospate (Acros Organics, USA) and 1 unit of glucose-6-phosphate dehydrogenase (Sigma, Germany).

Components	Concentration	Volume (µl)	Final concentration
Potassium phosphate buffer	1M	5	0.1M
βNADP	10mM	2.5	0.5mM
Glucose-6-phosphate	50mM	5	5mM
Glucose-6-phosphate dehydrogenase	0.4U/mL	2.5	1U

Table 2.5.1: The components of the standard assay mixture used for the enzyme assay

This was followed by mixing 1µl of [^{3}H]-oestrone (PerkinElmer, USA), specific activity of 94 Ci/mmol, with 1µl of unlabeled oestrone (Cayman, USA) at different concentrations (2.5, 5, 10, 20, 40 and 80µM), in the presence or absence of bimatoprost (gift from Dr David Woodward). The volume of ethanol was kept constant at a concentration of 4%v/v without bimatoprost and 5%v/v with bimatoprost.

The reaction started by the addition of 3µg of recombinant AKR1C3 (gift from Kikuko Watanbe) in a total volume of 50µl, topped up with distilled water, for 60 minutes at 37°C. The reaction was terminated by 250µl of cold ethyl acetate (Fisher, UK) (Penning et al., 2000). A small spoonful of anhydrous sodium sulphate (Acros

organics, USA) was added and mixed thoroughly to absorb water in the mixture. The extraction efficiency by ethyl acetate was 96%.

To aid visualisation, 1µl of unlabeled oestrone (Cayman, USA) and 17β-oestradiol (Sigma, Germany) (both at 10mM) were added as authentic markers on the thin layer chromatography (TLC) plate (Merk Millipore, USA). The organic phase (100µl) was blotted on a TLC plate and developed for 30 minutes in ethyl acetate:chloroform (1:4 v/v) (Fisher, UK) for the separation of oestrone and 17β-oestradiol. The TLC plate was sprayed with methanol:sulphuric acid (1:1 v/v) (Fisher, UK) and then heated at 110°C for 10minutes (Penning et al., 2000). This allows the visualisation of the oestrone and 17β-oestradiol bands as permanent yellow-brown bands on the TLC plate (Fig 2.51). The corresponding bands for oestrone (Rf value = 0.47) and 17β-oestradiol (Rf value = 0.24) were scraped off and placed in separate vials containing Opti-Fluor liquid scintillation cocktail (PerkinElmer, USA). The vials were placed in a Packard Tricarb 2100TR scintillation counter (PerkinElmer, USA) to measure counts per minute (cpm) values.

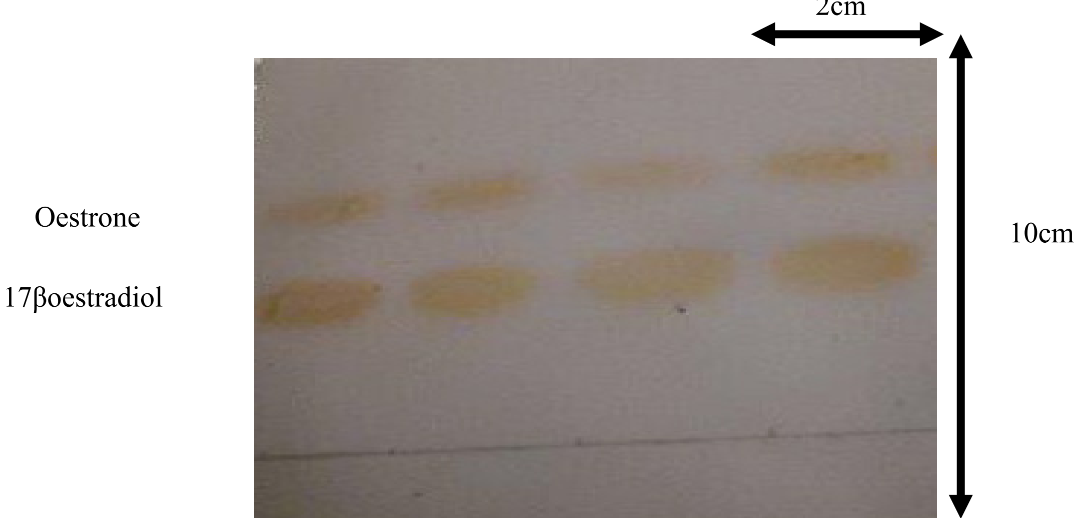


Fig 2.5.1: The bands of oestrone and 17β-oestradiol visualised by spraying methanol:sulphuric acid (1:1 v/v) on a TLC plate. The plate was heated at 110°C for 10 minutes which forms permanent yellow-brown bands.

2.6. Extracellular Flux Analysis

This method was conducted in collaboration with Dr Ayse Latif, who had helped with conducting the extracellular flux analysis using with the XF^{e}96 Analyser.

Extracellular flux (XF) glycolysis stress test and mitochondrial stress test were performed using an XF^{e}96 Analyser (Seahorse Bioscience, USA) according to the manufacturer's instructions. For each experiment performed, plates were prepared with endometrial cells and seeded at a density of $1.5x10^5$ cells seeded per ml. The XF glycolysis stress test assay medium (Seahorse Bioscience, USA) used in these experiments was supplemented with 2 mM L-Glutamine (Gibco, USA). On the other hand, the XF mitochondrial stress test assay medium (Seahorse Bioscience, USA) used in these experiments was supplemented with 10 mM Glucose (Gibco, USA), 2 mM L-Glutamine (Gibco, USA) and 1 mM sodium pyruvate (Gibco, USA) (Latif et al., 2014).

To measure the effect of oestrone and 17β-oestradiol treatments, cells were treated with 10nM oestrone and 1nM 17β-oestradiol for 24 hours. Each concentration was measured using 4 replicates.

For glycolysis, glucose was added, followed by oligomycin, which is a complex V inhibitor, allowing the measurements of basal glycolysis and maximum glycolytic activity (glycolytic capacity) by the cells. 2-deoxyglucose (2-DG) was then added to inhibit glycolysis to deduce the glycolytic reserve of cells (Ferrick et al., 2008). During glycolysis stress test, three sequential measurements of extracellular acidification rate (ECAR) were recorded at each treatment point. All three sequential

measurements were used to calculate glycolysis (=glucose response – basal ECAR), glycolytic capacity (=oligomycin response – basal ECAR) and glycolytic reserve (= glycolytic capacity - glycolysis) (Fig 2.6.1).

For mitochondrial respiration, the cells were treated with oligomycin to inhibit complex V, which allows the deduction of ATP production by oxidative phosphorylation. Carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP) is an electron uncoupler which allows the measurement of maximal respiration by cells. Antimycin A and rotenone inhibit electron transport chain which halts mitochondrial respiration to deduct the glycolytic capacity of cells (Ferrick et al., 2008). During mitochondrial stress test, three sequential measurements of oxygen consumption rate (OCR) were recorded at each treatment point. All three sequential measurements were used to calculate basal respiration (=basal OCR - non mitochondrial respiration), ATP production (=oligomycin response - basal respiration), maximal respiration (= FCCP response - basal respiration) and spare capacity (= maximal respiration - basal respiration) (Fig 2.6.1).

To normalise the data following metabolic measurement, cells were fixed with 10% trichloroacetic acid (Sigma, Germany) at 4°C for 1 hour, rinsed with H_2O and stained with sulforhodamine B (SRB) (Sigma, Germany) for 10 minutes. Excess dye was removed by washing the cells with 1% acetic acid. SRB bound to cell protein content was solubilised using a standard volume of 10mM Tris buffer (Sigma, Germany) and absorbance was read at 540 nm.

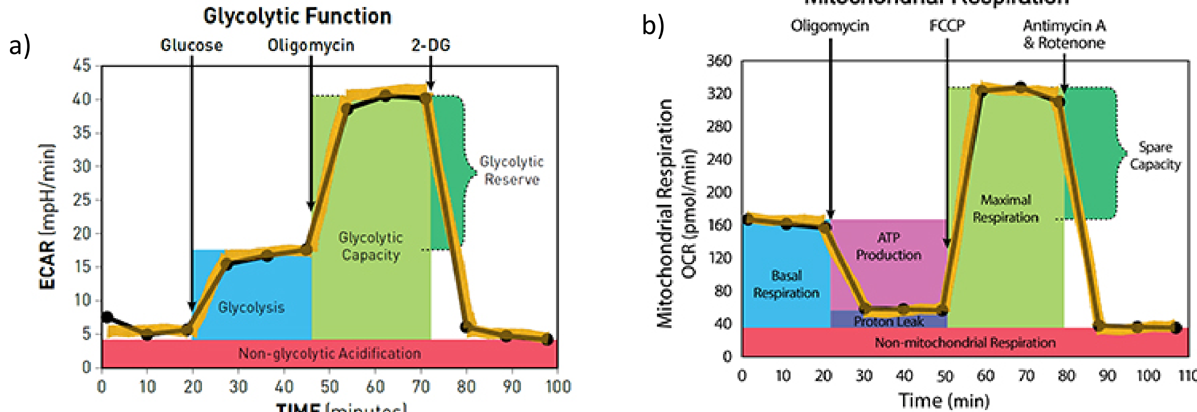


Fig 2.6.1: a) Typical curve for glycolytic stress test, showing the fundamental parameters of cellular glycolysis: glycolysis, glycolytic capacity, and glycolytic reserve. b) Typical curve for mitochondrial stress test, showing the fundamental parameters: basal respiration, ATP turnover, proton leak, and maximal respiration, or spare respiratory capacity.

2.7. Statistical analysis of data

GraphPad Prism Software (Version 6.04) was used for graphical representation of data. The data were represented as means ± standard error mean (SEM).

The data were first tested for normality using Kolmogorov-Smirnov Test. Log-transformation was conducted for non-parametric data. For calculating statistical difference between independent groups, Student's t-test was used for comparing two groups, whereas one-way analysis of variance (ANOVA) test was used for comparing two or more groups. Significance level or P-value of less than 0.05 was considered to be statistically different (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Pearson correlation was used for parametric data and Spearman correlation was used for non-parametric data to measure the value of correlation coefficient (r).

3. The gene expression of Aldo-keto reductases (1C1-1C3), prostaglandin E receptors (1-4) and prostaglandin F receptor in the human endometrium, endometrial lesion, omental fat and breast fat

3.1. Introduction

3.1.1. Background

The AKR1C enzymes are generally implicated in reproductive hormonal diseases in males and females. Inherently, these enzymes are found to be responsible for the activation and inactivation of sex hormones, affecting the access to their respective receptors (Penning, 2003). Hence, any imbalance that may occur would disturb the local hormonal milieu and thereby induce or contribute to the induction of diseases such as endometriosis, breast cancer, endometrial cancer and prostate cancer.

Moreover, there is a cross talk between the sex steroid hormones and prostaglandins. For instance, in endometriosis there is upregulation of COX-2 enzymes, resulting in upregulation of PGE_2 and $PGF_{2\alpha}$ release (Wang et al., 2012). Also, enzymes involved in the steroidogenic pathway are upregulated. For example, steroidogenic acute regulatory protein (StAR), cytochrome P450 17A1 (CYP17A1), 3β-hydroxysteroid dehydrogenase type 1 (HSD-3B1) and aromatase are all upregulated in the human endometriotic lesions. Additionally, treatment with PGE_2 induces the expression of these steroidogenic enzymes (Attar et al., 2009).

The aim of this chapter was to determine whether there is a dysregulation in the gene expression of AKR1C (1-3) enzymes in human eutopic endometrial tissues, endometriotic lesions and omental adipose tissue from patients with endometriosis or without endometriosis who were being investigated for other non-cancerous gynaecological problems. Breast adipose tissues from patients with breast cancer were also investigated, given the hormonal dependency of tumour progression and

survival. These tissues might give an insight into the imbalance of hormonal milieu and shed light on any common trends that may be seen in endometriosis and breast cancer which are both hormone dependent conditions.

In addition, we aim to determine any dysregulation in the gene expression of EP_{1-4} receptors and FP receptor in the aforementioned samples to determine any differences that might give an indication of abnormal cell signalling transduction, and in turn, influence the hormonal milieu in endometriosis and breast cancer.

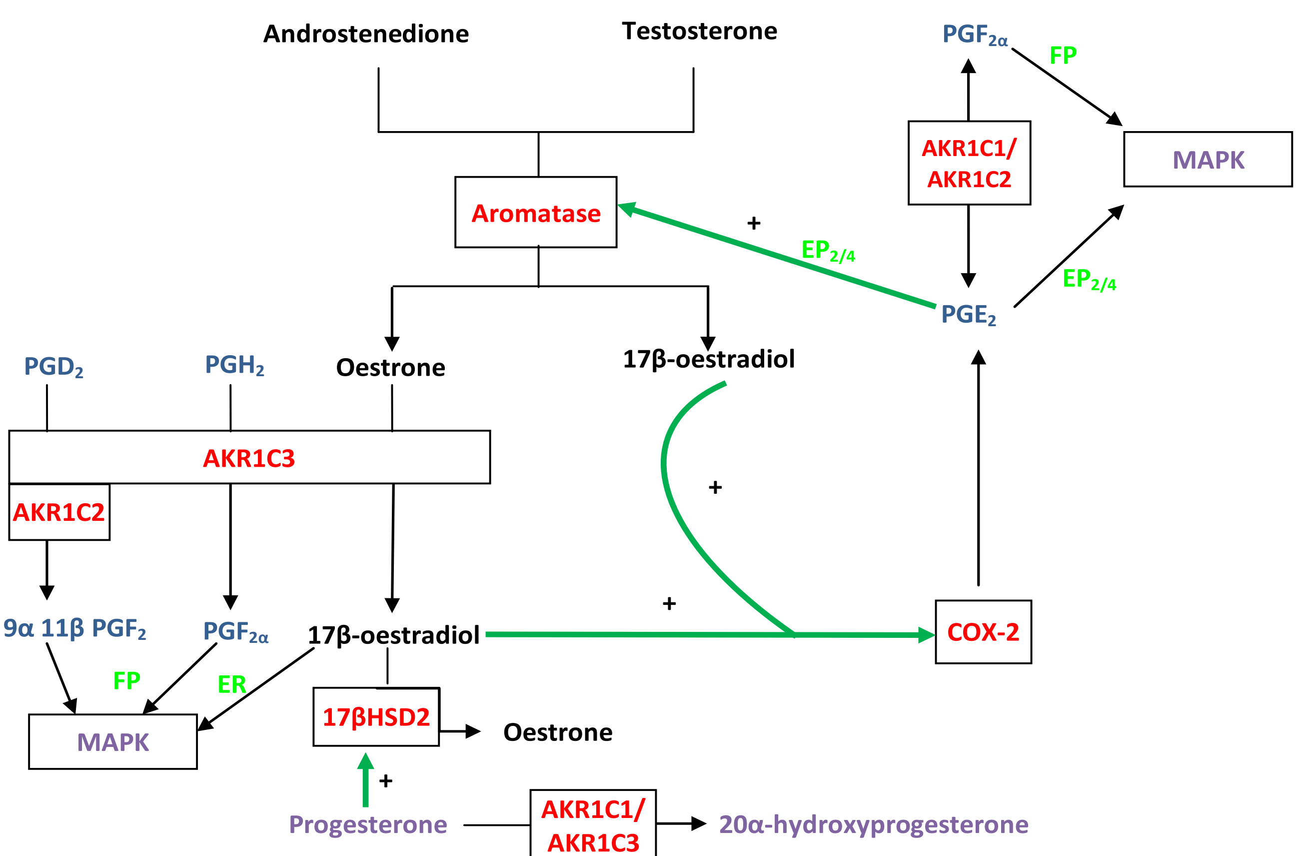


Fig 3.1.1: A schematic diagram showing the interrelationship between sex steroid hormones - 17β-oestradiol and progesterone - and PGE_2 and $PGF_{2\alpha}$ in the human reproductive system. Adapted from:(Casey et al., 1994; Hara et al., 1996; Bulun et al., 2001; Dozier et al., 2008; Penning and Byrns, 2009).

Abbreviations: **MAPK**: mitogen-activated protein kinases; **17βHSDs**: 17β hydroxysteroid dehydrogenase; **AKR**: Aldo keto reductase; **COX-2**: cyclooxygenase 2; **EP**: prostaglandin E receptor; **FP**: prostaglandin F receptor

3.1.2. Patient data

(a) Endometrial and omental adipose samples used in this chapter

Sample ID	Stage of cycle	Age	BMI	Pathology	Contraception	Medication	Parity
HP1	Luteal	53	24.8	Mild endometriosis	Copper IUD	None	5
HP6	Menses	44	24	Endometriosis, dysmenorrhoea, menorrhagia, fertility problems	None	None	1
HP10	Follicular	40	27	Mild endometriosis	None	None	2
HP11	Follicular	36	37	Fibroids	None	Citalopram	0
HP12	Follicular	36	24	Ovarian endometriosis	None	None	3
HP13	Follicular	48	28	Fibroids	Sterilisation	None	3
HP15	Luteal	56	35	Fibroids and Menorrhagia	None	gliclazide, metformin, enalapril, amlodipine, aspirin, pravastatin	0
HP16	Follicular	29	25	Cervical intra-epithelial neoplasia	None	None	0
HP19	Luteal	23	24	Pelvic pain	None	None	0
HP21	Menses	48	31.9	Fibroids	None	Iron, hyoscine, analgesics	3
HP23	Menses	38	28	Endometriosis and menorrhagia	None	Lisinopril	
HP24	Luteal	36	19.1	Stage IV endometriosis	None	Qvar, salbutamol	0
HP25	irregular	39	23	Pelvic pain and menorrhagia	Sterilisation	None	3
HP29	Irregular	43	30.1	Menorrhagia	None	Tranexamic acid	1
HP 31	Follicular	38	24.7	Endometriosis and Pelvic pain	None	None	3
HP32	Follicular	27	27.8	Endometriosis and Pelvic pain	None	None	1
HP37	Menses	28	N/A	Fertility problems	None	Creon, azithromycin, vitamins	0
HP39	Luteal	35	N/A	Dysmenorrhoea, menorrhagia, fertility problems	None	None	0
HP45	Luteal	20	28.9	Endometriosis, dysmenorrhoea	None	None	0
HP47	Follicular	45	24.8	Endometriosis	None	None	3

(b) Breast fat adipose tissues used in this chapter:

SAMPLE ID	Age	GRADE	ER status	PR status	HER2 status	Tumour size (mm)	NPI	NPI outcome	Ki67	PATHOLOGY
BB6RC08	45	2	pos	pos	neg	31	4.62	moderate	11-20%	IDC/DCIS
BB6RC20	32	3	neg	neg	neg	20	4.4	moderate		IDC
BB6RC21	41	3	pos	pos	neg	17	4.34	moderate	21-30%	IDC
BB6RC28	61	3	neg	neg	neg	17	4.34	moderate	31-40%	IDC/DCIS
BB6RC32	47	2	pos	pos	neg	16	4.32	moderate	11-20%	IDC/ILC/DCIS
BB6RC43	75	3	neg	neg	neg	30	6.6	poor	51-60%	IDC/DCIS
BB6RC45	41	3	pos	pos	neg	16	6.32	poor	41-50%	IDC
BB6RC50	62	3	pos	neg	neg	29	4.58	moderate	>60%	IDC/DCIS
BB6RC62	77	2	pos	pos	neg	25	3.5	moderate		IDC
BB6RC63	78	3	pos	pos	neg	28	5.56	poor	21-30%	ILC
BB6RC66	87	3	pos	neg	neg	35	5.7	poor	31-40%	ILC
BB6RC67	49	1	pos	pos	neg	15	2.3	excellent		Tubular
BB6RC111	67	3	neg	neg	neg	30	4.6	moderate	>60%	IDC
BB6RC132	50	2	pos	pos	neg	80	5.6	poor	15	ILC
BB6RC141	52	3	neg	neg	neg	165	7.3	poor	51-60%	Fungating IMC
BB6RC144	57	2	pos	pos	neg	48	5.96	poor	41-50%	ILC
BB6RC152	50	3	neg	neg	Not known	80	7.6	poor		IDC
BB6RC166	73	3	pos	neg	neg	26	6.52	poor		IDC
BB6RC188	81	3	pos	pos	neg	50	5	moderate	41-50%	IDC/DCIS

Abbreviations: ER: oestrogen receptor; PR: progesterone receptor; NPI: Nottingham Prognostic Index; IDC: Invasive ductal carcinoma; DCIS: Ductal carcinoma in situ; ILC: Invasive lobular carcinoma; Fungating IMC: Fungating invasive mammary carcinoma

3.2. Results

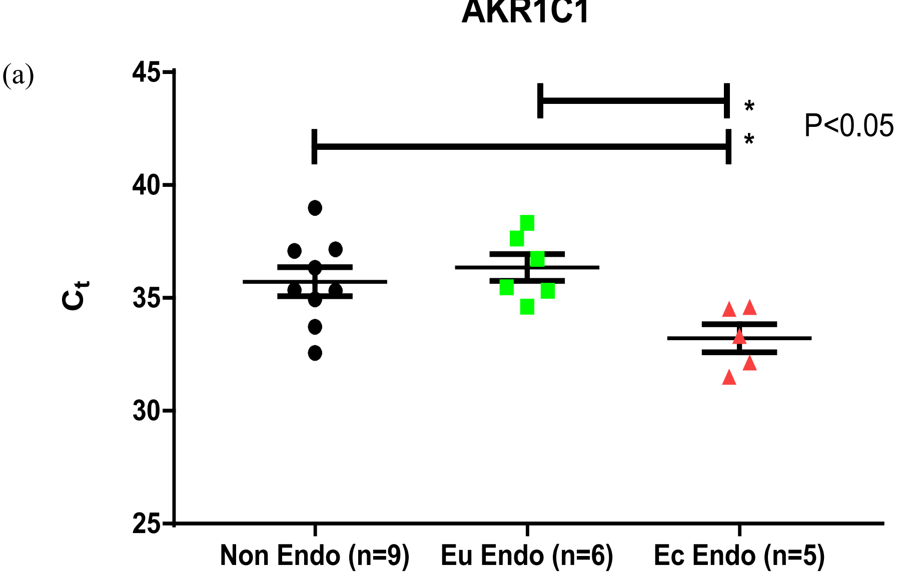
3.2.1. The gene expression of AKRs in the human endometrium

The gene expression of AKRs was investigated in the human eutopic endometrial tissues from non-endometriosis patients with other gynaecological conditions, and eutopic endometrial tissues and endometriotic lesions from endometriosis patients. These tissues were collected over a three year period.

The gene expression of AKR1C1 and AKR1C3 was strongly detected in the human endometrial tissues. However, AKR1C2 was below the detection limit, since the C_t values were above 40 cycles which indicates that the AKR1C2 expression is very low in endometrial tissues. Also, aromatase was not detected due to low gene expression in these tissues.

It is worth noting that AKR1C3 gene expression in endometriotic lesions only is the highest at the follicular phase. This trend was not seen in AKR1C1. Also, the level of AKR1C1 gene expression was much lower than that of AKR1C3 in the human endometrium.

(a)



(b)

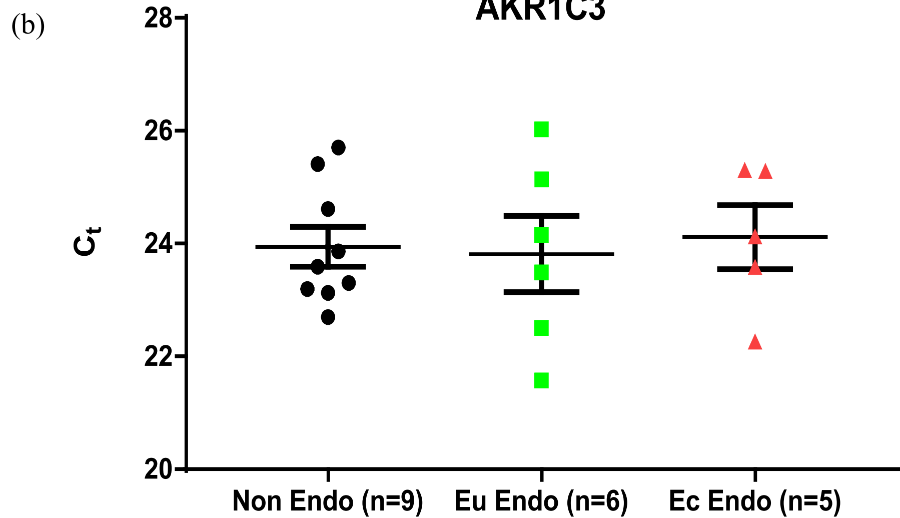


Fig 3.2.1a: The C_t values of (a) AKR1C1 and (b) AKR1C3 genes in human endometrial samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-Endo (●) = Eutopic endometrial samples from patients without endometriosis; Eu Endo (■) = Eutopic endometrial samples from patients with endometriosis; Ec Endo (▲) = Endometriotic lesions from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post-hoc* test; *P<0.05.

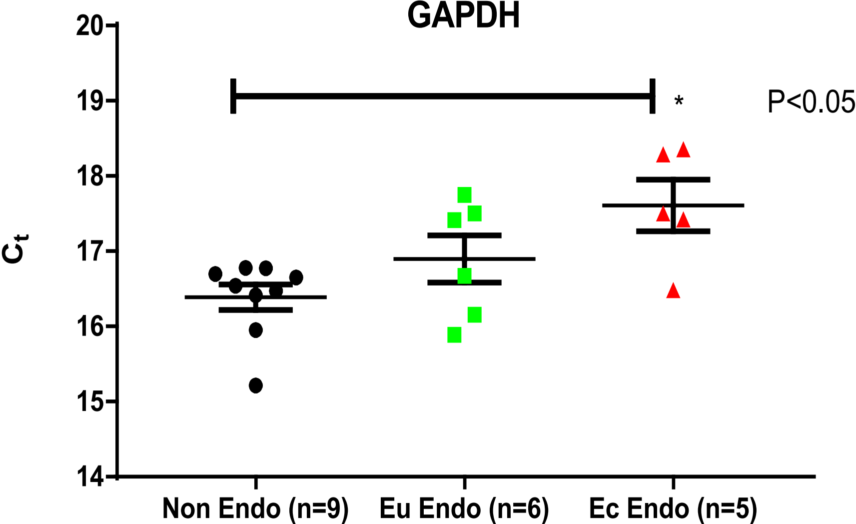


Fig 3.2.1b: The C_t values of glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) housekeeping gene in human endometrial samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-Endo (●) = Eutopic endometrial samples from patients without endometriosis; Eu Endo (■) = Eutopic endometrial samples from patients with endometriosis; Ec Endo (▲) = Endometriotic lesions from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post-hoc* test; *P<0.05.

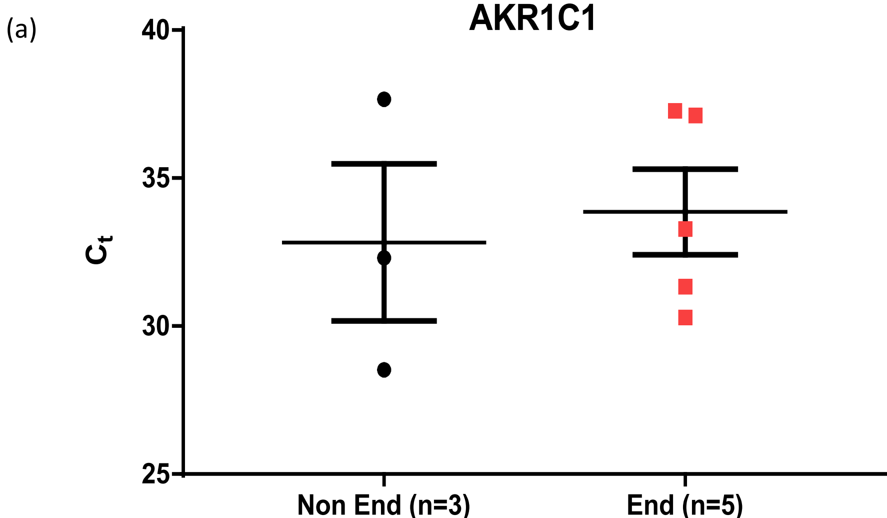
There was significant elevation of AKR1C1 gene expression in the endometriotic lesions from patients with endometriosis compared to eutopic endometrial tissues from patients with and without endometriosis (Fig 3.2.1a). However, the housekeeping gene, GAPDH, showed a statistical significant change in C_t values when compared with eutopic endometrial tissues from patients without endometriosis and endometriotic lesions from patients with endometriosis (Fig 3.2.1b). As a result, the comparison between these two biological groups was invalid, since GAPDH was used for normalisation and should not have varied between the two groups.

On the other hand, the C_t values of GAPDH did not significantly change between eutopic endometrial tissues from patients with endometriosis and endometriotic lesions from patients with endometriosis. Therefore, the data can be interpreted as significant elevation of AKR1C1 gene expression observed in endometriotic lesions from patients with endometriosis, compared to eutopic endometrial tissues from patients with endometriosis.

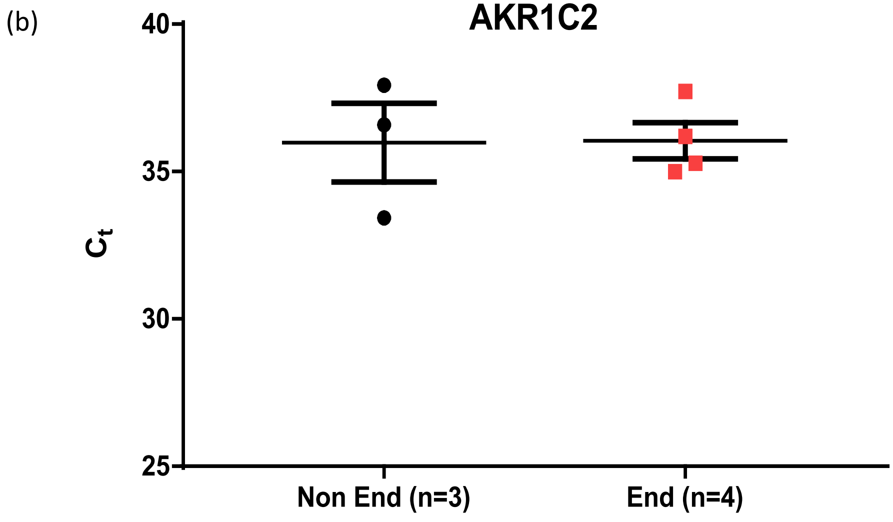
3.2.2. The gene expression of AKRs in the omental adipose tissue

The gene expression of AKRs was investigated in the omental adipose tissue from patients with and without endometriosis. The gene expression of all the AKRs was detected in the omental adipose tissue. The data did not show any significant difference between endometriosis and non-endometriosis patients. Aromatase was not detected in omental adipose tissues due to low gene expression. Again, AKR1C3 gene expression was the highest, compared to AKR1C1 and AKR1C2.

(a)



(b)



(c)

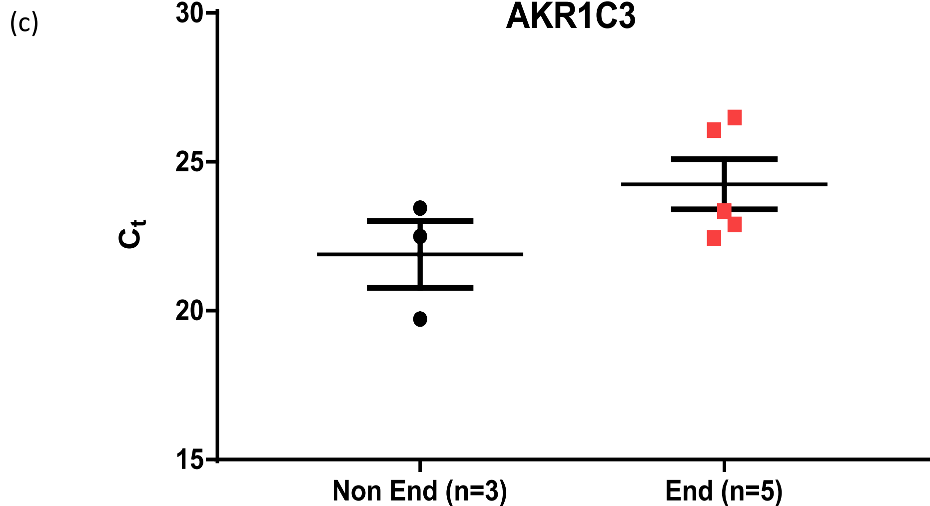


Fig 3.2.2a: The C_t values of (a) AKR1C1, (b) AKR1C2, (c) AKR1C3 genes in human omental adipose samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-End (●) = Omental fat samples from patients without endometriosis; End (■) = Omental fat samples from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

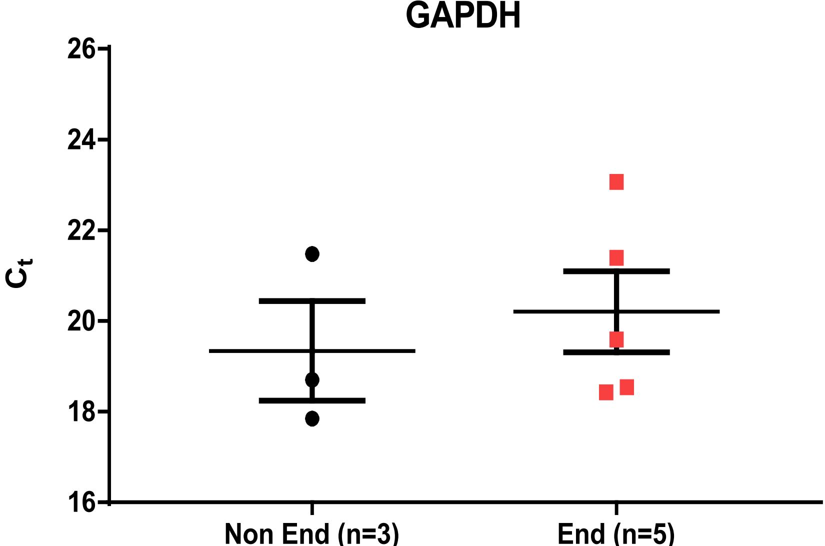


Fig 3.2.2b: The C_t values of glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) housekeeping gene in human omental adipose samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-End (●) = Omental fat samples from patients without endometriosis; End (■) = Omental fat samples from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

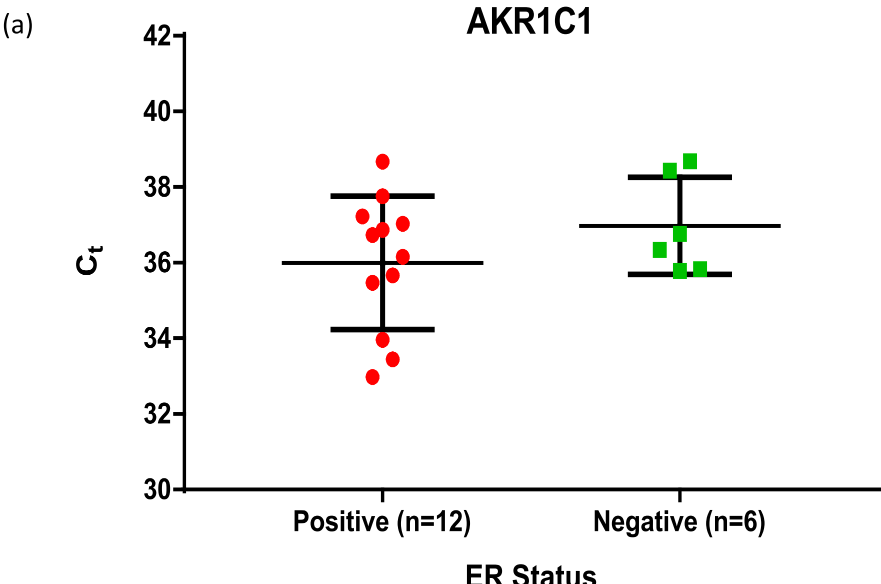
3.2.3. The gene expression of AKRs in the breast adipose tissue from breast cancer patients

The gene expression of AKRs was investigated in the breast adipose tissue from patients with breast cancer. The gene expression of all AKRs was detected in the breast adipose tissue, although the gene expression of AKR1C3 was the highest in the breast adipose tissue, compared to AKR1C1 and AKR1C2.

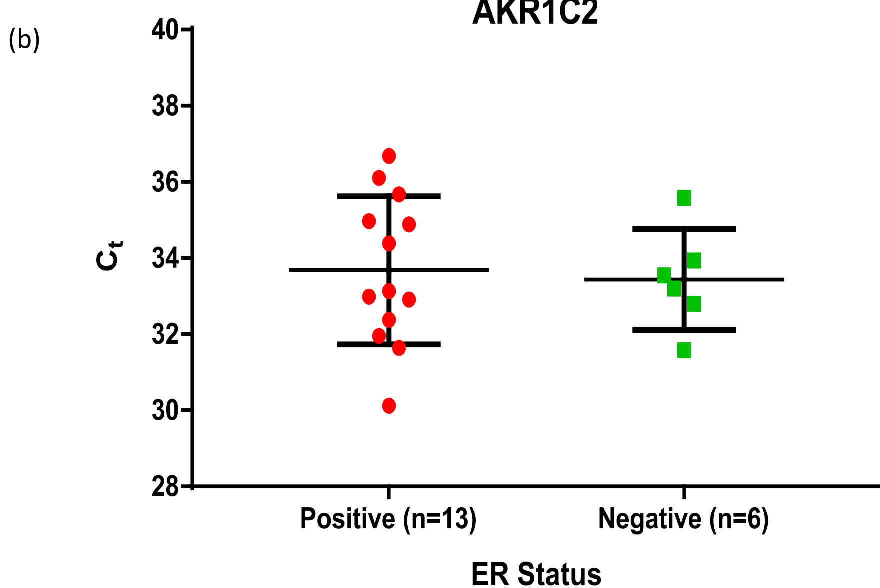
Since AKR1C enzymes are involved in the biosynthesis of oestrogen and metabolism of progesterone, the gene expression of AKR1C (1-3) was grouped according to the oestrogen receptor (ER) status and progesterone receptor (PR) status of primary tumours, which were determined by immunohistochemistry. Also, aromatase gene was not detected in all the breast adipose tissues, only detected in 3 out of 9 breast adipose tissue samples.

I. AKR1C gene expression in relation to oestrogen receptor status:

(a)



(b)



(c)

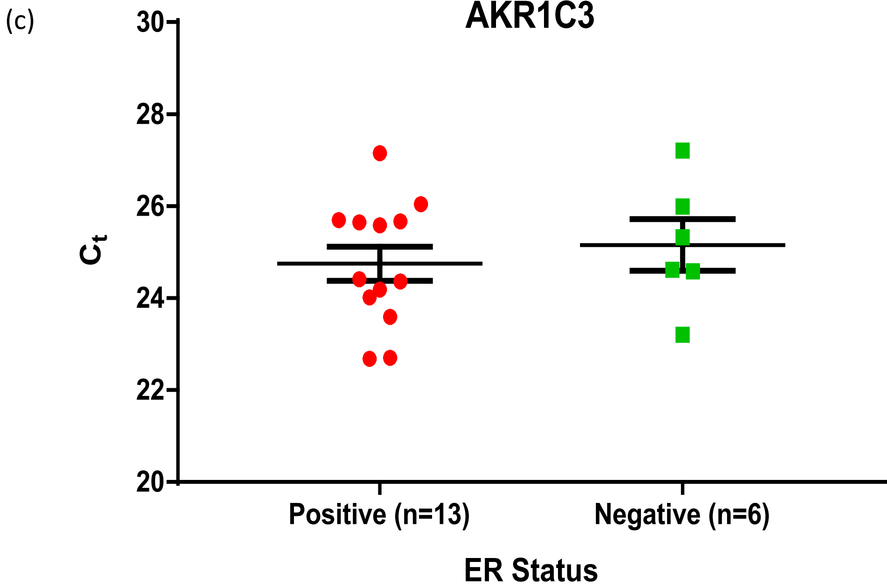


Fig 3.2.3a: The C_t values of (a) AKR1C1, (b) AKR1C2, (c) AKR1C3 genes in human breast adipose samples from patients with breast cancer according to oestrogen receptor (ER) status: ER-positive (●) and ER-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

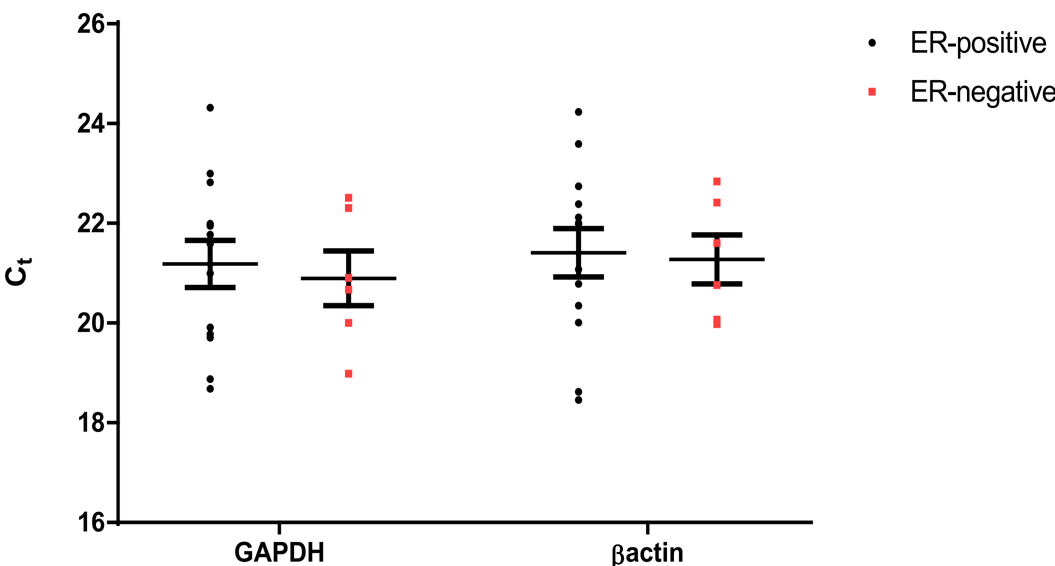
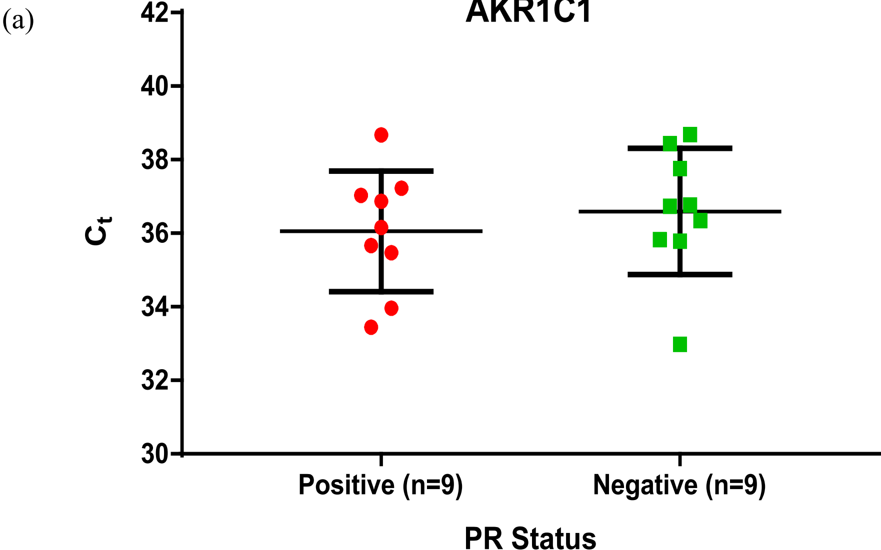


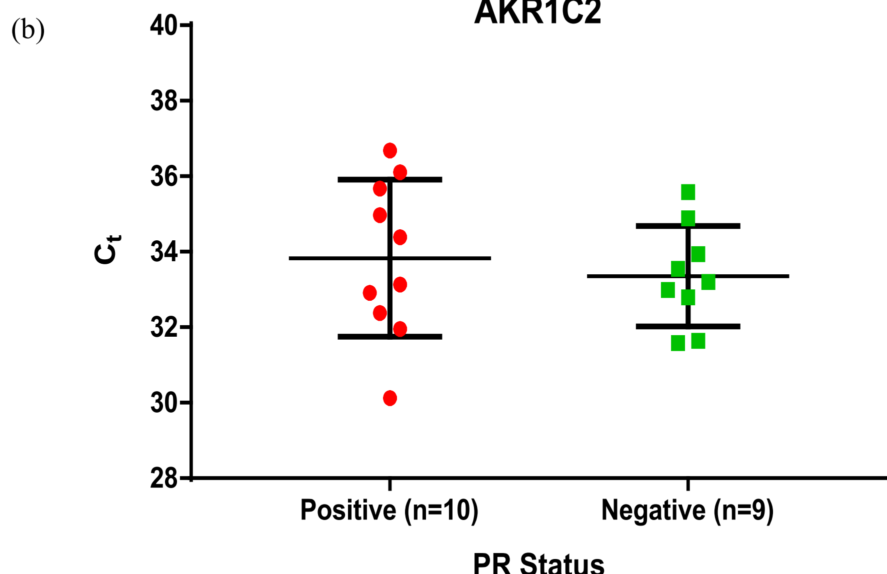
Fig 3.2.3b: The C_t values of GAPDH and β-actin housekeeping genes in human breast adipose samples from patients with breast cancer according to oestrogen receptor (ER) status: ER-positive (●) and ER-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using two-way ANOVA.

II. AKRs gene expression in relation to progesterone receptor status:

(a)



(b)



(c)

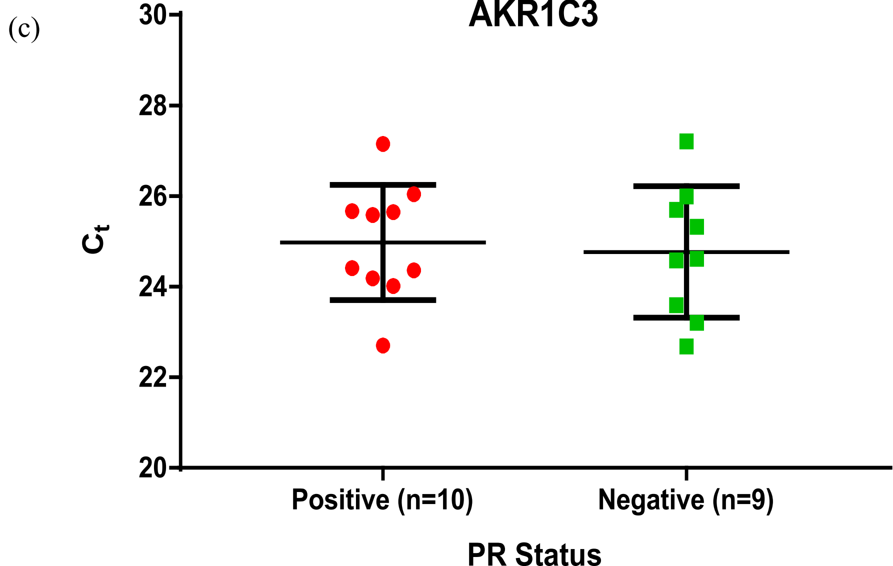


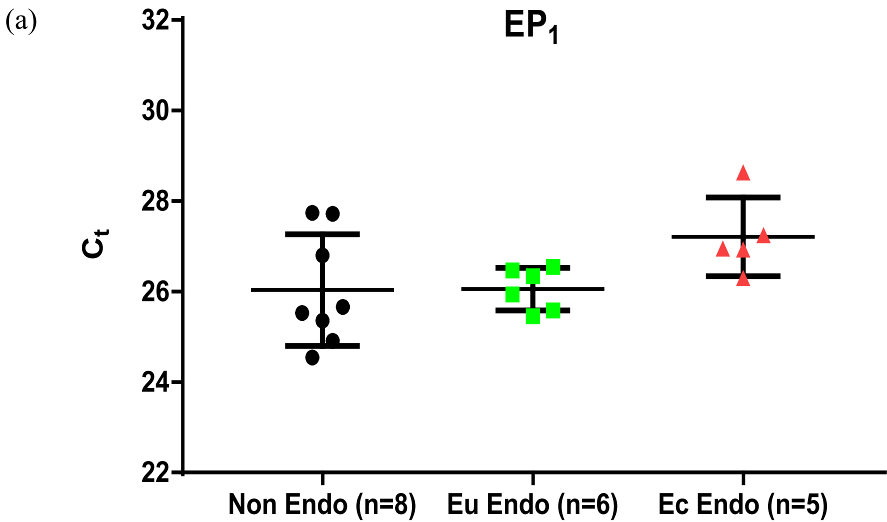
Fig 3.2.3c: The C_t values of (a) AKR1C1, (b) AKR1C2, (c) AKR1C3 genes in the human breast adipose tissues from patients with breast cancer, according to progesterone receptor (PR) status: PR-positive (●) and PR-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

Fig 3.2.3d: The C_t values of GAPDH and β-actin housekeeping genes in the human breast adipose samples from patients with breast cancer according to progesterone receptor (PR) status: PR-positive (●) and PR-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using two-way ANOVA.

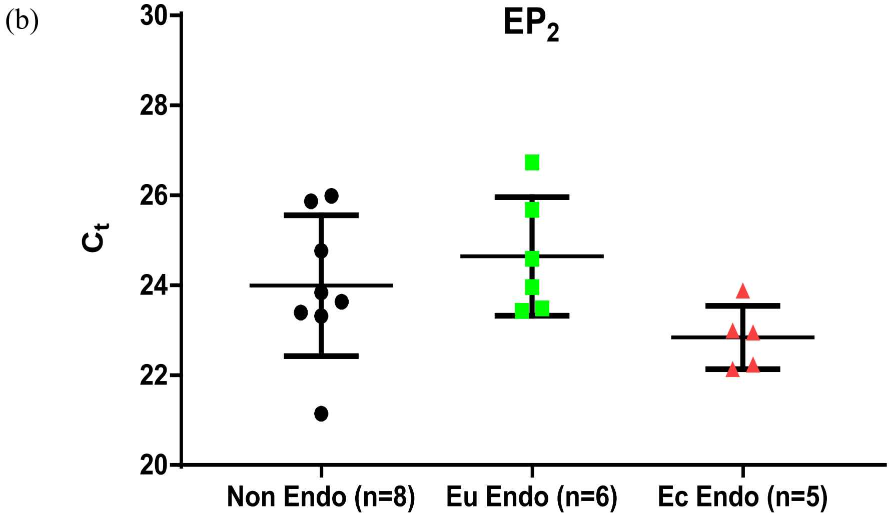
There was no significant difference in the gene expression of AKR1C1-3 enzymes in the breast adipose tissues when grouped according to the oestrogen receptor status, oestrogen-positive versus oestrogen-negative groups. This was also true when the data was regrouped according to the progesterone receptor status, progesterone-positive versus progesterone-negative groups.

3.2.4. The gene expression of EP_{1-4} and FP receptors in the human endometrium

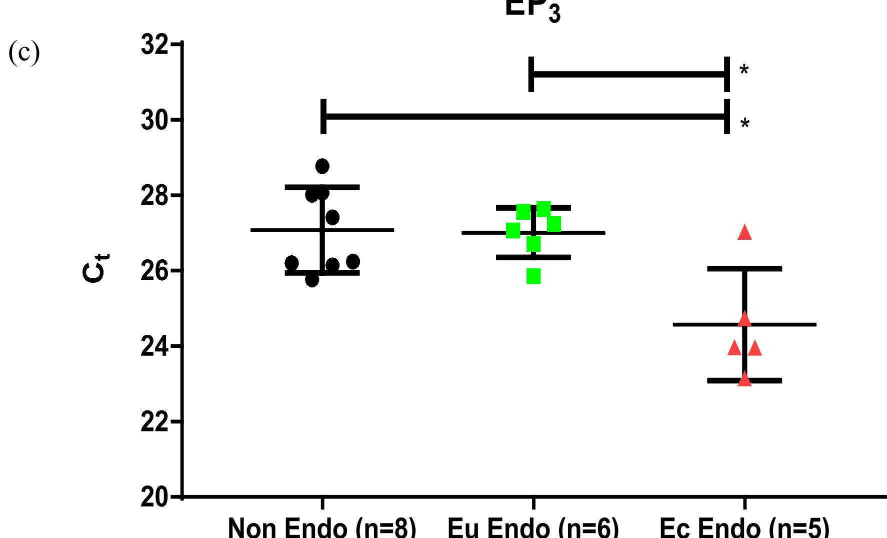
(a)



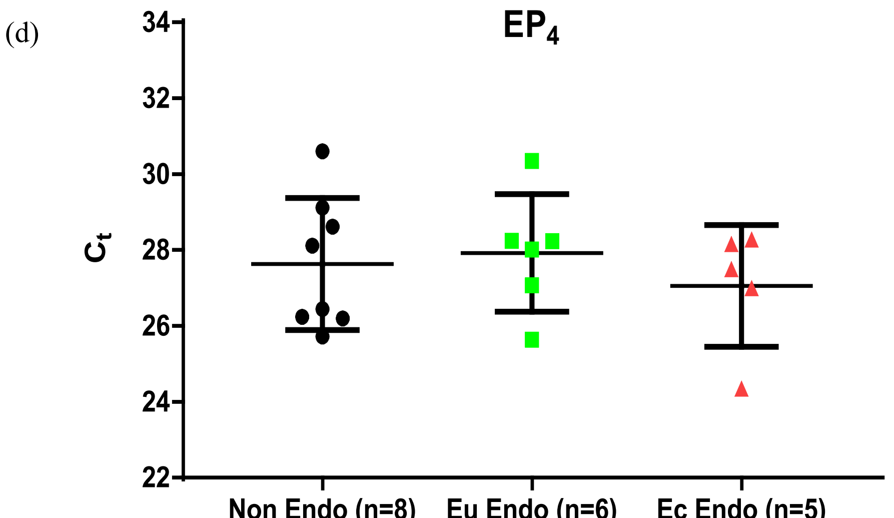
(b)



(c)



(d)



(e)

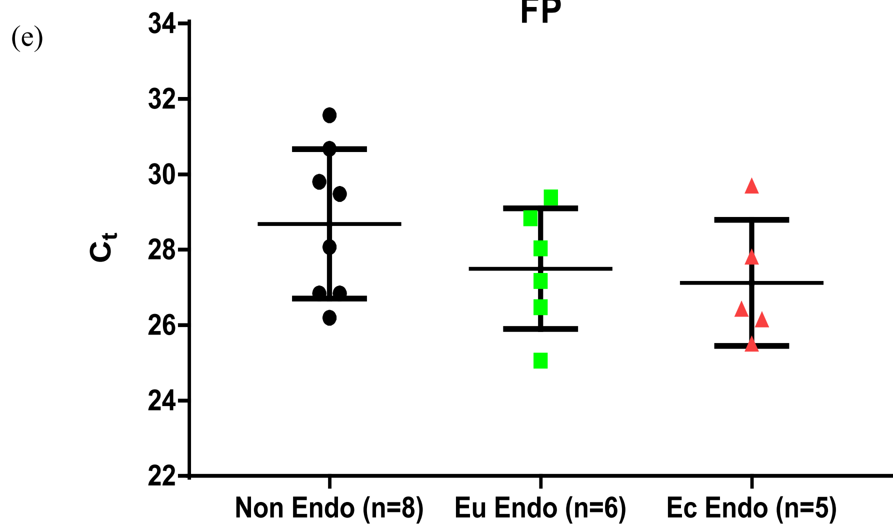


Fig 3.2.4a: The C_t values of (a) EP_1, (b) EP_2, (c) EP_3, (d) EP_4 and (e) FP receptor genes in human endometrial samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-Endo (●) = Eutopic endometrial samples from patients without endometriosis; Eu Endo (■) = Eutopic endometrial samples from patients with endometriosis; Ec Endo (▲) = Endometriotic lesions from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post-hoc* test; *P<0.05

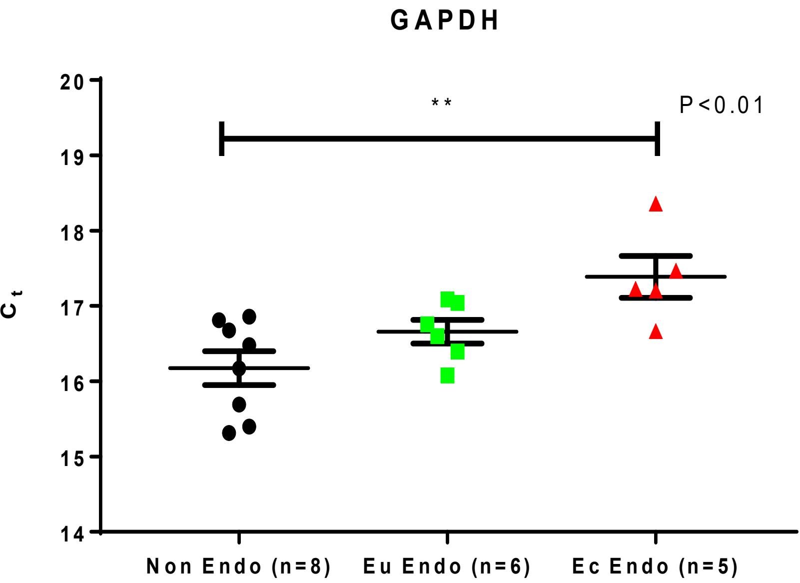


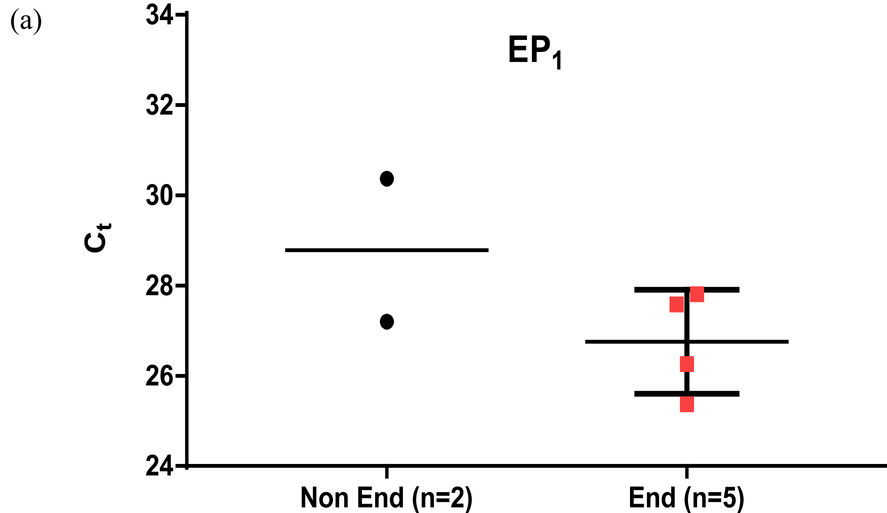
Fig 3.2.4b: The C_t values of GAPDH housekeeping gene in human endometrial samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-Endo (●) = Eutopic endometrial samples from patients without endometriosis; Eu Endo (■) = Eutopic endometrial samples from patients with endometriosis; Ec Endo (▲) = Endometriotic lesions from patients with endometriosis. Data are expressed as means ±SEM. Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post-hoc* test; **P<0.01

There was significant elevation of EP_3 gene expression in endometriotic lesions from patients with endometriosis compared to eutopic endometrial tissues from patients with and without endometriosis (Fig 3.2.4a). However, the housekeeping gene, GAPDH, showed a statistical significant change in C_t values when compared with eutopic endometrial tissues from patients without endometriosis and endometriotic lesions from patients with endometriosis (Fig 3.24b). Consequently, the comparison between these two biological groups was invalid, since GAPDH was used for normalisation and should not have varied between the two biological groups.

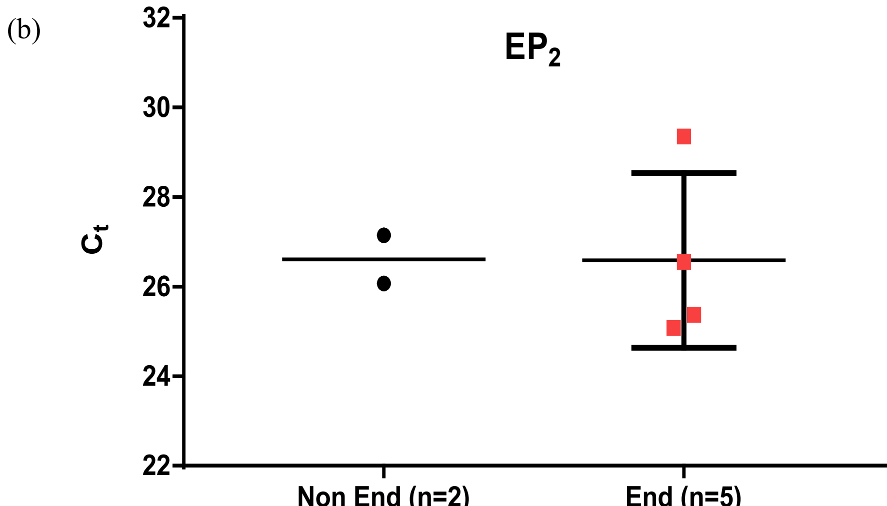
On the other hand, the C_t values of GAPDH did not significantly change between eutopic endometrial tissues from patients with endometriosis and endometriotic lesions from patients with endometriosis. As a result, the significant elevation of EP_3 gene expression observed in endometriotic lesions from patients with endometriosis compared to eutopic endometrial tissues from patients with endometriosis was valid as it was not affected by the changes of GAPDH.

3.2.5. The gene expression of EP_{1-4} and FP receptors in the human omental adipose tissue

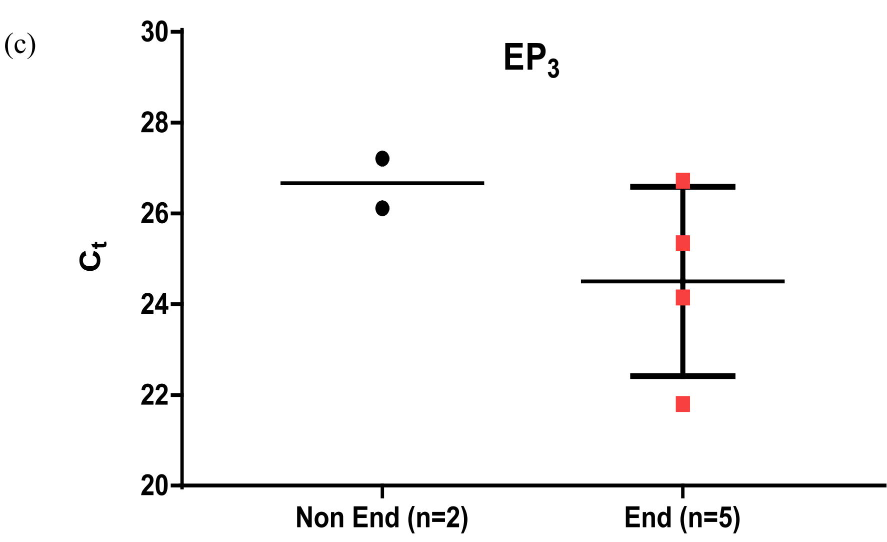
(a)



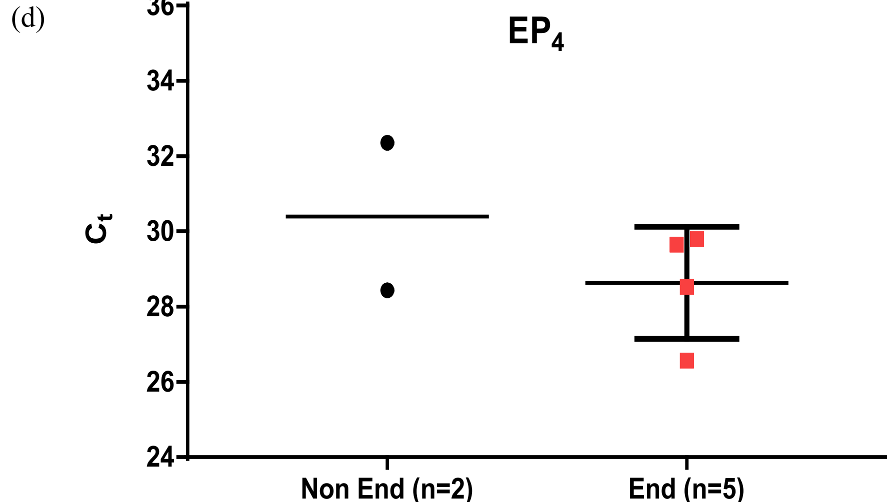
(b)



(c)



(d)



(e)

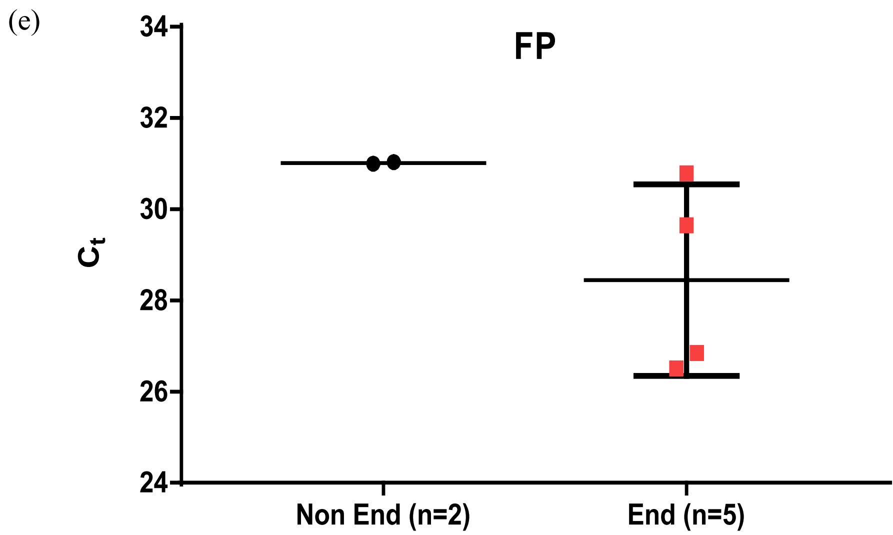


Fig 3.2.5a: The C_t values of (a) EP_1, (b) EP_2, (c) EP_3, (d) EP_4 and (e) FP receptor genes in the human omental adipose tissue samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non- End (●) = Omental fat from non-endometriosis patients; End (■) = Omental fat from endometriosis patients. Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

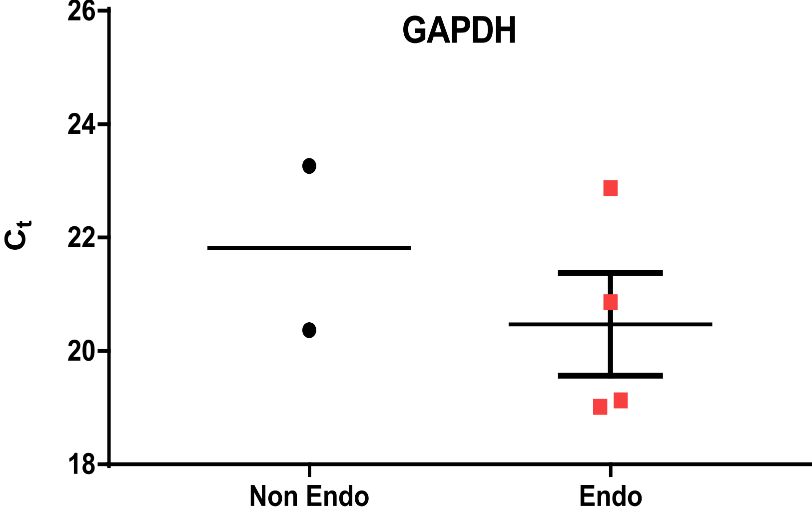


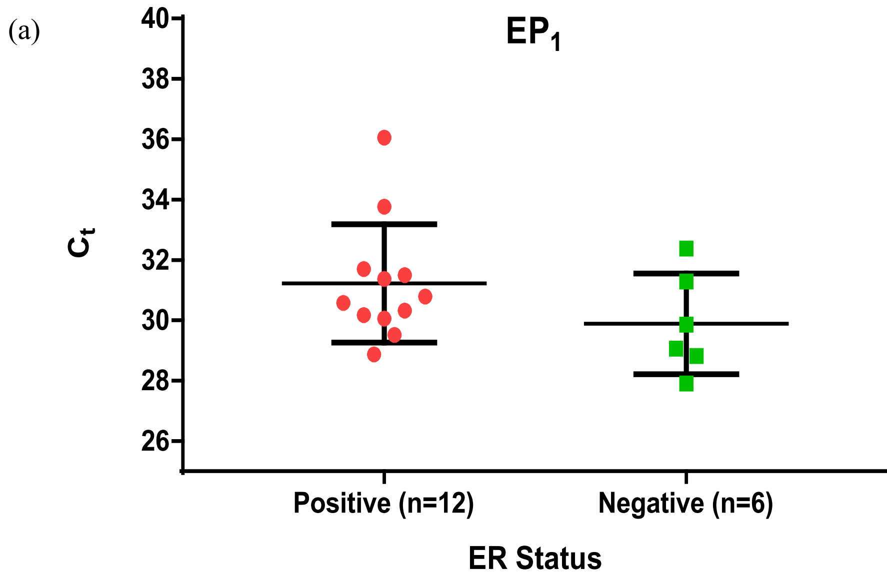
Fig 3.2.5b: The C_t values of GAPDH, housekeeping gene, in the human omental adipose tissue samples. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for quantitative real-time PCR (qRT-PCR). Non-End (●) = Omental fat from non-endometriosis patients; End (■) = Omental fat from endometriosis patients. Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

There was no significant difference in the gene expression of EP (1-4) and FP receptors in the omental adipose tissue from patients with and without endometriosis (Fig 3.2.5a). Unfortunately due to the low number of samples, it was not possible to find any statistically significant difference between the endometriosis and non-endometriosis groups.

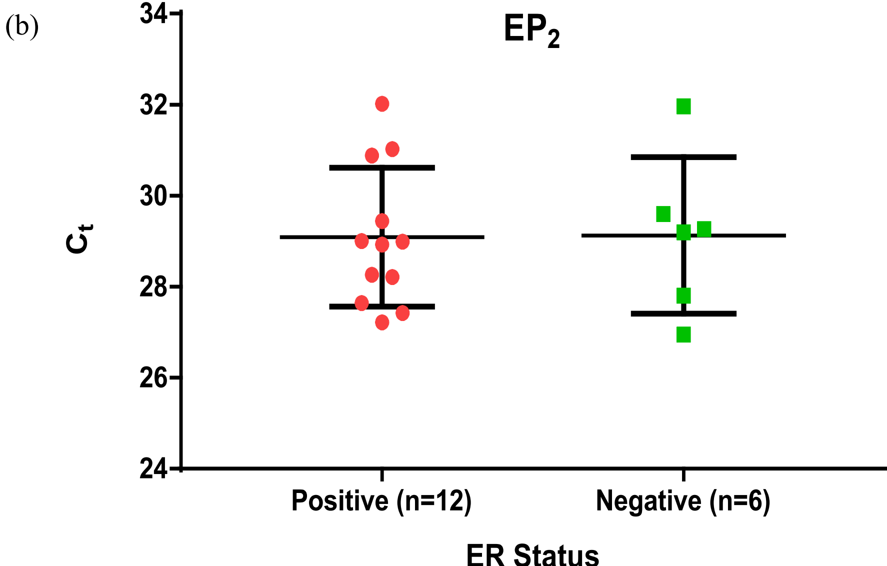
3.2.6. The gene expression of EP_{1-4} and FP receptors in the human breast adipose tissue from breast cancer patients

I. Gene expression according to the oestrogen receptor status:

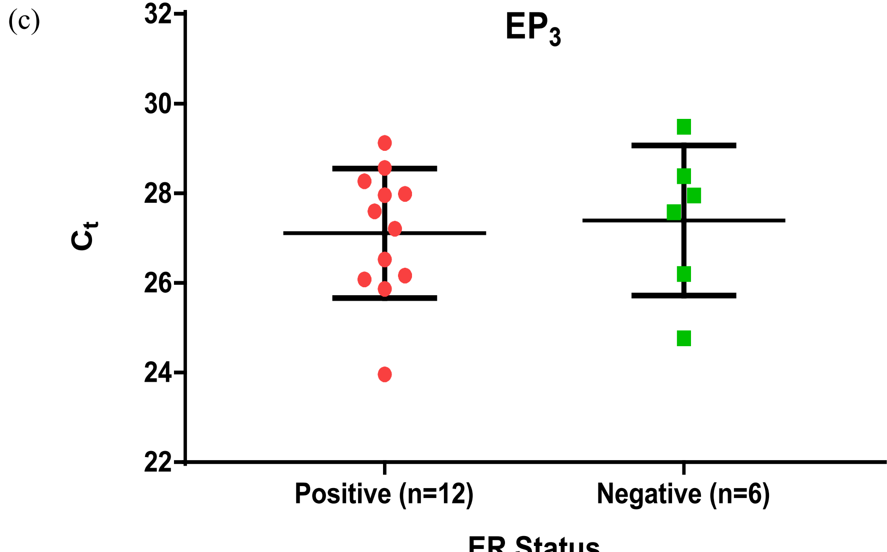
(a)



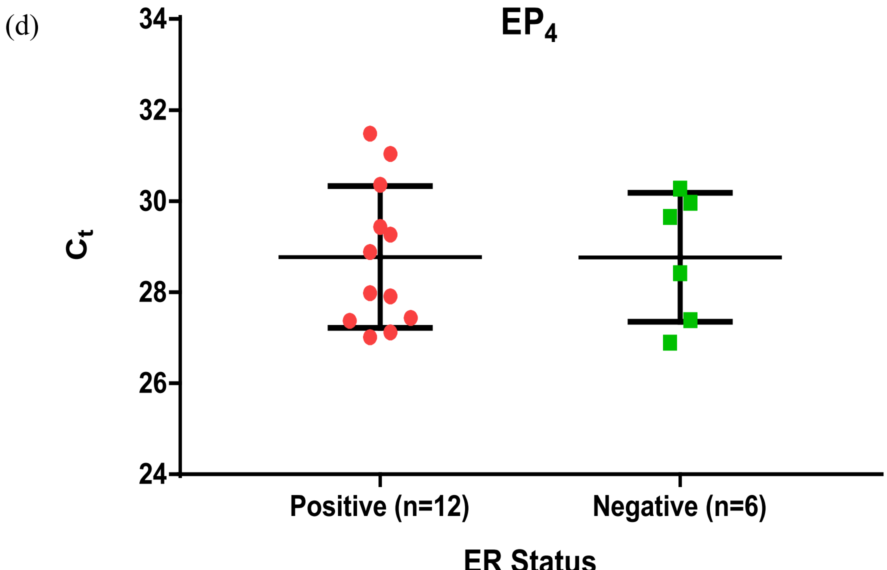
(b)



(c)



(d)



(e)

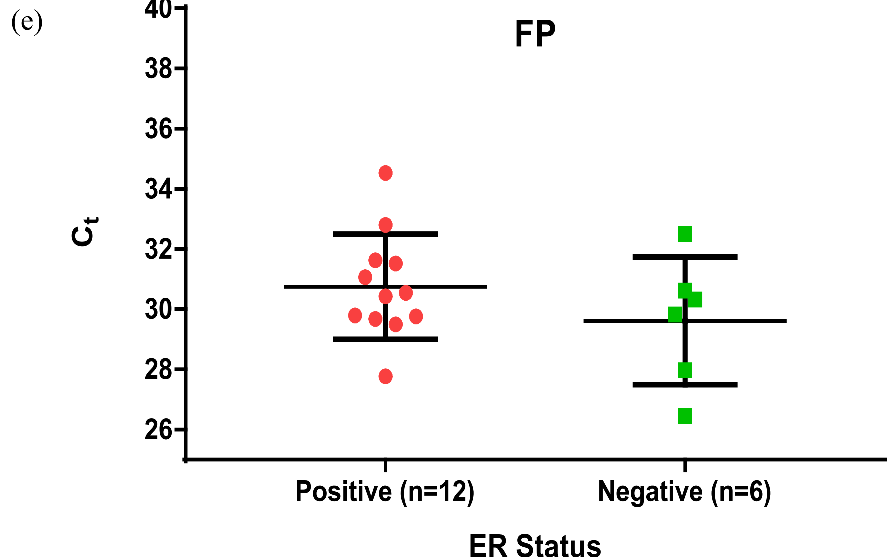


Fig 3.2.6a: The C_t values of (a) EP_1, (b) EP_2, (c) EP_3, (d) EP_4 and (e) FP receptor genes in the human breast adipose tissue samples from breast cancer patients according to ER status: ER-positive (●) and ER-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

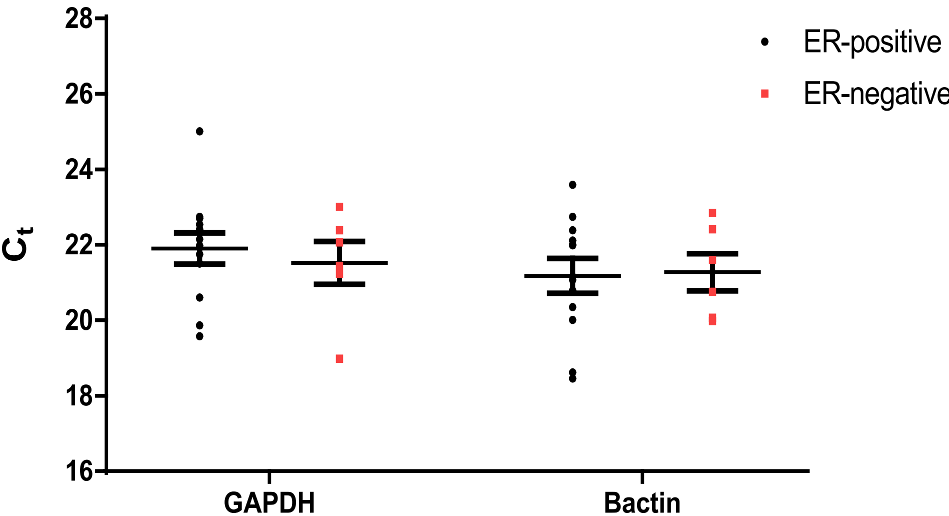
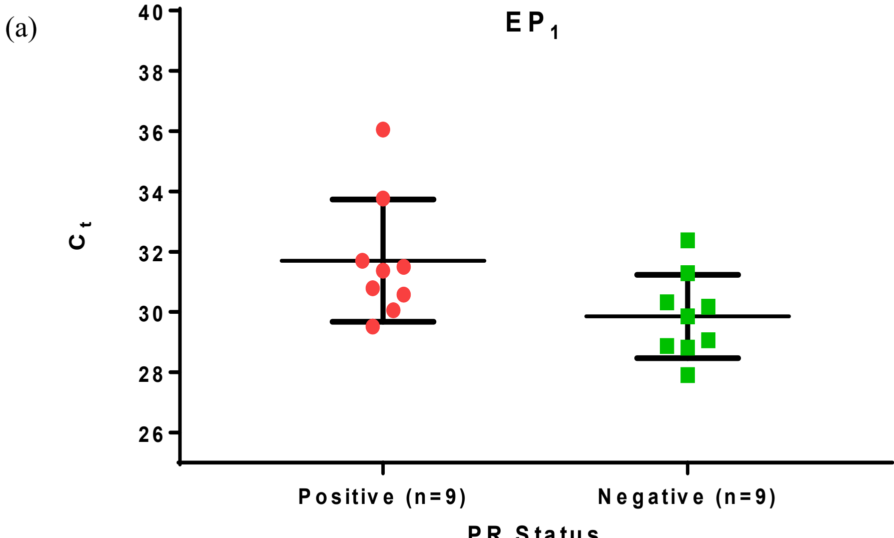


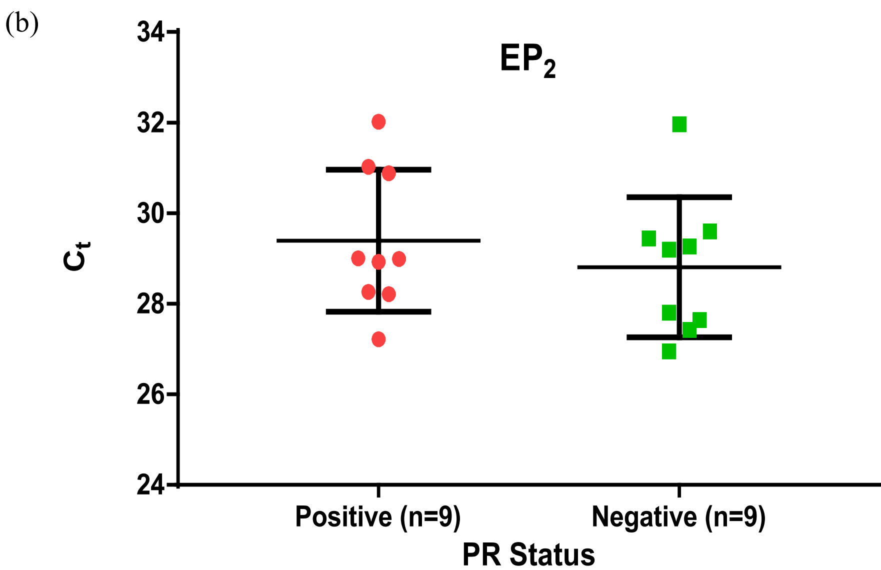
Fig 3.2.6b: The C_t values of GAPDH and β-actin housekeeping genes in the human breast adipose tissue samples from breast cancer patients according to ER status: ER-positive (●) and ER-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

II. Gene expression according to the progesterone receptor status:

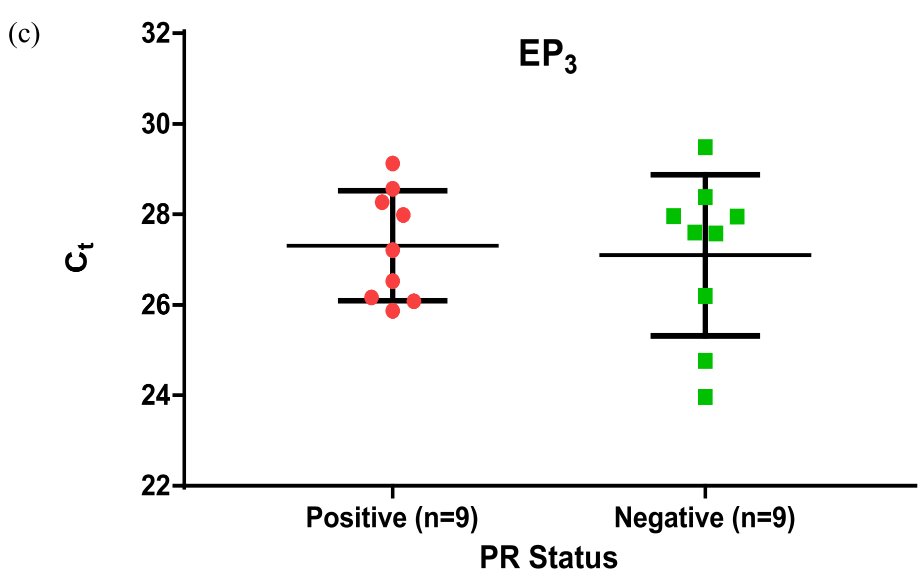
(a)



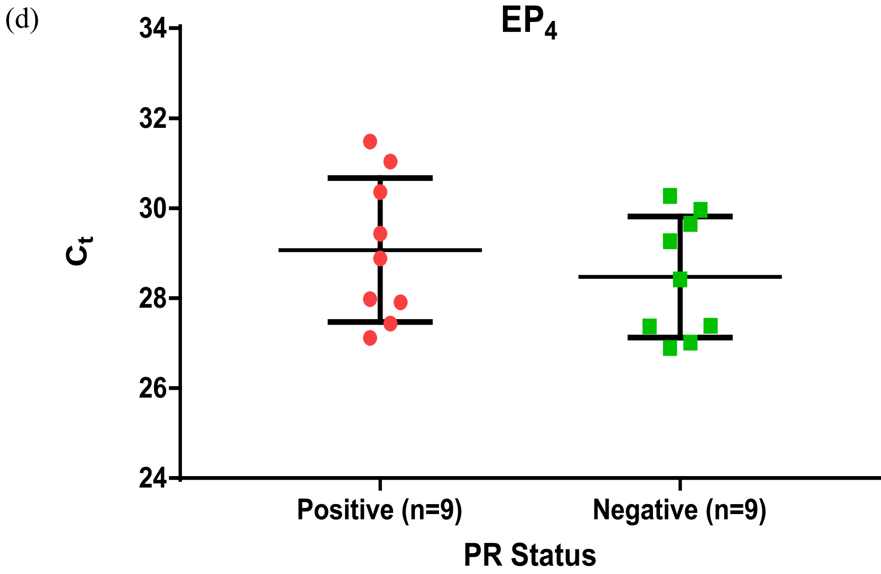
(b)



(c)



(d)



(e)

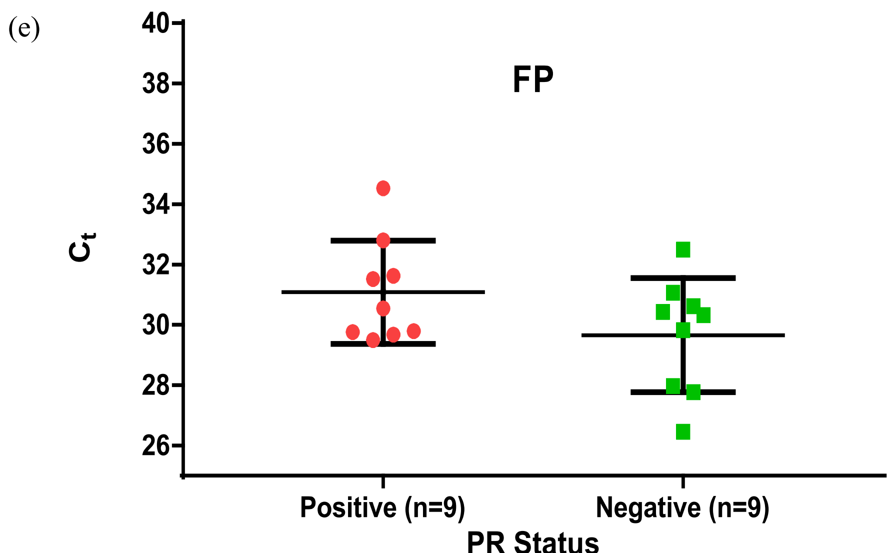


Fig 3.2.6c: The C_t values of (a) EP_1, (b) EP_2, (c) EP_3, (d) EP_4 and (e) FP receptor genes in the human breast adipose tissue samples from breast cancer patients according to PR status: PR-positive (●) and PR-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Relative gene expression was normalised against GAPDH and β-actin as housekeeping genes. Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

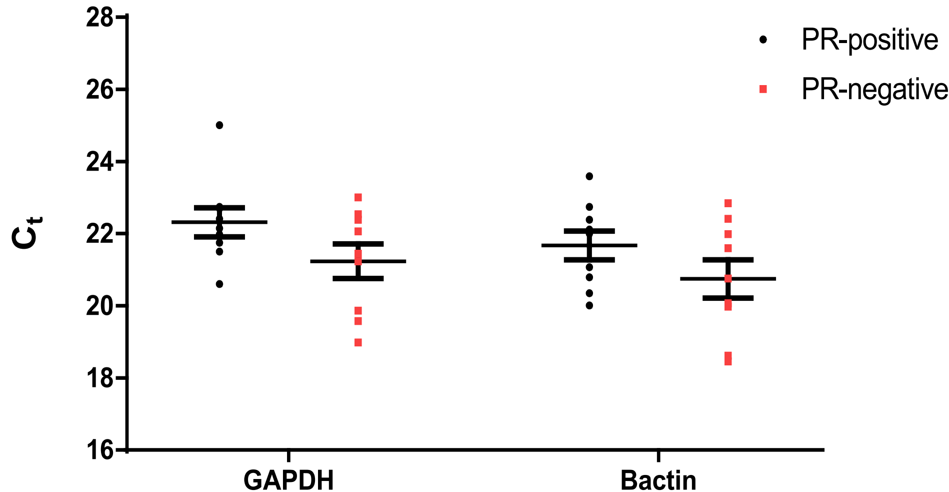


Fig 3.2.6d: The C_t values of GAPDH and β-actin housekeeping genes in the human breast adipose tissue samples from breast cancer patients according to PR status: PR-positive (●) and PR-negative (■). The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (5nmol) for quantitative real-time PCR (qRT-PCR). Data are expressed as means ±SEM. Statistical significance was calculated using Student's t-test.

There was no significant difference in the expression of EP $_{(1-4)}$ and FP receptors in the omental adipose tissues when the data was grouped according to ER and PR status (Fig 3.2.6a and 3.2.6d).

3.3. Discussion

3.3.1. The role of AKR1C (1-3) in hormone dependent diseases

I. The endometrium

There was a significant elevation of AKR1C1 ($P<0.05$) gene expression in the endometriotic lesions of patients with endometriosis compared to eutopic endometrial samples from patients with endometriosis. The housekeeping gene, GAPDH, was not altered between the two biological groups, indicating that the comparison between the endometriotic lesions from patients with endometriosis and eutopic endometrial tissue from endometriosis patients was valid. Also, the C_t values of all the samples were all below 40 cycles, indicating that they were all above the background noise.

It is worth noting that AKR1C2 was not detected in the human endometrium, unlike the omental adipose tissue and breast adipose tissue. This confirms that AKR1C2 gene is not expressed in the human endometrium. Furthermore, aromatase was not detected in the eutopic endometrial tissues and endometriotic lesions, which was supported by another group (Colette et al., 2009).

These results indicate that there is a local hormonal imbalance, at least at the genomic level. These results were further corroborated by other groups who have found similar results (Hevir et al., 2011; Rakhila et al., 2013). This is due to the increased translation of AKR1C1 and AKR1C3 which would enhance the depletion of progesterone combined with excessive production of 17β-estradiol via AKR1C3

in the endometriotic lesions. Consequently, this creates an oestrogenic environment which is required to support the ectopic endometrial lesions.

It is widely known that endometriotic lesions are capable of *de novo* biosynthesis of oestrogen through steroidogenic factor-1 (SF-1), which was exclusively found in the endometriotic lesions but not in eutopic endometrium (Zeitoun et al., 1999). The induction of SF-1 is caused by PGE_2 (Attar et al., 2009). The rate limiting step in the pathway is aromatase which would result in the aromatisation of androstenedione and testosterone to oestrone and 17β-oestradiol, respectively, depending on the availability of substrate.

To ensure high levels of 17β-oestradiol are produced and maintained in the local environment of the endometriotic lesions, the equilibrium between oestrone and 17β-oestradiol levels would need to be shifted towards 17β-oestradiol via the 17β-hydroxysteroid dehydrogenases (17β HSDs). Thus, 17β HSD type 1, which is one of the major enzymes responsible for the conversion of oestrone to 17β-oestradiol, was found to be upregulated in endometriotic lesions, whereas 17β-HSD type 2, which is responsible for the conversion of 17β-oestradiol to oestrone, was downregulated (Huhtinen et al., 2012). The role of 17β-oestradiol in endometriotic tissues is principally mitogenic. Aberrant levels of 17β-oestradiol have upregulated the expression of fibroblast growth factor-9 (FGF-9) and its cognate receptors in the stromal cells of endometriotic endometrial cells, causing proliferation, and hence, survival (Wing et al., 2003).

Also, the metabolism or inactivation of progesterone in the endometriotic lesions is a key contributing factor to the aberrant levels of 17β-oestradiol, as shown by the

upregulation of AKR1C1. Progesterone is responsible for reducing the mitogenic effects of 17β-oestradiol, mainly by metabolising 17β-oestradiol through the induction of 17βHSD type 2 which would convert 17β-oestradiol back to oestrone (Casey et al., 1994). At the receptor level, the upregulation of ERβ in endometriotic lesions suppresses the expression of ERα which in turn causes the suppression of PRB (Trukhacheva et al., 2009). Consequently, the endometriotic micro-environment would have the characteristics of excessive local 17β-oestradiol caused by progesterone resistance.

Additionally, progesterone and progestagens have been shown to inhibit the expression of MMP1 and MMP3, as well as angiogenic factors such as VEGFA, basic fibroblast growth factor (bFGF) and Cysteine-rich angiogenic inducer 61 (CYR61) in endometriotic lesions (Mönckedieck et al., 2009). This underpins the essential role of progesterone in balancing the local hormonal milieu and halting the progression of endometriosis. Currently, progestagens are used in the treatment for endometriosis for controlling pain symptoms associated with the disease (Prentice et al., 2000).

Not only does elevated ERβ expression create a pro-oestrogenic environment in endometriotic lesions, it also induces the expression of COX-2 enzyme which results in the biosynthesis of proinflammatory prostaglandins (Bulun et al., 2001).

It's also worth noting that endometrial tissue samples from patients taking aromatase inhibitors, selective oestrogen receptor modulator (SERMs) or gonadotrophin releasing hormone receptor analogues (GnRHAs) have downregulated gene expression of AKRs in endometriotic lesions (data not shown).

II. The omental adipose tissue

In the omental fat, there was no significant difference in the gene expression of AKR1C enzymes in samples taken from patients with endometriosis and patients without endometriosis (Fig 3.2.2). This might be due to the low sample number. However, omental fat has the capability of being a source of essential factors that provide a good environment for growth and survival of endometriotic tissues. Compared to the human endometrial tissues and endometriotic lesions, omental adipose tissues had much higher ΔC_t values.

Adipocytes and preadipocytes are capable of synthesising oestrone and 17β-oestradiol from circulating androstenedione and testosterone, respectively (Ackerman et al., 1981). Also, oestrogen receptors are distributed in adipose tissues through which oestrone and 17β-oestradiol exert their effect (Pedersen, Hansen, et al., 1996). Therefore, extragonadal 17β-oestradiol originating from adipose tissue surrounding endometriotic lesions can have a paracrine effect and may contribute to the progression of endometriosis. However, aromatase gene was not detected in the omental adipose tissues in this study, using different aromatase primers, which contradicts the previous reports.

A co-culture with adipocytes enhanced the proliferation of human endometrial glandular cells by increasing the expression of proliferation markers, such as Proliferating cell nuclear antigen (PCNA), cyclin D1 and Cyclin-dependent kinase 1 (CDK1). It also decreased apoptosis markers, such as BCL2-antagonist/killer 1(BAK1), which is mediated via TNF_{α} secreted from adipocytes (Hotamisligil et al., 1993; Nair et al., 2013). In addition, the peritoneal environment in endometriosis has marked differences, especially the presence of cytokines such as TNF_{α}, which is

found in the vast majority of patients with endometriosis but not in the peritoneal fluid from non-endometriosis patients (Overton et al., 1996). This would also result in the proliferation of ectopic endometrial cells and enhance their chance of survival.

Unfortunately, there is paucity in studies conducted on the role of omental fat in endometriosis. However, preliminary studies are emerging on endometrial cancer which characterise the active role of omental adipose tissues in maintaining the growth of endometrial tumour at a paracrine level. For example, a co-culture of preadipocytes with Ishikawa cells increased the rate of cell proliferation, as well as causing Ishikawa cells to secrete more VEGF (Linkov et al., 2014). This experiment can be extrapolated using ectopic endometrial cells to determine whether omental adipose tissues have an active role in endometriosis.

III. The breast adipose tissue

There was a no significant change in the gene expression of AKR1C1-3 enzymes in the breast adipose tissue from breast cancer patients regardless of the oestrogen or progesterone receptor status.

These findings do not correlate with previous reports on the breast tumours which reported significant downregulation of AKR1C1 compared to paired normal unaffected breast tissues (Ji et al., 2004; Wenners et al., 2015) and unpaired normal breast tissues (Lewis et al., 2004). However, these reports did not consider age, menopausal status, or receptor status in their comparison. These differences were apparent when the data were grouped according to hormone receptor status (ER or PR) (Fig: 3.2.3a-d & 3.2.6 a-d).

Nevertheless, there are more recent findings that corroborate the theory of “enhanced progesterone inactivation” in the progression of breast cancer. Mohammed et al. (2015) have reported that progesterone has an anti-proliferative effect by modulating the ERα action in the breast cancer cells. This was further confirmed using primary breast cancer tissue *ex vivo* which showed that progesterone reversed the proliferative effect of 17β-oestradiol.

There was no statistical difference in the gene expression of AKR1C2 when compared with breast adipose tissues from patients with breast cancer, regardless of ER or PR status. Conversely, AKR1C2 was downregulated in breast cancer tissues as reported by other groups (Ji et al., 2004; Lewis et al., 2004; Wenners et al., 2015).

In breast tissue, there are mixed reports about AKR1C3 expression from different groups. Lewis et al. (2004) reported that AKR1C3 was downregulated in tumour breast tissue, whereas Jansson et al. (2006) reported an upregulation of AKR1C3, as well as significant 5-year relapse risk in ER-positive breast cancer patients with high levels of expressed AKR1C3.

Moreover, the surrounding adipose tissues around the breast tumour may serve as a reservoir to provide the essential steroid hormones which facilitates the progression of tumour growth via modulating local levels of 17β-oestradiol and progesterone for the tumour.

Finally, aromatase gene was not detected in all the breast adipose tissues. This might be either due to improper primer designing, even though different primers were designed, or aromatase was below the detection limit in some of these tissue samples.

3.3.2. The role of EP_{1-4} and FP receptors in hormone dependent diseases

I. The endometrium

There was a significant elevation in the gene expression of EP_3 ($p<0.05$) in the endometriotic lesions compared to eutopic endometrial tissues from patients with endometriosis. These results were somewhat similar to the findings reported by Rakhila et al. (2015), who found significant elevation of EP_3, EP_4 and FP receptor gene expression in endometriotic lesions. These discrepancies might be due to different factors, such as primer design, the method used for data analysis, as well as the differences in ethnicity, age and health status of patients from which samples were taken. Also the number of samples used by Rakhila et al. (2015) was much higher compared to the sample number used in this study.

In addition to PGE_2 and $PGF_{2\alpha}$ being the most abundant prostanoids in the endometrium, they also have a role in uterine diseases, especially endometriosis (Jabbour and Sales, 2004). The PGE_2 effect mediated via the EP_2 receptor results in trans-activation of the epidermal growth factor receptor (EGFR) via cAMP-dependent protein kinase (PKA), as well as activation of mitogen-activated protein kinase (MAPK) pathway in Ishikawa cells (Sales, Maudsley, et al., 2004). Moreover, VEGF mRNA expression and secretion was elevated by PGE_2 via the EP_2 receptor (Sales, Maudsley, et al., 2004). The PGE_2-EP_2 interaction therefore results in the proliferation of the endometriotic lesions with survival facilitated by enhanced angiogenesis. Furthermore, EP_3 mediated action can result in the phosphorylation of fibroblast growth factor receptor-1 (FGFR-1) via the mobilisation of fibroblast

growth factor-2 (FGF-2) through the induction of matrix metalloproteinases (MMPs) which consequently results in FGF-2 release from the extracellular matrix, and in turn, phosphorylates FGFR-1 (Finetti et al., 2008). $PGF_{2\alpha}$ mediated effect via FP receptor has mitogenic and angiogenic effect on endometrial cells. FP receptor mediated effect resulted in activation of EGFR, via phospholipase Cβ (PLCβ), as well as MAPK pathway (Sales, Milne, et al., 2004). Furthermore, $PGF_{2\alpha}$-FP receptor interaction elevated the mRNA expression and secretion of VEGF (Sales et al., 2005).

II. The omental adipose tissue

Due to the low number of samples, there was no significant difference in the gene expression of EP_{1-4} and FP receptors in the omental fat from patients with and without endometriosis (Fig 3.2.6). Nevertheless, there was slight elevation in EP_1, EP_3, EP_4 and FP receptor gene expressions and slight decrease in EP_2 receptor gene expression in omental adipose tissue of endometriosis patients. The levels of EP_{2-4} and FP genes were higher in the omental adipose tissues compared to the human endometrial tissues and endometriotic lesions (Table 3.2.8).

FP receptor stimulation is widely known to inhibit the initial phases of preadipocyte differentiation into mature adipocytes (Casimir et al., 1996). However, the omental adipose tissue is capable of producing $PGF_{2\alpha}$ and can be modulated in response to a different range of mediators. For example, $PGF_{2\alpha}$ biosynthesis is elevated when omental adipose tissue is exposed to inflammatory mediators such as TNF_{α} and IL-1β (Michaud, Lacroix-Pépin, Pelletier, Veilleux, et al., 2014). Also, the omental

adipose tissue releases significant amounts of PGE_2 and $PGF_{2\alpha}$ compared to subcutaneous fat, indicating that omental adipose tissue may have other roles due to the proximity of the tissue to surrounding organs, including the reproductive organs (Michaud, Lacroix-Pépin, Pelletier, Daris, et al., 2014). Furthermore, the omental adipose tissue can promote angiogenesis *in vivo.* This is believed to be mediated by PGE_2, since the effect was abolished when indomethacin was administered (Silverman et al., 1988).

In conclusion, the link between omental adipose tissue and endometriosis is at its early stages, but there are few indications that may prompt conducting more research to be able to further understand the role of omental adipose tissue in endometriosis.

III. Breast adipose tissue

In the breast adipose tissue samples, there was no significant difference in the gene expression of EP_{1-4} or FP receptors between the ER-positive and ER-negative breast cancer patients. Also, no significant difference was found amongst PR-positive and PR-negative breast cancer patients (Fig 3.2.6a and 3.2.6c).

Nevertheless, PGE_2 has a synergistic effect on aromatase activity in human breast adipocytes, resulting in aromatisation of androgens to oestrogens. Also, inflammatory mediator TNF_α induced the release of PGE_2 due to the upregulation of COX-2 and PGE synthases (Karuppu et al., 2002). This indicates that the breast adipose tissues can act within a paracrine/autocrine system to influence the development and progression of the breast tumour. Moreover, p53, an aromatase suppressor, is inhibited by PGE_2 in human breast adipocytes and it is downregulated

in tumour associated human breast adipocytes (Wang et al., 2015). PGE_2 regulation of aromatase could be mediated by EP_1 and EP_2 receptors in the breast adipose tissues (Richards and Brueggemeier, 2003).

Furthermore, the effect of 9α,11β PGF_2 (a stereoisomer of $PGF_{2\alpha}$) in MCF-7 cells activated MAPK pathway and cAMP response element binding protein (CREB) (Yoda et al., 2015). Therefore, 9α, 11β PGF_2 produced in the neighbouring breast adipose tissues can have a paracrine effect on the breast cancer cells.

4. Bimatoprost as a potential aldo-keto reductase 1C3 inhibitor

4.1. Introduction

4.1.1. Background

Bimatoprost or 17-phenyl trinor prostaglandin $F_{2\alpha}$ ethyl amide is a prostamide $F_{2\alpha}$ analogue with a molecular formula of $C_{25}H_{37}NO_4$ and formula weight of 415.6 (Woodward et al., 2001). Bimatoprost (Lumigan©) was approved by the European Medicines Agency (EMA) in 2002 for the reduction of elevated intraocular pressure in chronic open-angle glaucoma and ocular hypertension in adults. [http://www.ema.europa.eu/docs/en_GB/document_library/EPAR__Product _Information/human/000391/WC500044435.pdf]. It was also approved in 2008 by the Food and Drug Administration (FDA) for the treatment of hypotrichosis of eyelashes (Fagien, 2015).

4.1.2. Bimatoprost mechanism of action

Bimatoprost, like prostamide $F_{2\alpha}$, reduces intraocular pressure by decreasing aqueous humour volume in the eye. Bimatoprost does not suppress aqueous humour production, but enhances the trabecular and uveoscleral outflow (Woodward et al., 2001). This is achieved by lowering the pressure-sensitive and pressure insensitive resistance, and hence enhance aqueous humour outflow (Christiansen et al., 2004).

4.1.3. Prostamides or prostaglandin ethanolamides

Prostamides are a group of lipids closely related to prostaglandins. They have ethanolamine attached to the carbonyl functional group, instead of a carboxylic acid. They are derived from anandamide, a neutral lipid, and are oxygenated by COX-2. Unlike arachidonic acid, anandamide is not oxygenated by COX -1 (Yu et al., 1997).

Interestingly, the prostamide biosynthesis pathway uses the same enzymes used in the prostaglandin pathway. Anandamide is converted by COX-2 to prostamide H_2, an unstable precursor, which can be converted to prostamide D_2, prostamide E_2 and prostamide $F_{2\alpha}$ via prostaglandin D synthases, prostaglandin E synthases and prostaglandin F synthases, respectively (Woodward et al., 2008).

The first prostamide to be discovered was prostamide E_2 in the human foreskin fibroblasts, which is also the major product of anandamide (Yu et al., 1997). Moreover, prostamides were found *in vivo* in the mouse (Weber et al., 2004).

The pharmacology of prostamides is quite distinct from that of prostanoids. Although the structures of the two are very similar, prostamide $F_{2\alpha}$ does not activate the prostanoid FP receptor (Matias et al., 2004). The action of prostamide E_2 on prostanoid EP receptor subtypes was significantly lower than that of PGE_2. Also, prostamide E_2 very weakly binds with vanilloid receptor 1 (VR1), indicating that prostamides are not involved in the endocannabinoid system either, unlike anandamide (Ross et al., 2002).

There are different opinions suggesting that prostamides act through their own distinct set of receptors or different isoforms of prostanoid receptors. This opinion

was based solely on investigating the pharmacology of prostamides through their agonists and antagonists. For example, bimatoprost and $PGF_{2\alpha}$ stimulated different populations of cells in the feline iris (Spada et al., 2005), whereas AGN204397 (prostamide F receptor antagonist) antagonised the contractile effect of prostamide $F_{2\alpha}$ and bimatoprost but not $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ (Woodward et al., 2008).

Fig 4.1.1: (A) Biosynthesis of prostamides; starting from the parent molecule, anandamide, which is converted to prostamide H_2 (PMH_2) by cyclooxygenase 2 (COX-2). PMH_2 is a precursor which can be converted to prostamide $F_{2\alpha}$ ($PMF_{2\alpha}$) by prostamide/prostaglandin F synthase (PMPGFS) and AKR1C3; to prostamide E_2 (PME_2) by prostaglandin E synthases (PGES); to prostamide D_2 (PMD_2) by prostaglandin D synthases (PGDS); to prostaglandin I_2 ethanolamide (PGI_2-EA) by prostacyclin synthase; or to thromboxane A_2 ethanolamide (TXA_2-EA) by thromboxane synthase (TXA-S). (B) The structural similarities between $PGF_{2\alpha}$, $PMF_{2\alpha}$ and bimatoprost.

4.1.4. Bimatoprost: a prostamide $F_{2\alpha}$ or prostaglandin $F_{2\alpha}$ analogue?

Bimatoprost has been described as a prostamide F receptor agonist, rather than a prostanoid FP receptor agonist, as it does not behave in a similar manner to prostaglandin $F_{2\alpha}$ (Woodward et al., 2008). It has been suggested that prostamides and their analogues, like bimatoprost, act on different set of receptors, called prostamide receptors, even though they are not fully characterised.

Initial investigations revealed that bimatoprost did not stimulate myometrial contractions in isolated rat, mouse and human uterine tissues. Furthermore, unlike $PGF_{2\alpha}$, bimatoprost did not relax the rabbit jugular vein (Chen et al., 2005). More importantly, the work by Woodward et al. (2003) found that pre-treatment with bimatoprost did not block the calcium response to $PGF_{2\alpha}$, which suggests that bimatoprost exerts its effect through a different pathway other than prostanoid FP receptor.

However, there is a different point of view that suggests bimatoprost is in fact a prostanoid FP receptor agonist, but a weak one, and is converted in the body to 17-phenyl $PGF_{2\alpha}$, which is a potent prostanoid FP receptor agonist (Camras et al., 2004). Bimatoprost and 17-phenyl $PGF_{2\alpha}$ displaced tritiated prostaglandin $F_{2\alpha}$ at the prostanoid FP receptor at K_i of 6310 nM and 83 nM, respectively. Moreover, bimatoprost and 17-phenyl $PGF_{2\alpha}$ activated prostanoid FP receptor at EC_{50} of 2220 nM and 39 nM, respectively. (Sharif et al., 2001, 2003).

In summary, this topic will remain a controversial one until the putative prostamide receptors are fully characterised and their mechanism of action is understood.

4.1.5. Bimatoprost: An AKR1C3 inhibitor

As mentioned earlier, upregulation of AKR1C3 expression is found to be implicated in many malignant diseases such as breast cancer, as well as endometriosis (Smuc et al., 2009; Byrns et al., 2010). For that reason, it is important to investigate the effect of such dysregulation and develop inhibitors that can aid in elucidating the aetiology of these conditions and form the foundation of a novel target in the treatment of such diseases.

Bimatoprost is a licensed drug, with a well-documented safety profile. Hence, it is prudent to reposition the drug for use in conditions where AKR1C3 activity is implicated, since it can reach the market much quicker and cheaper compared to novel drugs.

Bimatoprost, as an AKR1C3 inhibitor, was first discovered by Koda et al. (2004). It inhibited the PGD_2 11-ketoreductase activity at $IC_{50} = 5\mu M$, which converts PGD_2 to 9α,11β PGF_2, and PGH_2 9, 11-endoproxidase reductase activity at $IC_{50} = 6\mu M$, which converts PGH_2 to $PGF_{2\alpha}$. The inhibition of bimatoprost was reported as non-competitive, suggesting that it binds to the free enzyme and the substrate-enzyme complex. This was further corroborated by the work of Komoto et al. (2006) who showed that bimatoprost was bound to the active site of AKR1C3.

The catalysis of oestrone, testosterone and progesterone reduction via AKR1C3 could be important in the aetiology of sex hormone dependent diseases. In this chapter, the effect of bimatoprost was investigated on oestrone reduction to 17β-oestradiol via AKR1C3.

4.2. Results

4.2.1. AKR1C3 kinetic assay

Firstly, the time course of enzymatic reaction had to be optimised for oestrone, which had to be at the initial rate where the enzyme-substrate complex did not change. The time course for the reaction was set at 60 minutes, as shown below.

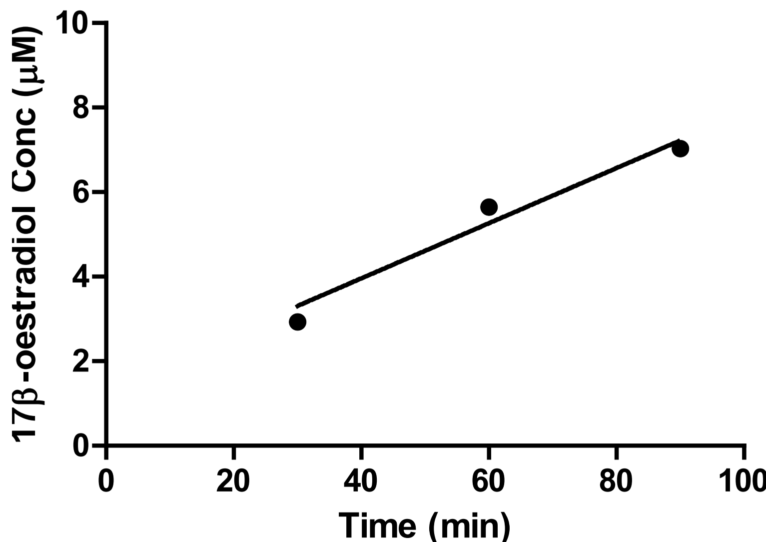


Fig 4.2.1a: A mixture of 2nmol of oestrone and 0.01nmol (1μCi) of [^{3}H] oestrone were added to the standard assay mixture. The reaction started by adding 4μg of AKR1C3 and terminated at different time points after: 30, 60 and 90 minutes at 37°C.

The concentration of enzyme was also optimised to ensure that the enzyme reaction velocity was in the linear range. The amount of enzyme used in subsequent reactions was 3µg of enzyme as shown below (Fig 4.2.1b).

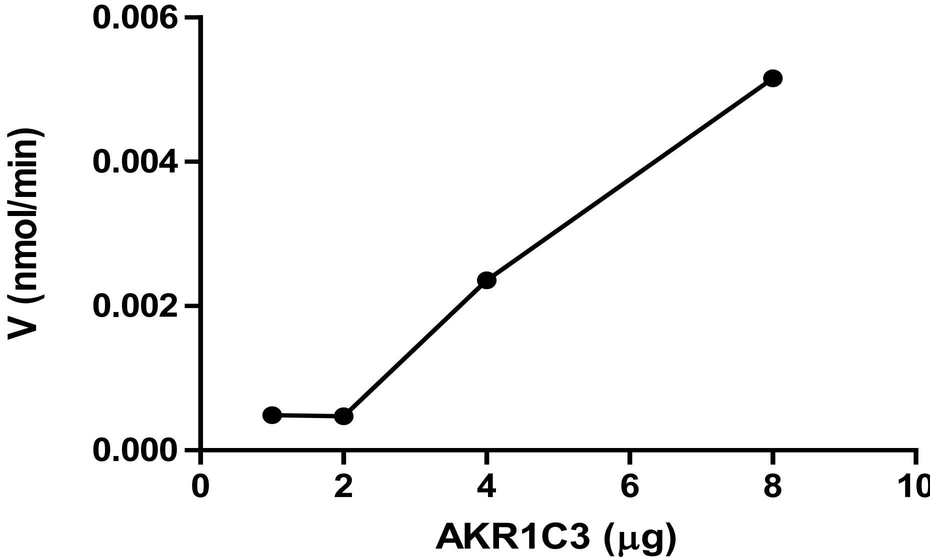


Fig 4.2.1b: A mixture of 2nmol of oestrone and 0.01nmol (1µCi) of [^{3}H] oestrone were added to a standard assay mixture. The reaction started by adding different amounts of AKR1C3: 1, 2, 4 and 8 µg of AKR1C3. The enzymatic reaction was terminated after 90 minutes at 37°C.

I. Determination of the kinetic parameters for oestrone

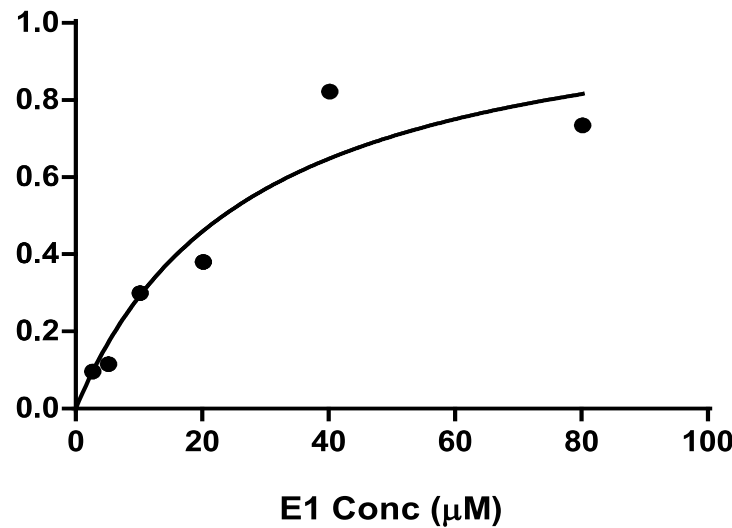


Fig 4.2.1c: The Michaelis-Menten curve (R^2=0.6) for the catalysis of oestrone to 17β-oestradiol via AKR1C3. 1μl [^{3}H] oestrone mixed with 1μl of unlabeled oestrone at different concentrations. The reaction started when 3μg of AKR1C3 was added and incubated for 60 minutes at 37°C. Data were expressed as means of 3 repeats

Substrate	**Oestrone**
	Mean (± SE)
(a) K_M (μM)	28.02 (±19.18)
(b) Vmax (nmol/min/mg of protein)	1.101 (±0.32)
(c) K_{cat} (min^{-1})	0.6608 (±0.1943)
(d) K_{cat}/K_M ($min^{-1}.mM^{-1}$)	0.0235

Table 4.2.1: Kinetic parameters for the substrate oestrone: (a) Michaelis constant (K_M), (b) Maximum velocity (Vmax), (c) Turnover number (K_{cat}), (d) catalytic efficiency (Kcat/Km). Data were expressed as means ± S.E. of 3 repeats

II. The effect of bimatoprost on AKR1C3 catalysis

Bimatoprost at different concentrations (1000nM and 5000nM) was added to the standard assay mixture and the purified AKR1C3. The mixture was pre-incubated for 30 minutes at 37ºC, allowing the bimatoprost to interact with AKR1C3. The mixture was then added to a mixture of 1µl (1µCi) of [^{3}H] oestrone and unlabeled oestrone at concentrations of 2.5, 5, 10, 20, 40 and 80µM. The mixture was then incubated for a further 60 minutes at 37ºC. The total concentration of ethanol was 5% v/v.

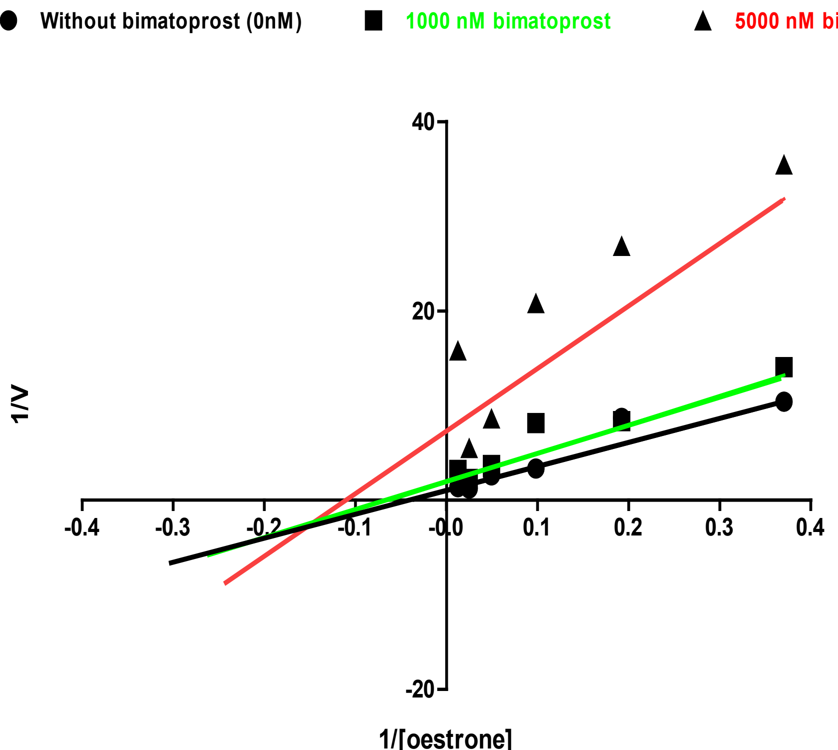


Fig 4.2.1d: The Lineweaver-Burk plots for oestrone only (●), oestrone plus 1000nM bimatoprost (■) and oestrone plus 5000nM bimatoprost (▲). 1µl [^{3}H] oestrone mixed with 1µl of unlabeled oestrone at different concentrations. The reaction started when 3µg of AKR1C3 was added and the mixture was incubated for 60 minutes at 37ºC. Data were expressed as means of 3 repeats.

Bimatoprost appeared to have inhibited the catalysis of oestrone reduction to 17β-oestradiol via AKR1C3. This inhibition, as indicated by the Lineweaver-Burk plot, is a mixed inhibition which means that bimatoprost inhibits the free enzyme and the enzyme substrate complex at different affinities.

III. Determining the inhibitory constant (Ki) value of bimatoprost

For mixed inhibition, the value of K_i was calculated to measure the affinity of bimatoprost at the free enzyme. This was determined using the slope of the Lineweaver-Burk plots for oestrone alone, oestrone plus 1000nM bimatoprost and oestrone plus 5000nM bimatoprost. The values of the slope were plotted against the concentration of bimatoprost. The K_i value was 2884nM (see Fig 4.2.1e).

The value of αK_i was calculated to measure the affinity of bimatoprost at the enzyme-substrate complex. This was calculated using the reciprocal of Vmax (1/Vmax) for oestrone only, oestrone plus 1000nM bimatoprost and oestrone plus 5000nM bimatoprost. These values were plotted against the concentration of bimatoprost. The αK_i value was 732nM (see Fig 4.2.1f) (Copeland, 2000).

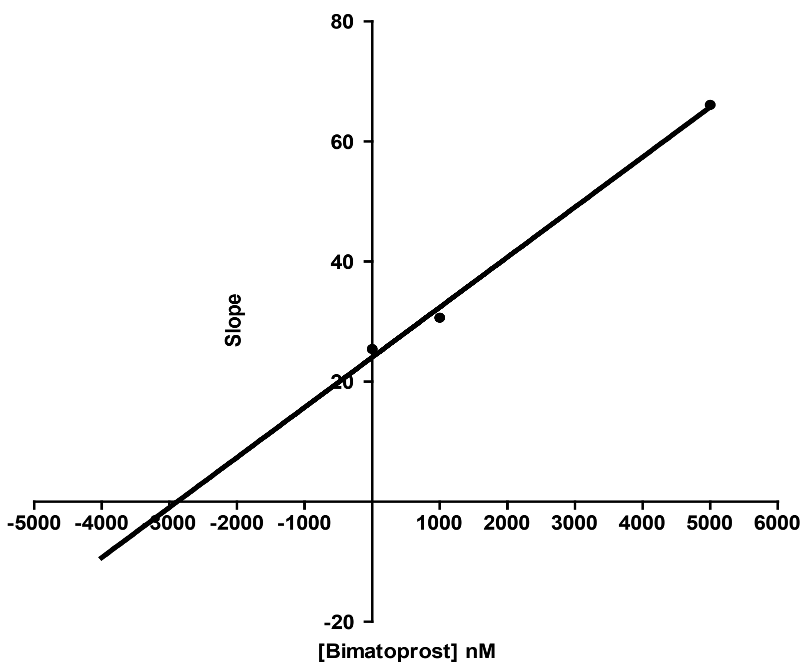


Fig 4.2.1e: The slopes of curves determined by the Lineweaver-Burk plot were plotted against bimatoprost concentration. The x-intercept represents the value of (-Ki). Hence, Ki = 2884 nM

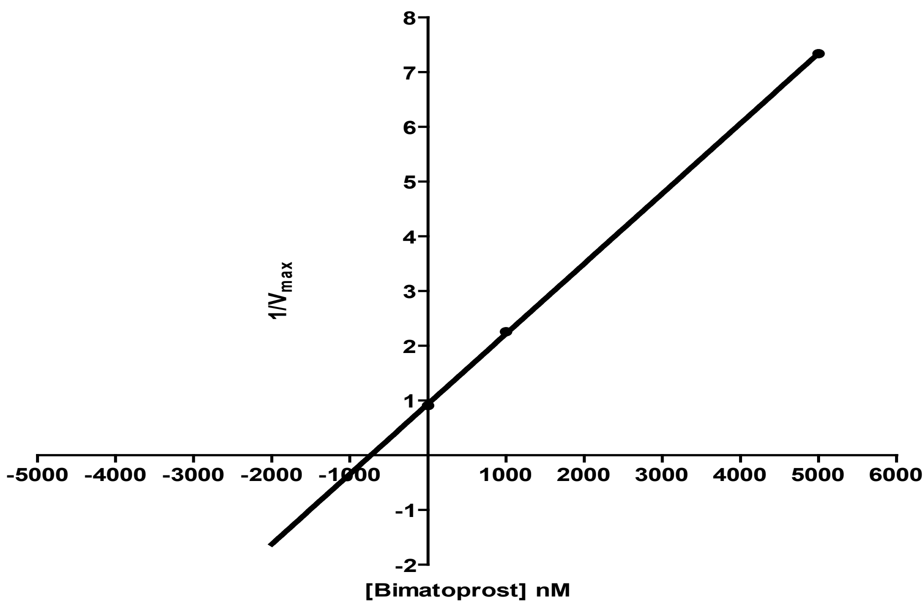


Fig 4.2.1f: The reciprocal of Vmax values were plotted against bimatoprost concentration. The x-intercept represents the value of (-αKi), i.e. uncompetitive inhibitor constant. αKi = 732nM

The results have confirmed the inhibitory effect of bimatoprost, as previously reported by another research group (Koda et al., 2004). The type of inhibition by bimatoprost appeared to be mixed for oestrone as determined by the inhibitor constants (K_i and αK_i). Since K_i = 2884nM and αKi = 732nM, the value of α = 0.25, indicating that bimatoprost favours the uncompetitive inhibition over competitive inhibition. This means that bimatoprost favours binding to oestrone-AKR1C3 complex over the free AKR1C3.

4.2.2. Patient data

Sample ID	Stage of cycle	Age	BMI	Pathology	Contraception	Medication	Parity
HP17	Irregular	29	26	Mild endometriosis, pelvic pain, backache	Mirena	Codeine, thyroxine, citalopram, lansoprazole	2
HP18	Follicular	27	24	Bartholin's cyst	None	None	0
HP21	Menses	48	31.9	Fibroids	None	Iron, hyoscine, analgesics	3
HP 31	Follicular	38	24.7	Endometriosis and Pelvic pain	None	None	3
HP32	Follicular	27	27.8	Endometriosis and Pelvic pain	None	None	1
HP37	Menses	28	N/A	Fertility problems	None	Creon, azithromycin, vitamins	0
HP39	Luteal	35	N/A	Dysmenorrhoea, menorrhagia, infertility	None	None	0
HP40	Follicular	31	24.8	Endometriosis, dysmenorrhoea, menorrhagia	None	Pregabalin, oxycontin, amitriptyline, hyoscine	3

Table 4.2.2: The patient information used in this chapter

4.2.3. The effect of bimatoprost on the production of 17β-oestradiol in eutopic endometrial cells

The concentration and length of treatment were optimised to ensure that the conditions were sensitive enough to detect changes when bimatoprost and oestrone were added.

Endometrial cells at a cell density of $0.5x10^5$ to $1x10^5$ cells/ml were plated on a 12-well plate and incubated for up to 2-4 days - depending on cell density - until reaching up to 85% confluence. The cells were incubated at 37°C at 5%CO_2 in the basal medium containing DMEM/F12-GLUTAMAX-I medium, supplemented with 10% *v/v* FBS, 1% *v/v* NEAA and 1% *v/v* pen-strep.

The basal medium was aspirated and the cells were washed twice with DPBS before adding any treatments to the cells. Treatment with oestrone (E1) at concentrations ranging from 1pM to 1μM were prepared in serum free and phenol red free DMEM/F12 media and incubated for 12, 24 and 48 hours. The conditioned media was collected and stored at -80°C for analysis. 17β-oestradiol was measured in the conditioned media using a Cayman ELISA kit (Fig 4.2.3a).

The viability of endometrial cells was assessed using MTT assay for 0.01μM oestrone and 1μM bimatoprost, alone and combined. Treatment with 1μM bimatoprost for 48 hours had significantly enhanced cell viability ($P<0.05$) (Fig 4.2.3b).

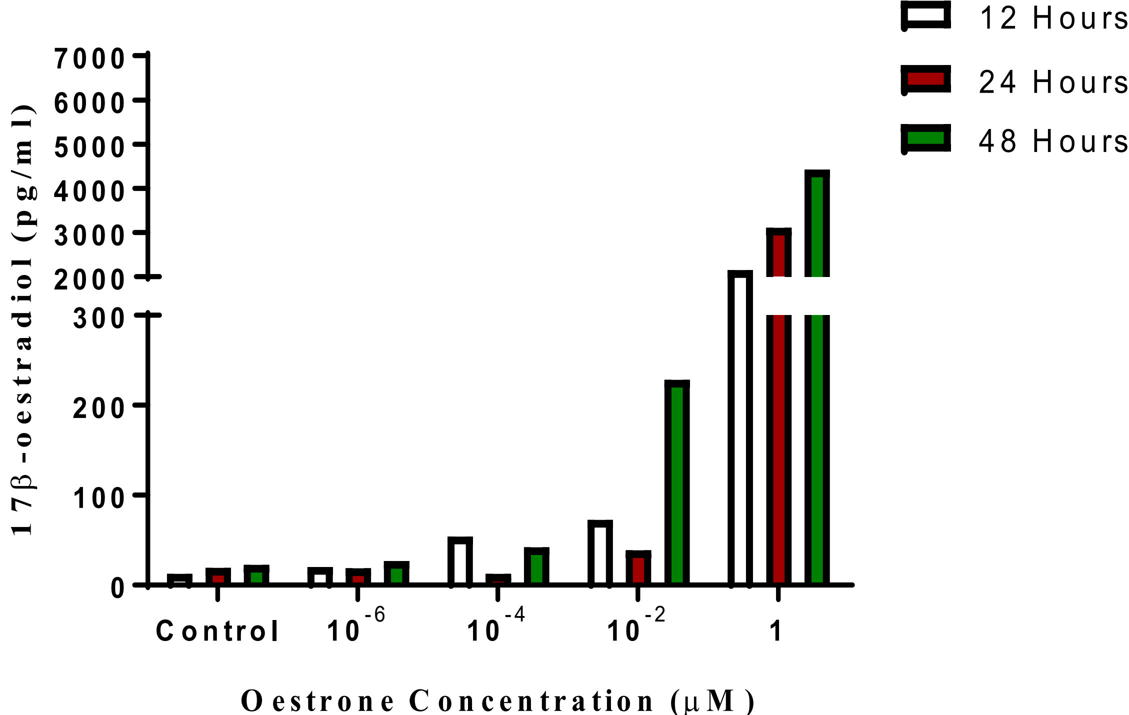


Fig 4.2.3a: Eutopic endometrial cells treated with oestrone (E1) from 1pM (10^{-6}μM) to 1μM for 12, 24 and 48 hours. Conditioned media was used to measure 17β-oestradiol by ELISA. The concentration 10^{-2}μM (0.01μM) and 48 hour time point were chosen as they are more sensitive to changes. Data are expressed as means (n=2).

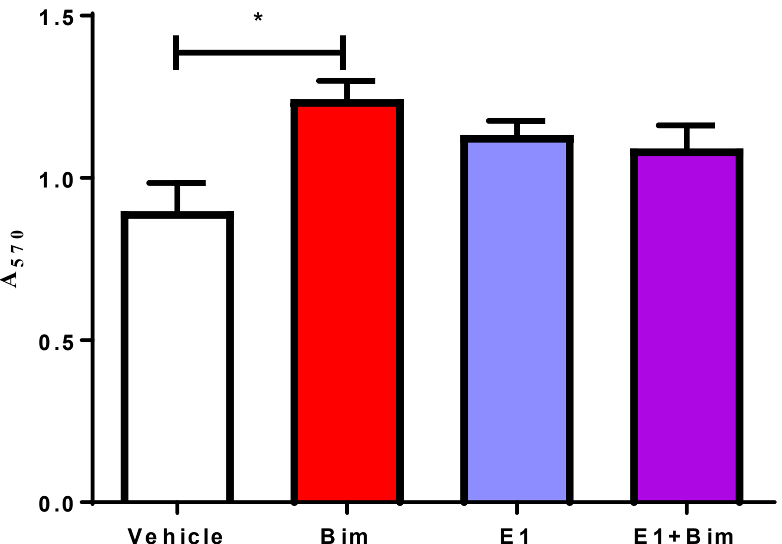


Fig 4.2.3b: Eutopic endometrial cells were treated with vehicle, 1μM bimatoprost (Bim), 0.01μM oestrone (E1) and bimatoprost at 1μM with 0.01μM oestrone for 48 hours. Following the treatment, MTT was added to the cells for 3 hours at 37°C. MTT was removed and DMSO was added to solubilise formazan crystals. Data are expressed as means±SEM (n=3). Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post-hoc* test $*P<0.05$

Eutopic endometrial cells were treated with 0.01µM oestrone, 1µM bimatoprost, and concomitantly treated with 0.01µM oestrone and 1µM bimatoprost for 48 hours.

The results showed that bimatoprost alone did not reduce the production of 17β-oestradiol, due to the low basal oestrone concentration. Oestrone addition appeared to have increased the production of 17β-oestradiol. A significant reduction in the production of 17β-oestradiol was shown when bimatoprost was co-treated with oestrone (p=0.04), as a result of AKR1C3 inhibition in the eutopic endometrial cells, as determined by ELISA (Fig 4.2.3c).

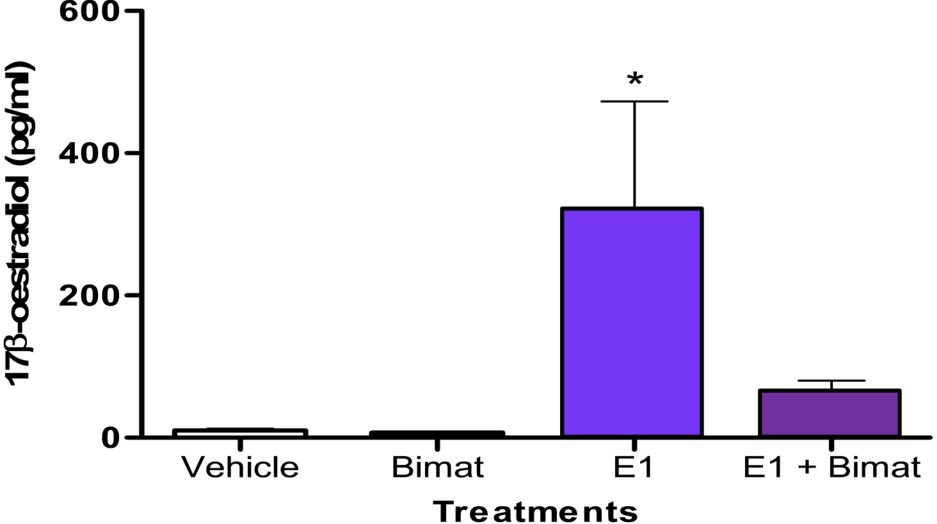


Fig 4.2.3c: Eutopic endometrial cells treated with vehicle, 1µM bimatoprost (bimat), 0.01µM oestrone (E1), as well as co-treatment of 0.01µM E1 with 1µM bimatoprost (E1+Bimat) for 48 hours. Conditioned media was used to measure 17β-oestradiol by ELISA. Data are expressed as means±SEM (n=4). **P<0.05* E1 compared to E1+Bimat.

4.2.4. The effect of bimatoprost on the production of 9α, 11β PGF_2 in eutopic endometrial cells

The cells were washed twice with DPBS before adding any treatments to the cells. Treatment with PGD_2 at concentrations ranging from 0.01μM to 10μM were prepared in serum free and phenol red free DMEM/F12 media and incubated for 3 hours to ensure the experiment was conducted within the linear region when bimatoprost was tested (Fig 4.2.4a). Thereafter, the endometrial cells were treated for 3, 6, 12 and 24 hours with 1μM PGD_2 to determine optimum time point. 9α, 11β PGF_2 was measured in the conditioned media using a Cayman ELISA kit (Fig 4.2.4b).

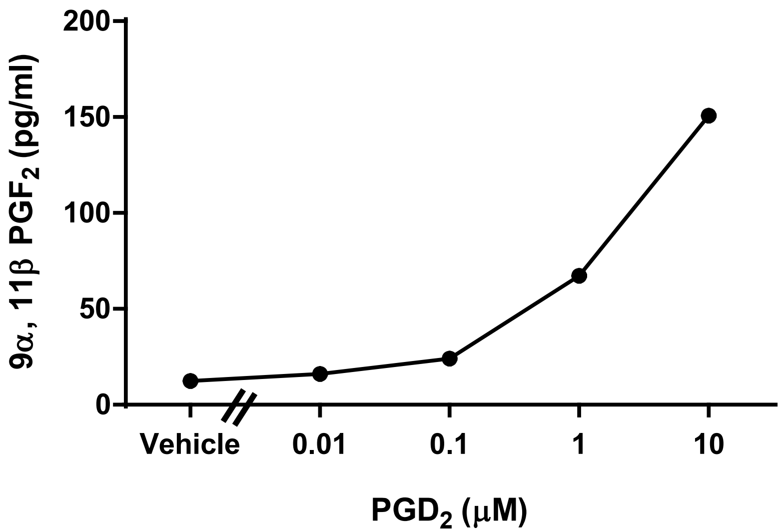


Fig 4.2.4a: Eutopic endometrial cells treated with PGD_2 at different concentrations from 0.01μM to 10μM for 3 hours. Conditioned media was used to measure 9α,11β PGF_2 by ELISA. The concentration (1μM) was used as it was more sensitive to changes. Data are expressed as means (n=2)

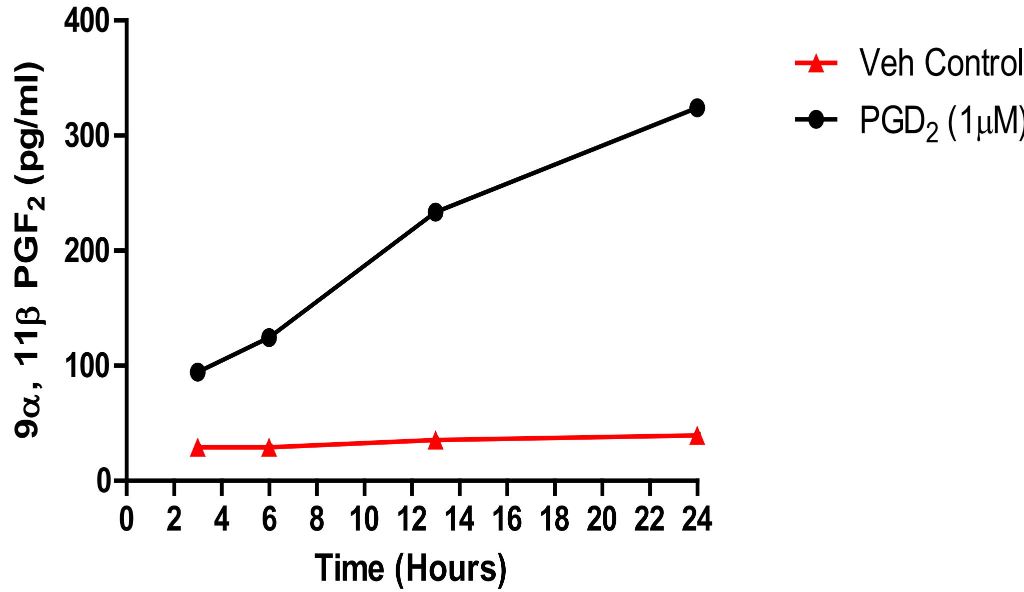


Fig 4.2.4b: Eutopic endometrial cells treated with PGD_2 (1 μM) at different time points: 3, 6, 13 & 24 hours. Conditioned media was used to measure 9α, 11β PGF_2 by ELISA. The 6-hour time point was used as it was more sensitive to changes. Data are expressed as means (n=3).

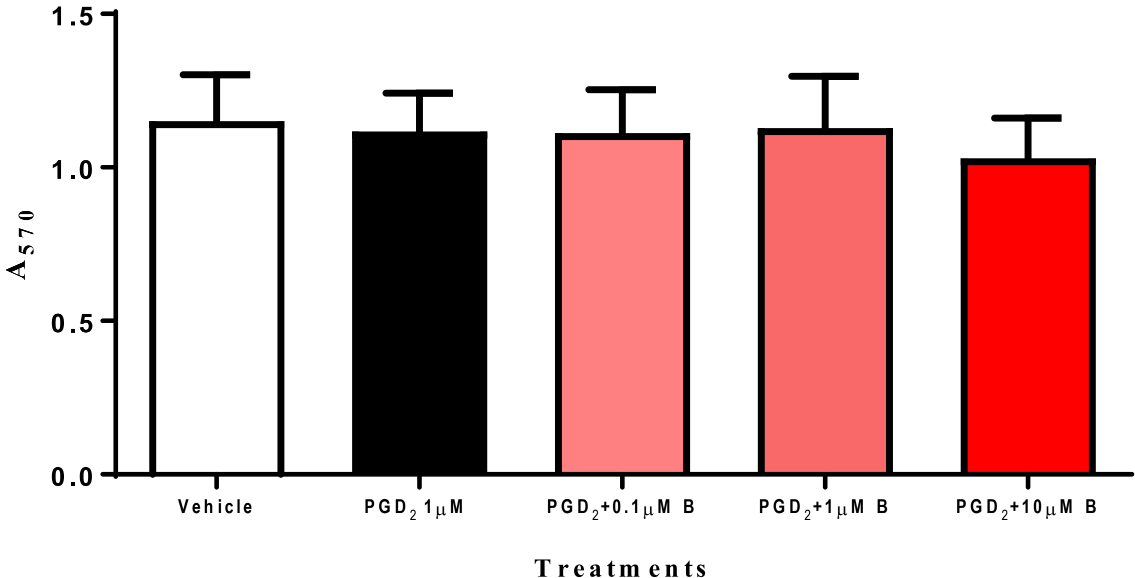


Fig 4.2.4c: Eutopic endometrial cells obtained treated with vehicle and 1μM PGD_2, as well as concomitant treatment of 1μM PGD_2 with 0.1μM, 1μM and 10 μM bimatoprost for 6 hours. Following the treatment, cells were incubated with MTT (0.5mg/ml) for 3 hours at 37°C. MTT was removed and DMSO was added to solubilise formazan crystals. Data are expressed as means±SEM (n=6).

The viability of endometrial cells was assessed using MTT assay when treated with 1μM PGD_2 alone, as well as concomitant treatment with increasing concentrations of bimatoprost from 0.1μM to 10μM for 6 hours (Fig 4.2.4c).

The results showed that PGD_2 had increased the production of 9α,11β PGF_2, but this production was reduced in a dose-dependent manner when the cells were co-treated with bimatoprost (Fig 4.2.4d).

A negative control for 1μM PGD_2 was tested to discern whether the increase in 9α, 11β PGF_2 was induced via AKR1C3 or it was as a result of cross-reactivity with the ELISA kit. The cross-reactivity with PGD_2 did not exceed 3%, which was negligible, when compared to endometrial cells treated with PGD_2. This confirmed that PGD_2 was predominantly produced via AKR1C3 catalysis.

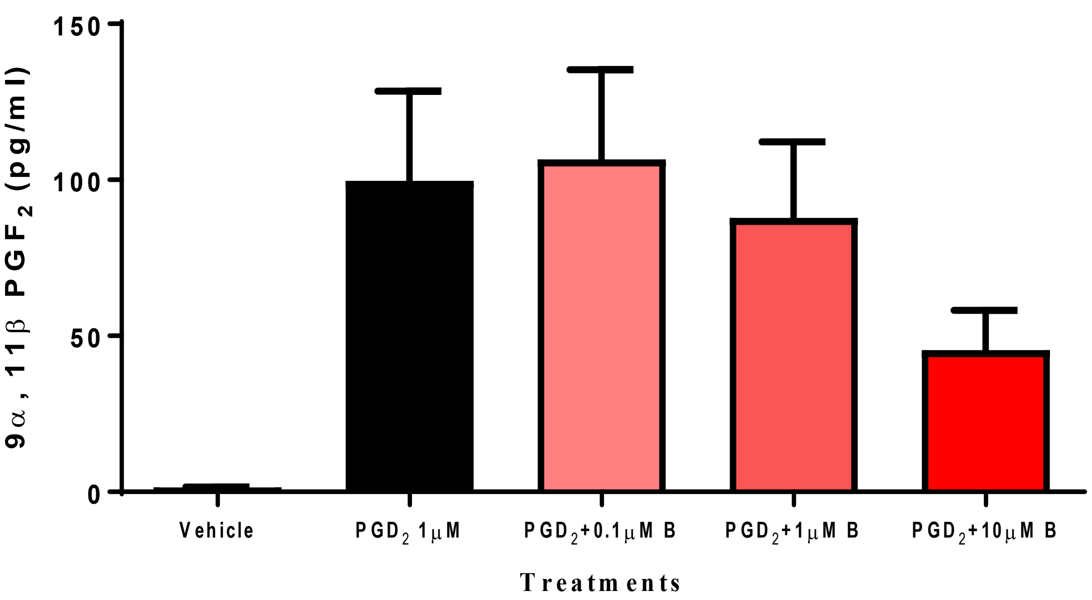


Fig 4.2.4d: Eutopic endometrial cells obtained treated with vehicle (ethanol) and 1μM PGD_2, as well as concomitant treatment of 1μM PGD_2 with 0.1μM, 1μM and 10 μM bimatoprost for 6 hours. Conditioned media was used for measuring 9α 11β PGF_2 by ELISA. Data are expressed as means±SEM (n=6).

4.2.5. The role of bimatoprost and $PGF_{2\alpha}$ on the gene expression of AKR1C3

The actions of bimatoprost and $PGF_{2\alpha}$ had been examined in eutopic endometrial cells to discern whether $PGF_{2\alpha}$ (which is one of AKR1C3 products) and bimatoprost have any regulatory effect on the gene expression of AKR1C3.

The results showed that neither bimatoprost nor $PGF_{2\alpha}$ had any regulatory effect on the gene expression of AKR1C3 enzyme in eutopic endometrial cells ($p = 0.1136$).

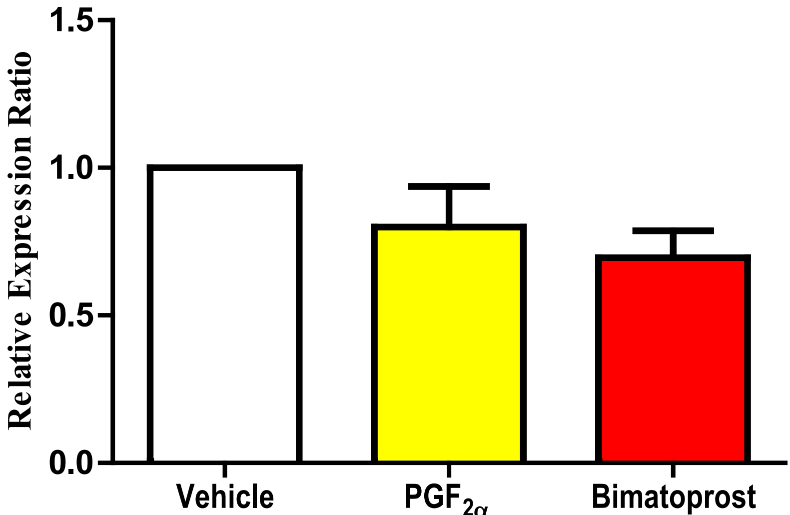


Fig 4.2.5: The relative gene expression of AKR1C3 in eutopic endometrial cells treated with $PGF_{2\alpha}$ and bimatoprost (both at 1µM) for 48 hours. RNA was isolated using the TRIzol method. Total RNA (1µg) was converted into cDNA, followed by using 1µl of cDNA (50ng) for quantitative real-time PCR (qRT-PCR). Relative gene expression was normalised against β-actin as a housekeeping gene. Data are expressed as means±SEM (n=5).

4.3. Discussion

Oestrone had a K_M value of 28.02µM, which was higher by 3 fold when compared to the findings reported by Byrns et al. (2010) which reported a K_M value of 9µM. Collectively, these K_M values indicate that oestrone is not the preferred substrate for AKR1C3, as they are too large compared with other substrates (Table 1.3.1). This suggests that AKR1C3 might not be the optimal pathway for inhibiting the production of 17β-oestradiol.

It is worth noting that this is the first time bimatoprost was investigated as an inhibitor of the AKR1C3 substrate, oestrone, using recombinant AKR1C3 and human eutopic endometrial cells. Bimatoprost was found to be a mixed inhibitor as it inhibited the free AKR1C3 and the AKR1C3-oestrone complex, resulting in the inhibition of oestrone conversion to 17β-oestradiol.

This was consistent with previous findings by Koda et al. (2004) who found that bimatoprost inhibited the production of PGD_2 and PGH_2 to their respective products by AKR1C3. The inhibition was reported as non-competitive inhibition for both substrates, indicating that bimatoprost also binds the free AKR1C3 and AKR1C3-substrate complex.

Bimatoprost inhibition of 17β-oestradiol production in human endometrial cells would result in the repression of endometriosis progression, as a result of reduction in mitogenesis, angiogenesis and inflammation in the endometrial lesions which are normally mediated via 17β-oestradiol (McLaren et al., 1996; Akoum et al., 2001). However, this result would have to be interpreted with caution due to the high cross reactivity with oestrone (>70-80%) when 17β-oestradiol ELISA kit was used. Also,

the eutopic endometrial cells had to be stimulated with oestrone, as the basal 17β-oestradiol levels were often below the detection range.

Although these data were from eutopic endometrial tissues, from patients with and without endometriosis, a reduction in 17β-oestradiol production would give an insight into the beneficial effect of such reduction in endometriotic lesions. This is due to an imbalance of 17β-oestradiol:oestrone ratio in endometriotic lesions as a result of significant changes in gene expression of local 17β-HSDs. For example, the gene expression of 17β-hydroxysteroid dehydrogenase type 2, which is the enzyme responsible for the oxidation of 17β-oestradiol to oestrone, was found to be significantly lower in endometriotic lesion. On the other hand, the gene expression of 17β-HSDs responsible for the reduction of oestrone to 17β-oestradiol was found to be elevated in endometriotic lesions (Huhtinen et al., 2012). Therefore, inhibition of AKR1C3 would contribute to restoring this balance. This was also reinforced by the upregulation of AKR1C3 gene expression in the endometriotic tissues, compared to eutopic endometrial tissues.

Furthermore, not only AKR1C3 is involved in oestrogen biosynthesis, it also metabolises progesterone to 20α-hydroxyprogesterone and testosterone to dihydrotestosterone. As for progesterone, AKR1C3 contributes to the enhanced resistance to progesterone. As reported by Hevir et al. (2011), there is enhanced progesterone metabolism, together with downregulation of PRB, resulting in the loss of protective actions of progesterone through downregulation of 17β-HSD type 2 which is responsible for metabolising 17β-oestradiol.

Presumably, bimatoprost also inhibits the metabolism of progesterone, which would result in the elevation of progesterone levels. This would rebalance the ratio of progesterone:20α-hydroxyprogesterone and ultimately restore the protective role of progesterone via induction of 17β-HSD type 2 (Attia et al., 2000).

Bimatoprost inhibited the production of 9α, 11β PGF_2 in a dose-dependent manner. This was consistent with other findings by Dozier et al. (2008) who have also found that bimatoprost inhibited 9α,11βPGF_2 production in the human luteinising granulosa cells.

The IC_{50} of PGD_2 inhibition was 5µM as determined by Koda et al. (2004), whereas bimatoprost approximately reduced the production of 9α, 11β PGF_2 by half at 10µM in human endometrial cells. This indicates that the action of bimatoprost was affected in cell culture.

9α,11β PGF_2, a stereoisomer of $PGF_{2\alpha}$, is the primary metabolite of PGD_2, which is biologically active, and has very similar biological functions to $PGF_{2\alpha}$ (Liston and Roberts, 1985). Therefore, it is clear that 9α, 11βPGF_2 acts on the prostanoid FP receptor, and thus, have the same role in endometriosis as $PGF_{2\alpha}$. It is worth noting that actual concentration of 9α,11βPGF_2 present in the endometrium has not been determined yet due to the difficulty in separating the two stereoisomers.

Bimatoprost did not have any regulatory effect on the gene expression of AKR1C3 (Fig 4.2.5). This indicates that bimatoprost only has an inhibitory effect on the active site of the enzyme without having a negative feedback loop on the gene expression.

$PGF_{2\alpha}$-FP receptor interaction involves a mitogenic effect through cross communication with epidermal growth factor receptor (EGFR), which switches on the mitogen activated protein kinase (MAPK) signalling pathway. Although this was investigated in Ishikawa cells and endometrial adenocarcinoma explants, $PGF_{2\alpha}$ might have a similar proliferative role in endometriosis (Sales, Milne, et al., 2004). Also, the FP prostanoid receptor antagonist, AL8810, caused a decline in the number of endometriotic lesions as well as the size of lesions in a mouse model of endometriosis (Ahmad et al., 2015).

Bimatoprost, at 1µM concentration, had a mitogenic effect on endometrial cells after 48 hour treatment (Fig 4.2.2b). This effect was diminished when oestrone was concomitantly added. This mitogenic effect was not expected to occur in the endometrium, but it was potentially mediated through a different pathway. Bimatoprost upregulated the gene expression levels of connective tissue growth factor (CTGF) and cysteine-rich angiogenic protein 61 (Cyr61) in the cat iris, whereas it upregulated only Cyr1 gene expression in the human ciliary smooth muscle cells (Liang et al., 2003). These key proteins are involved in the proliferation and angiogenesis, and could explain the enhanced proliferation in endometrial cells as a result of bimatoprost treatment.

One limitation with the use of bimatoprost as an inhibitor of AKR1C3 in tissues capable of *de novo* synthesis of oestrogen, such as endometriotic lesions, would be the increase of local testosterone concentrations which is a substrate of aromatase, leading to the aromatisation of testosterone back to 17β-oestradiol via aromatase. However, this possible increase in local 17β-oestradiol would be counteracted because of the presence of 5α reductase type 1 and type 2 in both, eutopic and

ectopic endometrial tissues, which have greater affinity to testosterone and consequently convert the excess testosterone to dihydrotestosterone (Carneiro et al., 2008).

Enzyme	Substrate	Product	K_M (μM)	Reference
5α reductase type 1	Testosterone	dihydrotestosterone	10	(Aggarwal et al., 2010)
5α reductase type 2	Testosterone	dihydrotestosterone	0.0545	(Wang et al., 2010)
Aromatase	Testosterone	17β-oestradiol	0.21	(Kellis and Vickery, 1987)

Table 4.3.1: The Michaelis-Menten constants of testosterone by 5α reductase type 1 and 2, and aromatase.

In conclusion, the rationale for targeting AKR1C3 enzyme in endometriosis was to decrease the local concentrations of 17β-oestradiol, $PGF_{2\alpha}$ and 9α 11β PGF_2, which contribute to dysmenorrhoea, proliferation and inflammation. In addition, the restoration of local concentrations of progesterone which would have a synergistic effect, by further decreasing the levels of 17β-oestradiol and antagonising the angiogenic and proliferative effects 17β-oestradiol in the ectopic and eutopic endometrial (Moyer and Felix, 1998).

5. The interaction between prostaglandin E_2, Prostaglandin $F_{2\alpha}$ and 17β-oestradiol in the human endometrial cells

5.1. Introduction

5.1.1. Background

The endometrium is responsive to sex hormones, such as 17β-oesdtradiol and progesterone, as well as prostaglandins, mainly PGE_2 and $PGF_{2\alpha}$. Although they are separate entities, they interact and influence each other in the endometrium (Smith et al., 1984). In endometriotic lesions, there is aberrant expression of enzymes responsible for the biosynthesis of 17β-oestradiol, PGE_2 and $PGF_{2\alpha}$, including their respective receptors (Fig 3.2.1a & 3.2.4a) (Huhtinen et al., 2012; Rakhila et al., 2013). Thus, the dysregulation of these enzymes and receptors in endometriotic lesions would ultimately lead to altered levels of 17β-oestradiol, PGE_2 and $PGF_{2\alpha}$.

AKR1C1 is responsible for the conversion of progesterone to 20α-hydroxyprogesterone, as well as PGE_2 to $PGF_{2\alpha}$ interconversion. AKR1C3 is responsible for the conversion of oestrone to 17β-oestradiol, progesterone to 20α-hydroxyprogesterone, PGD_2 to 9α,11β PGF_2 and PGH_2 to $PGF_{2\alpha}$. AKR1C1 was upregulated in endometriotic lesions (Fig 3.2.1a).

This upregulation suggests that PGE_2 might be one of the main candidates for enhancing the gene expression of AKR1C1. This was due to the fact that PGE_2 can induce the expression of SF-1 and aromatase in endometriotic lesions, creating a pro-oestrogenic micro-environment with enhanced progesterone metabolism (Attar et al., 2009). In addition, the inducible mPGES-1 is strongly expressed in endometriotic lesions, compared to eutopic endometrium (Chishima et al., 2007). Similarly, it is imperative to investigate which EP receptor subtype(s) are more involved in creating

this pro-oestrogenic environment in endometriotic lesions. This would allow the development of much more selective pharmacological agents which target the most implicated EP receptor subtypes.

Since $PGF_{2\alpha}$ is one of the most abundant prostaglandins in the uterus and a major product of AKR1C3, it could be linked with enhancing AKR1C1 and AKR1C3 gene expression in endometriotic lesions, as higher levels of $PGF_{2\alpha}$ are found in endometriotic lesions (Khan et al., 2013).

On the other hand, ectopic endometrial cells exhibit similar traits to tumour cells, such as migration, invasion, angiogenesis and immune suppression. Therefore, ectopic cells might undergo a similar metabolic change similar to that of tumour cells, such as a shift in cell metabolism from mitochondrial oxidative phosphorylation to glycolysis. Since endometriotic lesions are responsive to oestrogen, it would be interesting to investigate whether a shift in cell metabolism could be altered when ectopic endometrial cells were stimulated with oestrone and 17β-oestradiol.

Therefore, the aim of this chapter was to investigate whether PGE_2 or $PGF_{2\alpha}$ could induce a positive feedback loop in the eutopic and ectopic endometrial cells, and thereby regulating the expression of AKR1C1 and AKR1C3 in the cells. Moreover, selective EP receptor agonists were used to determine which EP receptor subtype(s) is predominant in inducing 17β-oestradiol synthesis in ectopic endometriotic lesions. Finally, glycolytic activities and mitochondrial respiration were assessed in eutopic and ectopic endometrial cells to investigate any shift in cell metabolism at basal level and after oestrone and 17β-oestradiol stimulation.

5.1.2. Patient data

Sample ID	Stage of cycle	Age	BMI	Pathology	Contraception	Medication	Parity
HP10	Follicular	40	27	Mild endometriosis	None	None	2
HP12	Follicular	36	24	Ovarian endometriosis	None	None	3
HP13	Follicular	48	28	Fibroids	Sterilisation	None	3
HP14	Irregular	25	25	High grade cervical intra-epithelial neoplasia	Copper IUD	None	0
HP15	Luteal	56	35	Fibroids, menorrhagia	None	Gliclazide, metformin, enalapril, pravastatin, aspirin, amlodipine	
HP16	Follicular	29	25	Cervical intra-epithelial neoplasia	None	None	0
HP17	Irregular	29	26	Mild endometriosis, pelvic pain, backache	Mirena	Codeine, thyroxine, citalopram, lansoprazole	2
HP18	Follicular	27	24	Bartholin's cyst	None	None	0
HP19	Luteal	23	24	Pelvic pain	None	None	0
HP24	Luteal	36	19.1	Stage IV endometriosis	None	Qvar, salbutamol	0
HP 31	Follicular	38	24.7	Endometriosis and Pelvic pain	None	None	3
HP32	Follicular	27	27.8	Endometriosis and Pelvic pain	None	None	1
HP39	Luteal	35	N/A	Dysmenorrhoea, menorrhagia, infertility	None	None	0
HP40	Follicular	31	24.8	Endometriosis, dysmenorrhoea, menorrhagia	None	Pregabalin, oxycontin, amitriptyline, hyoscine	3
HP42	Luteal	43	30.9	BRCA mutation carrier	None	Analgesia, amitriptyline, steroid injections	N/A
HP45	Luteal	20	28.9	Endometriosis (left round ligament), dysmenorrhoea, menorrhagia	None	None	0
HP47	Follicular	45	24.8	Endometriosis	None	None	3
HP50	Amenorrhoea	50	22	Endometriosis (uterosacral), dysmenorrhoea, menorrhagia	None	Oxycontin, gabapentin, HRT, diuretics, senna, Zoladex	3

Table 5.1.1: The patient information for the human endometrial samples assayed in this chapter

5.2. Results

5.2.1. The effect of PGE_2 and $PGF_{2\alpha}$ on the local release of PGE_2, $PGF_{2\alpha}$ and 17β-oestradiol from human eutopic endometrial explants

Eutopic endometrial explants (10-15mg) were placed into 0.4µm well inserts in duplicates in 24-well plate in DMEM/ F12 Glutamax-I media (Gibco, USA), supplemented with 10% v/v foetal calf serum (Gibco, USA) and 1% v/v penicillin-streptomycin (Sigma, Germany) and incubated for 24 hours at 37°C, 5% CO_2 incubator (Chapter 2: 2.2 Explant culture). Treatments with vehicle (0.01%v/v ethanol), 1µM PGE_2 (see Fig 5.2.2a for optimisation) and 1µM $PGF_{2\alpha}$ in DMEM/F12 phenol red free media, supplemented with 1% v/v charcoal stripped foetal calf serum, were added. The treatments were replaced every 24 hours over 48 hours (Dudley et al., 1992).

The media collected on the second day (48 hour time point was the optimum time period for observing differences between different treatments) and were analysed using ELISA technique for measuring the concentrations of PGE_2, 17β-oestradiol and $PGF_{2\alpha}$ released by endometrial tissues. The concentrations were normalised to the weight of tissue. This experiment had taken almost 12 months to complete.

	Average %B/B_0	Concentration (pg/ml)	[a]Corrected Concentration (pg/ml)	[b]Corrected Concentration (pg/ml)	[c]Concentration (pg/mg)
PGE_2 (with tissue)	25.1	372.6	745170.2	150162.2	10010.8
PGE_2 (without tissue)	29.6	297.5	595008	0	0

[a]= The concentration was multiplied by 2000 to account for dilution.

[b]= The concentration of PGE_2 with tissue was subtracted from the concentration of PGE_2 without tissue.

[c]= The concentration of PGE_2 was normalised using the weight of endometrial tissue (15mg)

Table 5.2.1: An example of the method used to calculate the net PGE_2 release by endometrial explants

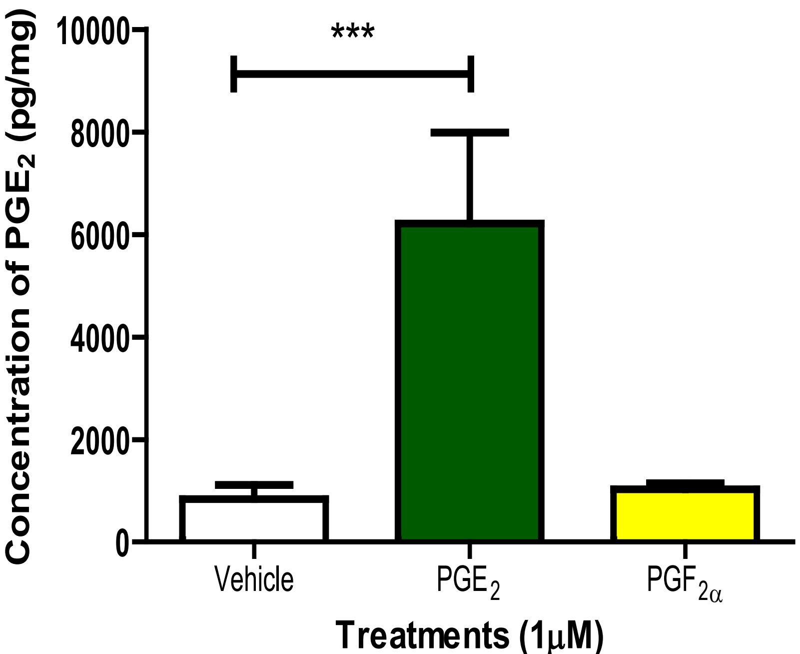


Fig 5.2.1a: PGE_2 released by eutopic endometrial explants treated with vehicle, 1μM PGE_2 and 1μM $PGF_{2\alpha}$ for 48 hours. Conditioned media at the 48 hour point was used to measure PGE_2 using ELISA. The data are expressed as means ± SEM (n=6). Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post hoc* test; *** $P < 0.001$

Note: The net PGE_2 released by endometrial tissues was calculated by subtracting from 1μM PGE_2 (negative control) added to an adjacent well without endometrial tissues.

	Average %B/B_0	Concentration (pg/ml)	[a]Corrected Concentration (pg/ml)	[b]Corrected Concentration (pg/ml)	[c]Concentration (pg/mg)
$PGF_{2\alpha}$ with tissue	23.9	3834.3	383427	-37901.2	-2526.7
$PGF_{2\alpha}$ without tissue	22.9	4213.3	421328.2	0	0

[a]= The concentration was multiplied by 100 to account for dilution.

[b]= The concentration of $PGF_{2\alpha}$ with tissue was subtracted from the concentration of $PGF_{2\alpha}$ without tissue.

[c]= The concentration of $PGF_{2\alpha}$ was normalised using the weight of endometrial tissue (15mg)

Table 5.2.2: An example of the method used to calculate the net $PGF_{2\alpha}$ release by endometrial explants

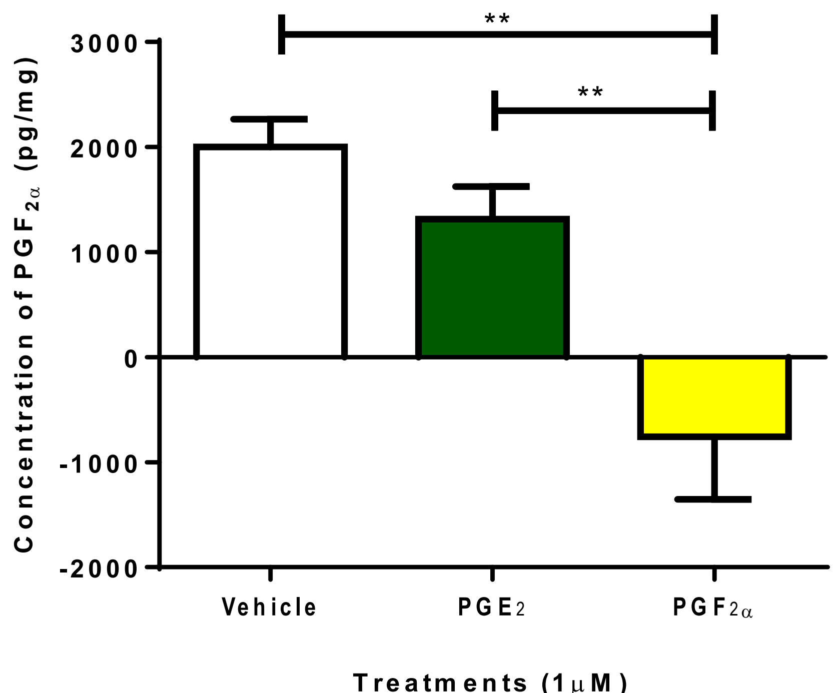


Fig 5.2.1b: $PGF_{2\alpha}$ released by eutopic endometrial explants treated with vehicle, 1μM PGE_2 and 1μM $PGF_{2\alpha}$ for 48 hours. Conditioned media at the 48 hour point was used to measure $PGF_{2\alpha}$ using ELISA. The data are expressed as means ± SEM (n=5). Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post hoc* test; **$P<0.01$

Note: The net $PGF_{2\alpha}$ released by endometrial tissue was calculated by subtracting from 1μM $PGF_{2\alpha}$ (negative control) added to an adjacent well without endometrial tissues.

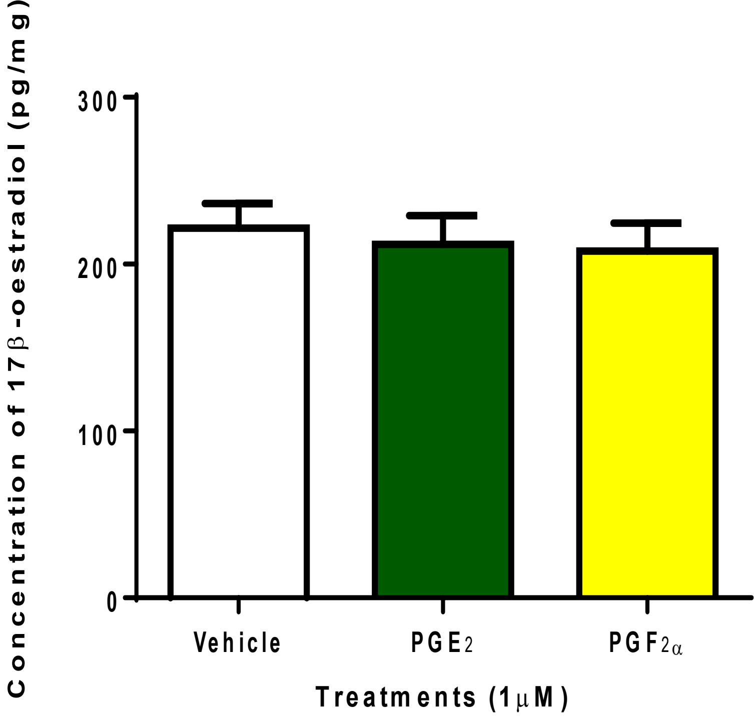


Fig 5.2.1c: 17β-oestradiol released by eutopic endometrial explants treated with vehicle, 1μM PGE_2 and 1μM $PGF_{2\alpha}$ for 48 hours. Conditioned media at the 48 hour point was used to measure 17β-oestradiol ELISA. The data are expressed as means ± SEM (n=6). Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post hoc* test.

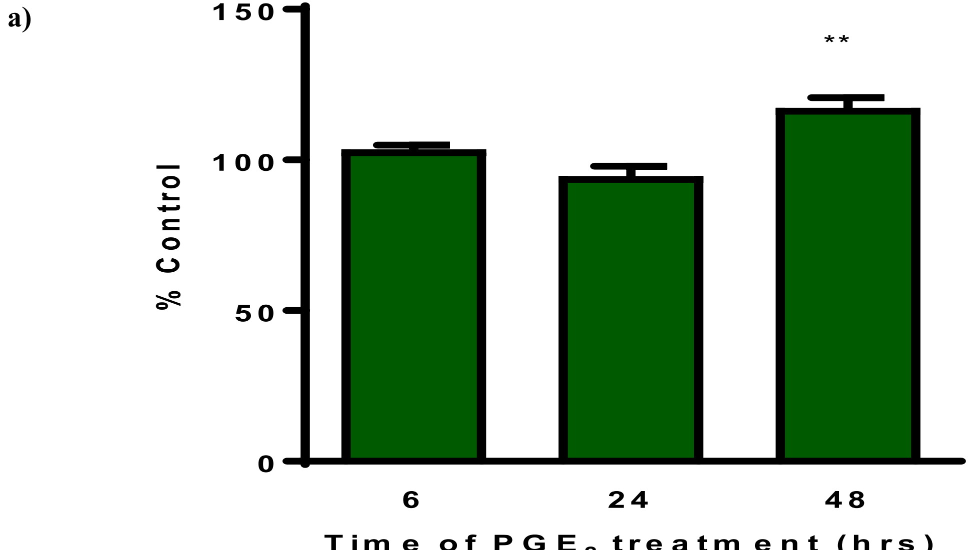
The treatment with PGE_2 resulted in an increased production of PGE_2 in the endometrial tissues (P=0.0003) (Fig 5.2.1a). However, this was not the case with $PGF_{2\alpha}$ treatment which resulted in a significant reverse of $PGF_{2\alpha}$ release (P=0.001) from endometrial tissues (Fig 5.2.1b). The level of 17β-oestradiol was not affected by PGE_2 or $PGF_{2\alpha}$ (Fig 5.2.1c).

5.2.2. The effects of PGE_2 and $PGF_{2\alpha}$ on the gene expression of AKR1C1 and AKR1C3

Due to limited number of tissues available, it was not possible to continue using endometrial explants. Instead, human endometrial cells were used to allow conducting more experiments from limited number of endometrial tissues.

In this experiment, human eutopic endometrial cells were cultured in 60mm culture dishes (Corning, USA) in DMEM/F12 media Glutamax-I (Gibco, USA), containing 10% v/v foetal calf serum (Sigma, UK), 1% v/v penicillin-streptomycin (Sigma, UK) and 1% NEAA (Sigma, Germany). The cells were incubated until they reached 80 – 90% confluence. When confluent, the cells were treated for 48 hours with control containing 0.01%v/v ethanol (vehicle), 1μM PGE_2 and 1μM $PGF_{2\alpha}$ in DMEM/F12 media without phenol red (Gibco, USA), containing 1%v/v charcoal stripped and heat inactivated foetal calf serum (Gibco, USA).

a)



b)

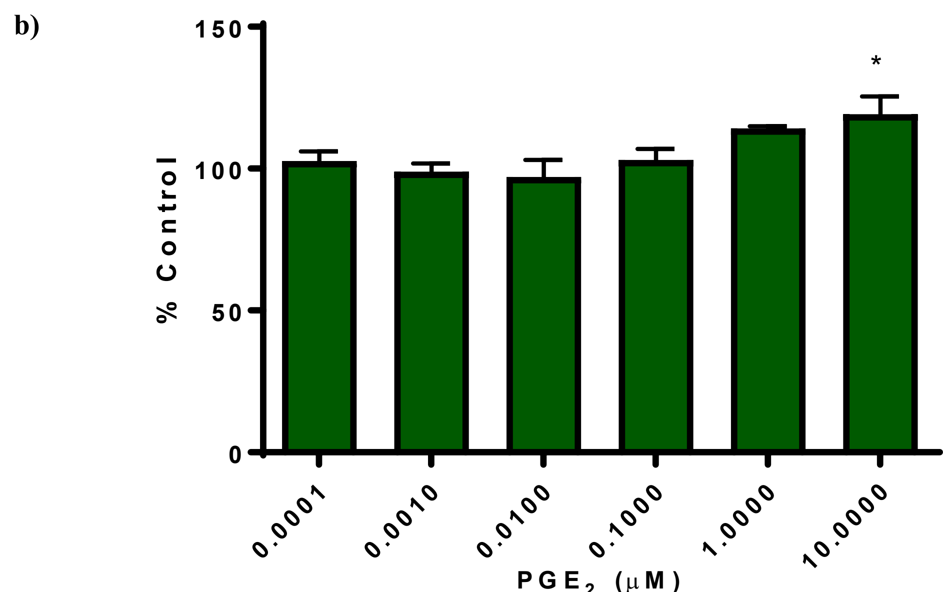
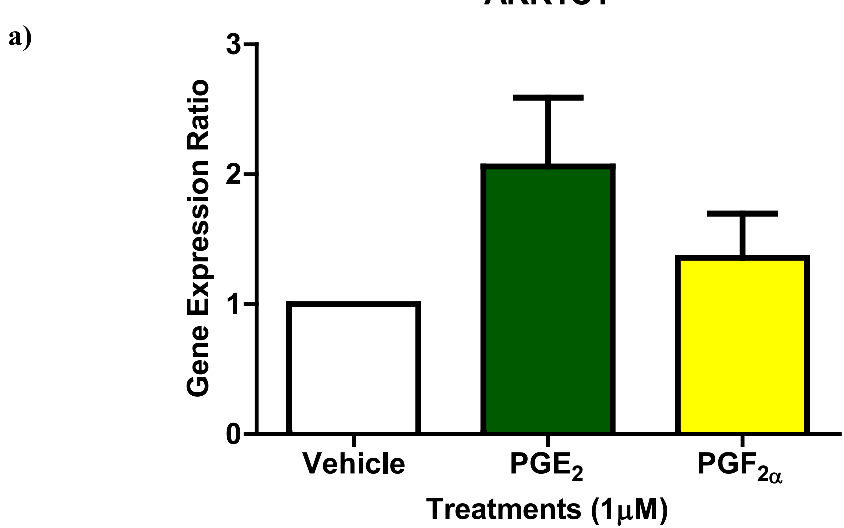


Fig 5.2.2a: Human eutopic endometrial cells treated with (a) 1µM PGE_2 for 6, 24 and 48 hours and (b) PGE_2 at a range of concentrations (0.0001µM to 1µM) for 48 hours. Conditioned media was aspirated and replaced with 0.5mg/ml MTT for 3 hours at 37°C. Formazan salts were solubilised in DMSO and the plate was read at 570nm using microplate reader. The data are expressed as means ± SEM (n=3). Statistical significance was calculated using one-way ANOVA, followed by Dunnett's *post hoc* test;*P<0.05 and **P<0.01 compared to control.

a)



b)

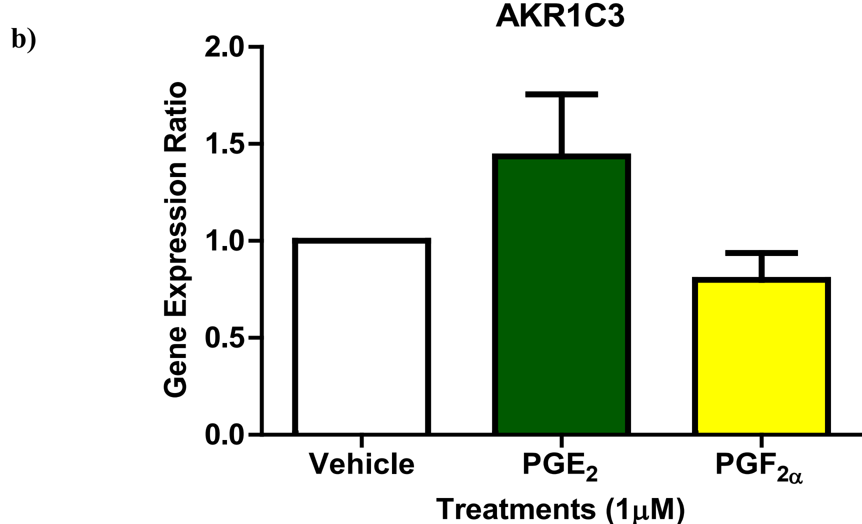


Fig 5.2.2b: Eutopic endometrial cells treated with vehicle (n=10), 1μM PGE_2 (n=10) and 1μM $PGF_{2\alpha}$ (n=5) for 48 hours. The RNA samples were extracted using TRIzol method. Total RNA (1μg) was converted to cDNA, followed by using 1μl of cDNA (50nmol) for qRT-PCR. Relative gene expression for (a) AKR1C1 and (b) AKR1C3 was calculated relative to β-actin. Data are expressed as means ± S.E.M and one-way ANOVA was performed for statistical significance.

From the results of MTT assay, 48 hour treatment was chosen at 1μM for PGE_2 (Fig 5.2.2a) and $PGF_{2\alpha}$. The treatment of human eutopic endometrial cells with 1μM PGE_2 and $PGF_{2\alpha}$ did not affect the gene expression of AKR1C1 and AKR1C3 (Fig 5.2.2b). However, PGE_2 upregulated AKR1C1 by 2-fold and AKR1C3 by 1.4-fold, and therefore, selective EP receptor agonists were used to further delineate the role

of the different EP receptor subtypes in regulating the gene expression of AKR1C1 and AKR1C3, since signal transductions differ between the different EP receptor subtypes.

5.2.3. The effect of the selective EP receptor agonists on the gene expression of AKR1C1 and AKR1C3

Name of agonist	Colour	Binding affinity (nM)				Reference
		EP_1	EP_2	EP_3	EP_4	
PGE_2	(green)	9.1	4.9	0.33	0.8	(Young et al., 2004)
ONO-D1-004	(orange)	150	>10000	>10000	>10000	(Suzawa et al., 2000)
Butaprost	(purple)	-	110	-	-	(Kiriyama et al., 1997)
Sulprostone	(pink)	107	>100000	0.35	7740	(Abramovitz et al., 2000)
L-902688	(brown)	>12000	3200	2800	0.38	(Young et al., 2004)

Table 5.2.3: The binding affinities of PGE_2 and EP receptor agonists to EP receptor subtypes (1-4).

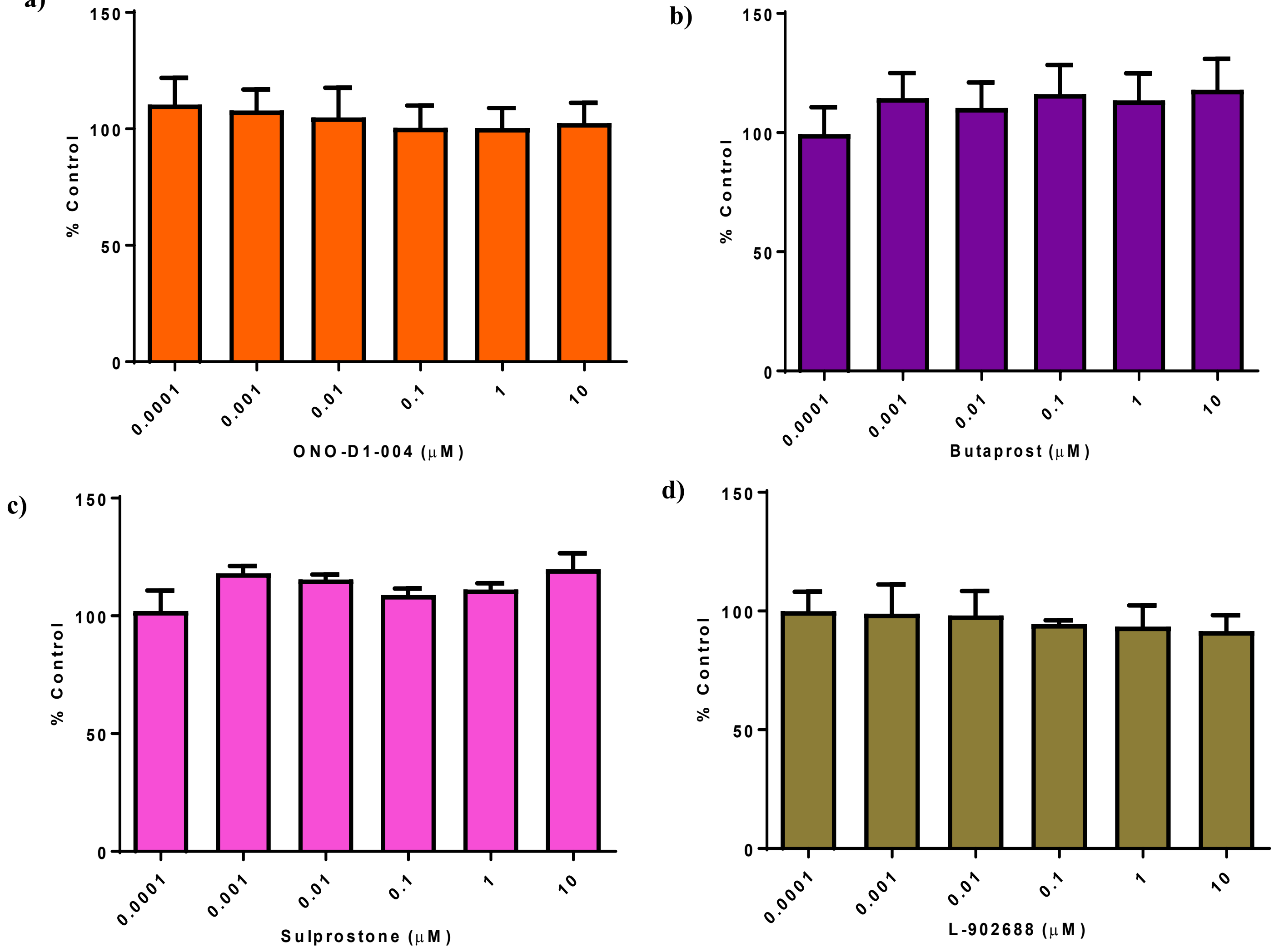


Fig 5.2.3a: Human eutopic endometrial cells treated with (a) ONO-D1-004 (■), (b) butaprost (■), (c) sulprostone (■) and (d) L-902688 (■) at a range of concentrations (0.0001μM to 10μM) for 48 hours. Conditioned media was aspirated and replaced with 0.5mg/ml MTT for 3 hours at 37°C. Formazan salts were solubilised in DMSO and the plate was read at 570nm using microplate reader.The data are expressed as means ± SEM (n=3). Statistical significance was calculated using one-way ANOVA.

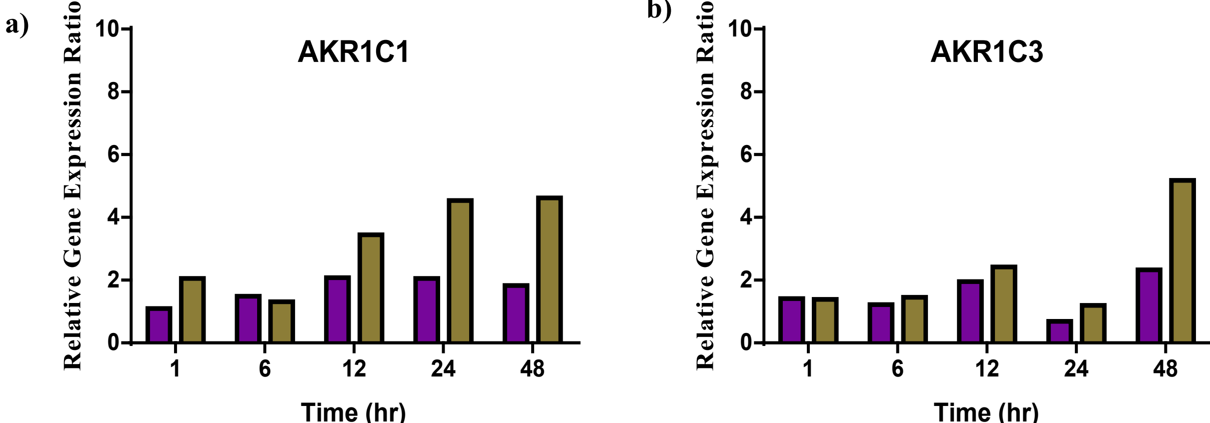
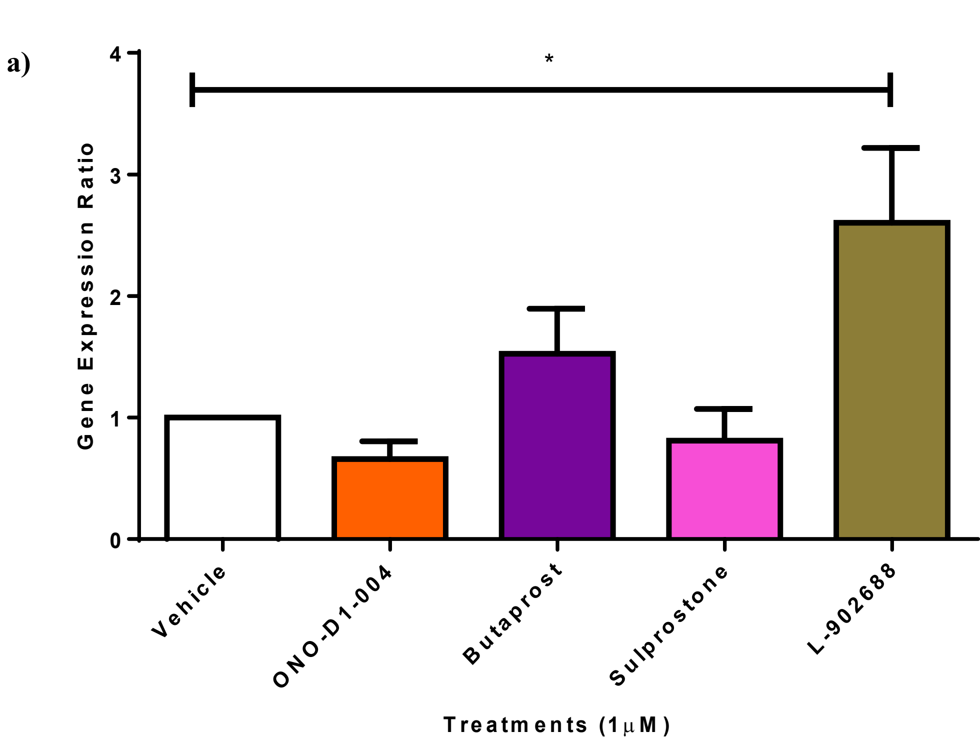


Fig 5.2.3b: Eutopic endometrial cells treated with 1μM butaprost (■) and 1μM L-902688 (■) for 1, 6, 12, 24 and 48 hours. The RNA samples were extracted using TRIzol method. Total RNA (1μg) was converted to cDNA, followed by using 1μl of cDNA (50nmol) for qRT-PCR. Relative gene expression for (a) AKR1C1 and (b) AKR1C3 was calculated relative to β-actin. Data are expressed as means (n=2).

The selective EP receptor agonists were assessed for cytotoxicity using MTT assay at different concentrations (Fig 5.2.3a). The upregulation of gene expression of AKR1C1 and AKR1C3 was optimised at different time points using butaprost and L-902688 (Fig 5.2.3b). Hence, the treatment for 48 hours was the best time point for investigating the different EP agonists and at a concentration of 1μM.

L-902688, which is selective for the EP_4 receptor, significantly upregulated the gene expression of AKR1C1 ($P=0.04$) and AKR1C3 ($P=0.003$), whereas other selective EP receptor agonists did not have any statistically significant regulatory effect on AKR1C1 and AKR1C3 gene expression (Fig 5.2.3c). This indicates that only EP_4 receptor activation is involved in upregulating AKR1C1 and AKR1C3 gene expression.

a)



b)

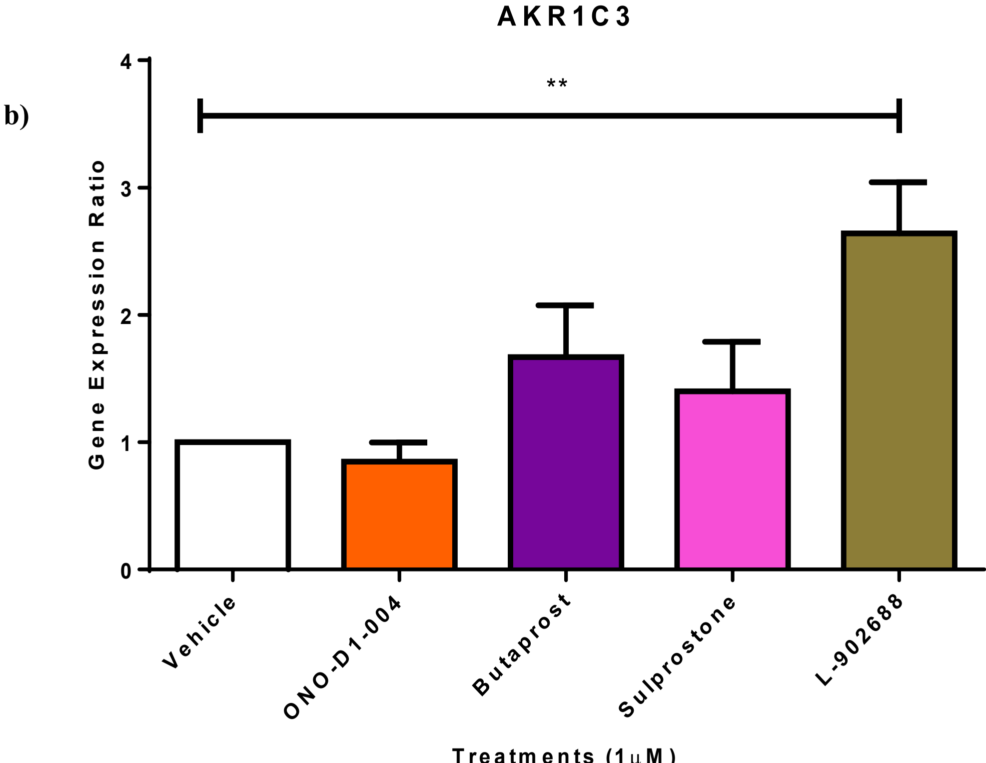


Fig 5.2.3c: Eutopic endometrial cells treated with vehicle, 1µM ONO-D1-004 (■) (n=4), 1µM butaprost (■) (n=10), 1µM sulprostone (■) (n=4) and 1µM L-902688 (■) (n=10) for 48 hours. The RNA samples were extracted using TRIzol method. Total RNA (1µg) was converted to cDNA, followed by using 1µl of cDNA (50nmol) for qRT-PCR. Relative gene expression for (a) AKR1C1 and (b) AKR1C3 was calculated relative to β-actin. Data are expressed as means ± SEM and one-way ANOVA was performed for statistical significance, followed by Dunnett's *post hoc* test. *P<0.05 and **P<0.01 compared to vehicle.

5.2.4. The effect of 48 hour treatment with PGE_2 and selective EP receptor agonists on the production of 17β-oestradiol in ectopic endometrial cells

Endometrial cells were cultured in 12-well plates in DMEM/F12 with Glutamax-I media, supplemented with 10% v/v foetal calf serum (Sigma, UK), 1% v/v penicillin-streptomycin (Sigma, UK) and 1%v/v NEAA (Sigma, Germany). The cells were incubated until they reached 80 – 90% confluence. When confluent, the cells were washed with DPBS (Sigma, Germany) and treated for 48 hours with vehicle, containing 0.01% v/v ethanol, PGE_2, ONOD1004, butaprost, sulprostone and L-902688, all at 1μM, in DMEM/F12 media without phenol red (Gibco, USA). Foetal calf serum was eliminated in order to avoid measuring any exogenous 17β-oestradiol found in the serum. However, 17β-oestradiol release by eutopic endometrial cells fell below the detection limit (6.6 pg/ml), and therefore, only ectopic endometrial cells were used in this experiment, as they were above the detection limit of the assay.

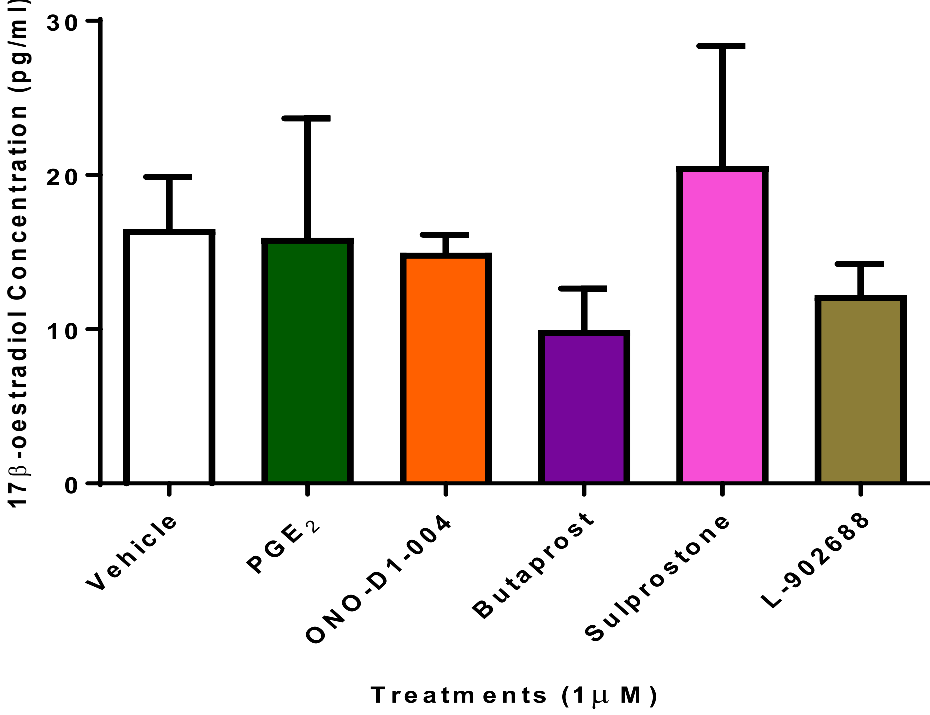


Fig 5.2.4: 17β-oestradiol release from ectopic endometrial cells obtained from endometriotic lesions of patients with endometriosis following the treatment with vehicle, 1μM PGE_2 (■), 1μM ONO-D1-004 (■), 1μM butaprost (■), 1μM sulprostone (■) and 1μM L-902688 (■) for 48 hours. Conditioned media were used to measure 17β-oestradiol using ELISA. The data are expressed as means ± SEM (n=4). One-way ANOVA was used for statistical significance.

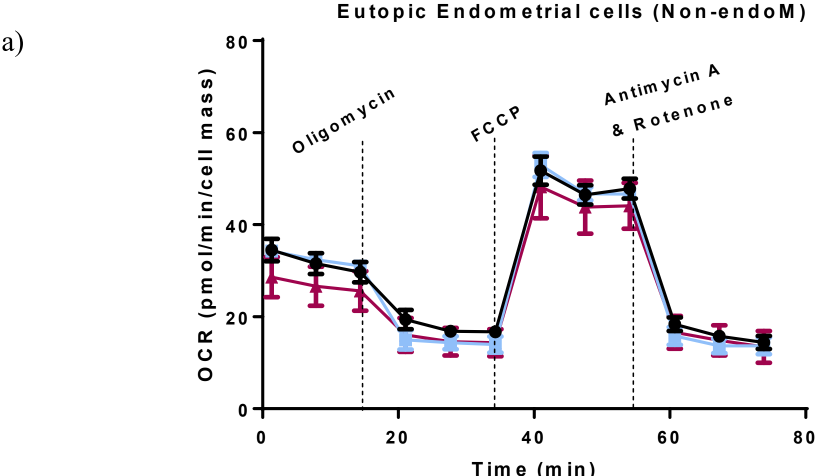
The release of 17β-oestradiol was not significantly different than vehicle when ectopic endometrial cells were treated with PGE_2 and EP receptor agonists (Fig 5.2.4). This indicates that activation of the EP receptors did not influence the release of 17β-oestradiol in ectopic endometrial cells.

5.2.5. The effect of oestrone and 17β-oestradiol on the mitochondrial respiration and glycolysis of the endometrial cells

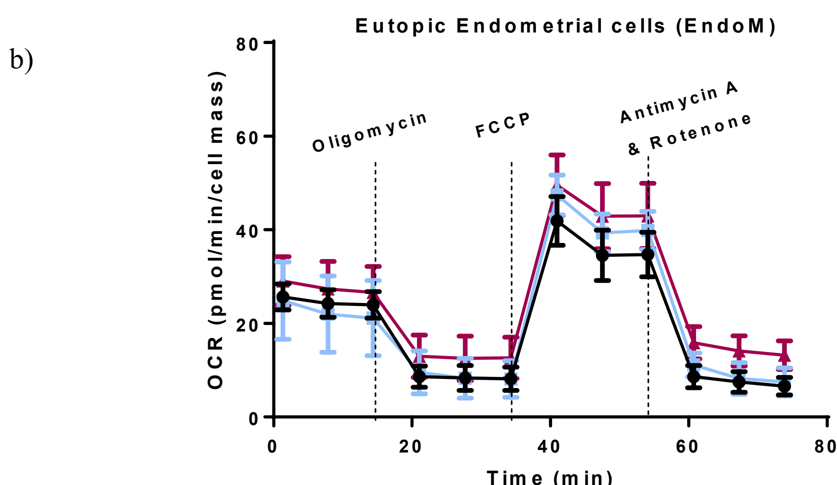
Endometrial cells (eutopic and ectopic) were cultured in 96-well plates at a density of 1.5x10^{5}cells/ml and incubated overnight at 37°C, 5% CO_2. A preliminary experiment was conducted by treating endometrial cells with DMEM/ F12 media only (control), 1nM 17β-oestradiol and 10nM oestrone for 24 hours, followed by monitoring mitochondrial respiration via oxygen consumption rate (OCR) and glycolysis via extracellular acidification rate (ECAR) using the Seahorse XF-96 Extracellular Flux Analyser (Agilent Technologies, USA) to investigate whether 17β-oestradiol and oestrone affect the metabolism of endometrial cells.

I. Mitochondrial respiration:

a)



b)



c)

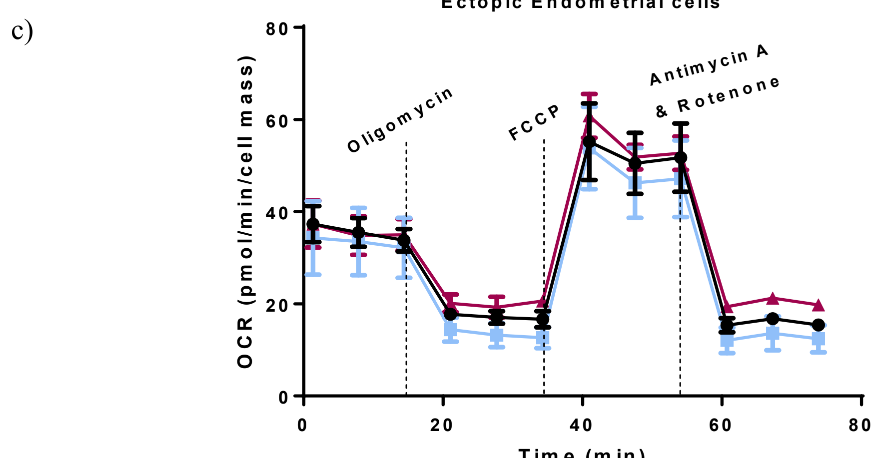
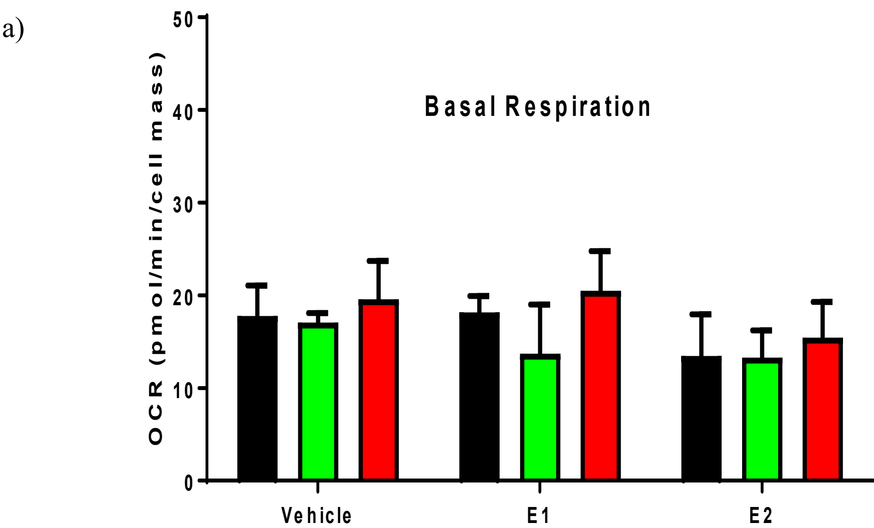
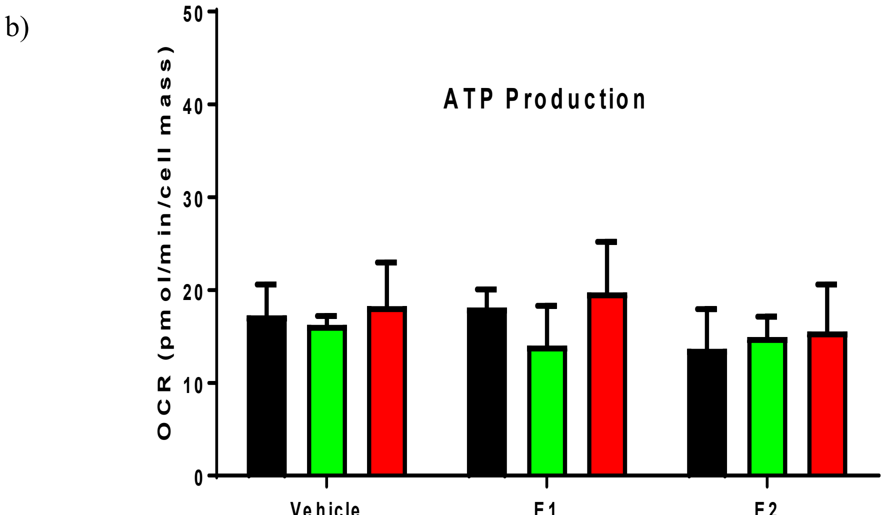


Fig 5.2.5a: Oxygen consumption rate (OCR) of (a) eutopic endometrial cells from female patients without endometriosis, (b) eutopic endometrial cells from patients with endometriosis and (c) ectopic endometrial cells from endometriotic lesions of patients with endometriosis treated with DMEM/F12 (Vehicle) (●), 10nM oestrone (■) and 1nM 17β-oestradiol (▲) for 24 hours. OCR was monitored using Seahorse XF-96 Extracellular Flux Analyser with the sequential injections of oligomycin (1μM), carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP) (0.25μM) and Antimycin A (0.5μM) & Rotenone (0.5μM) at the indicated time point into each well, after baseline rate measurement. The data are expressed as means ± SEM (n=3/group).

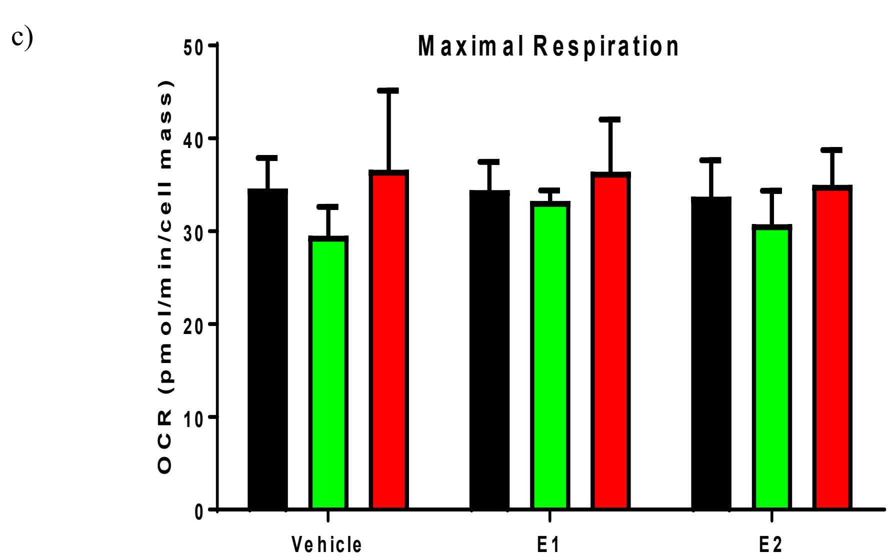
a)



b)



c)



d)

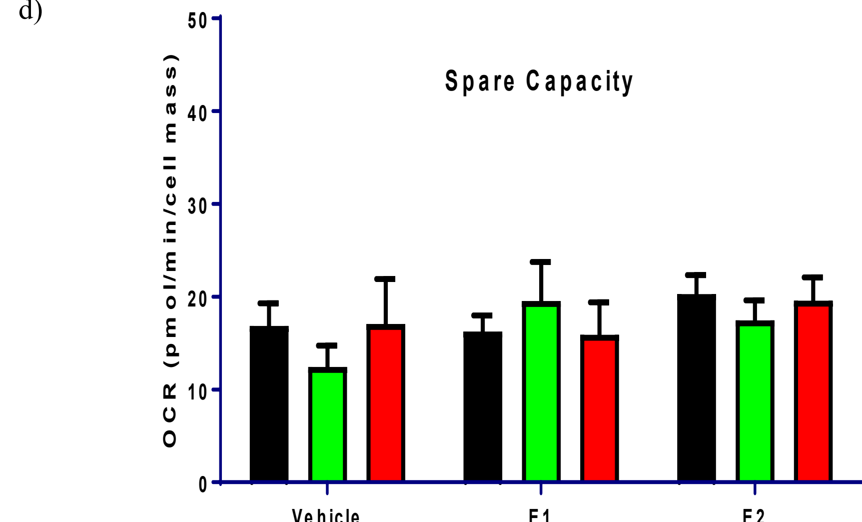
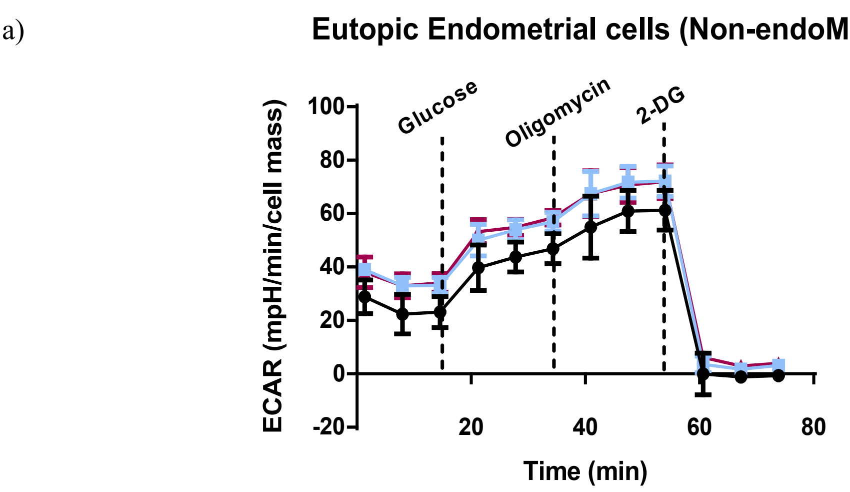


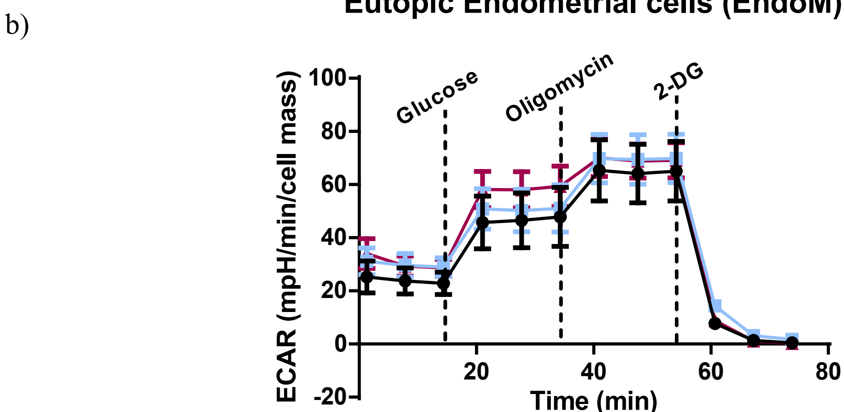
Fig 5.2.5b: Eutopic endometrial cells from female patients without endometriosis (■), eutopic endometrial cells from patients with endometriosis (■) and ectopic endometrial cells from endometriotic lesions of patients with endometriosis (■) were treated with DMEM/F12 (Vehicle), 10nM oestrone (E1) and 1nM 17β-oestradiol (E2) for 24 hours. Key parameters of mitochondrial functions: (a) basal respiration, (b) ATP production, (c) maximal respiration and (d) spare capacity were measured using Seahorse XF Extracellular Flux Analyser. The data are expressed as means ± SEM (n=3).

II. Glycolysis:

a)



b)



c)

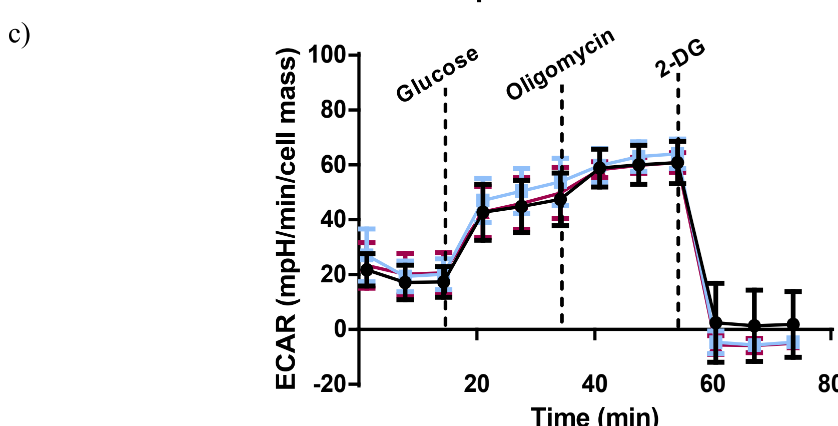
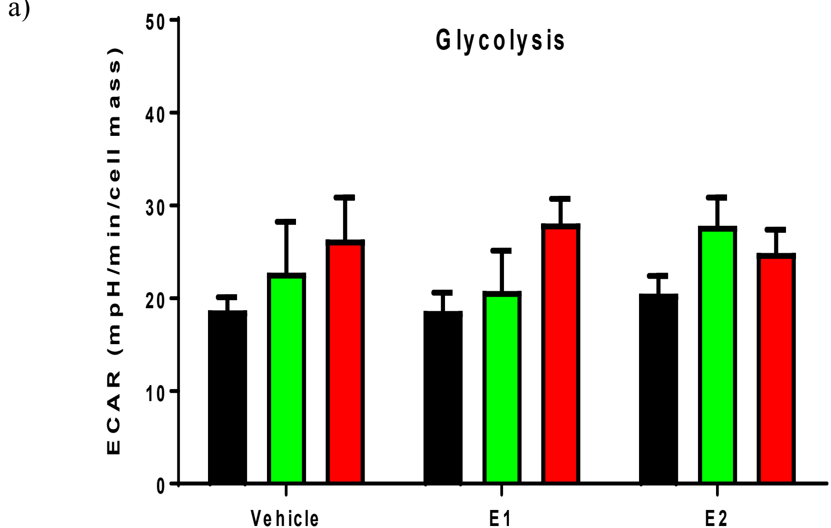
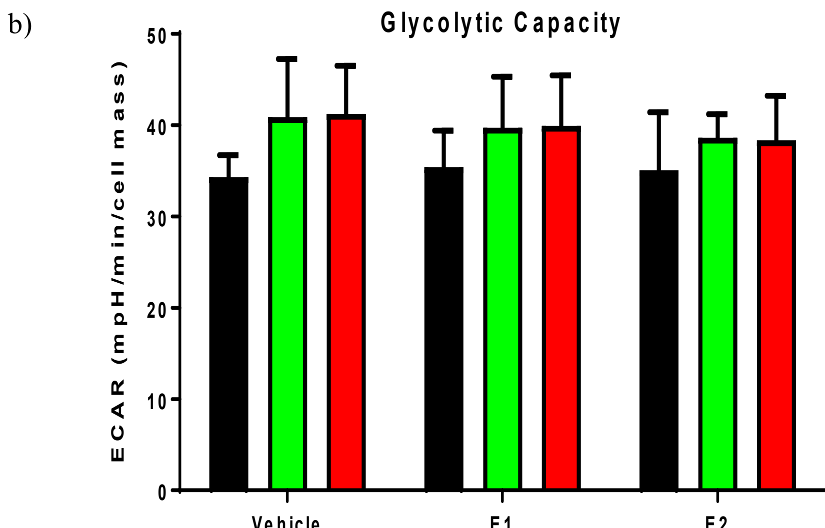


Fig 5.2.5c: Extracellular acidification rate (ECAR) of (a) eutopic endometrial cells from female patients without endometriosis, (b) eutopic endometrial cells from patients with endometriosis and (c) ectopic endometrial cells from endometriotic lesions of patients with endometriosis treated with DMEM/F12 (Vehicle) (●), 10nM oestrone (■) and 1nM 17β-oestradiol (▲) for 24 hours. ECAR was monitored using Seahorse XF-96 Extracellular Flux Analyser with the sequential injections of glucose (10mM), oligomycin (1µM) and 2-deoxyglucose (2-DG) (50mM) at the indicated time point into each well, after baseline rate measurement. The data are expressed as means ± SEM (n=3/group).

a)



b)



c)

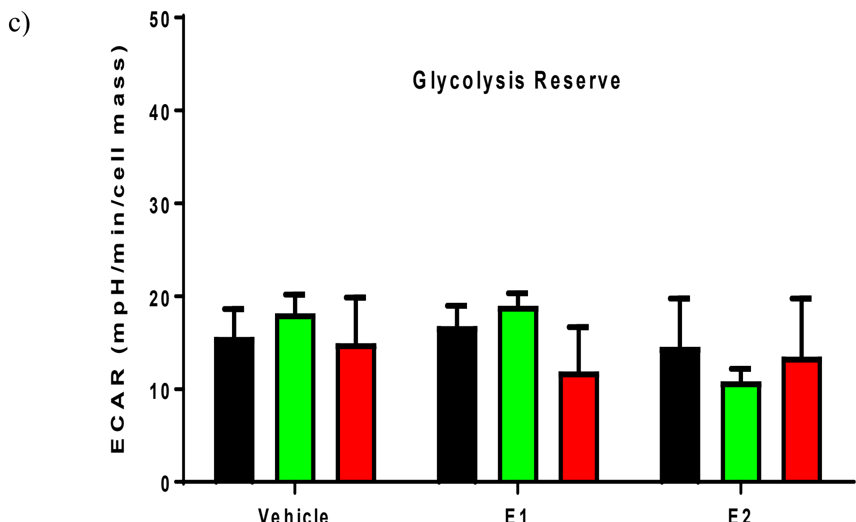


Fig 5.2.5d: Eutopic endometrial cells from female patients without endometriosis (■), eutopic endometrial cells from patients with endometriosis (■) and ectopic endometrial cells from endometriotic lesions of patients with endometriosis (■) were treated with DMEM/F12 (Vehicle), 10nM oestrone (E1) and 1nM 17β-oestradiol (E2) for 24 hours. Key parameters of glycolytic flux: (a) glycolysis, (b) glycolytic capacity and (c) glycolysis capacity were measured using Seahorse XF Extracellular Flux Analyser. The data are expressed as means ± SEM (n=3).

The OCR basal line of eutopic and ectopic endometrial cells did not alter, except eutopic endometrial cells from non-endometriosis patients which had lower basal line when treated with 17β-oestradiol (Fig 5.2.5a). On the other hand, the basal line of ECAR was higher for eutopic endometrial cells treated with oestrone and 17β-oestradiol from patients without endometriosis (Fig 5.2.5c).

Generally, the ectopic endometrial cells exhibited higher, yet not statistically significant, glycolysis flux and mitochondrial respiration compared to eutopic endometrial cells from patients with and without endometriosis (Fig 5.2.5b & 5.2.5d). Unfortunately, treatments with oestrone and 17β-oestradiol for 24 hours did not seem to alter mitochondrial respiration or glycolysis.

5.3. Discussion

The treatment of eutopic endometrial tissues with PGE_2 resulted in approximately 6-fold increase in the production of PGE_2 (P=0.0003) (Fig 5.2.1). This positive feedback loop underlines the importance of PGE_2 in a condition such as endometriosis. The mechanism by which over-production of PGE_2 occurs is due to different pathways, but mainly through the induction and up regulation of COX-2 which is the rate limiting enzyme for prostaglandin synthesis. Also, COX-2 is upregulated in eutopic endometrial tissues and endometriotic lesions of patients with endometriosis (Cho et al., 2010; Rakhila et al., 2015).

PGE_2 upregulated COX-2 in a dose dependent manner via Ras-MAPK cascade in HCA-7 cells (Wang et al., 2005). In cells of the macrophage lineage, PGE_2 upregulated the expression of NF-κB which has a positive effect on COX-2 (Camandola et al., 1996). *In vivo* studies showed that NF-κB, a ubiquitous transcription factor, can induce the expression of COX-2 directly (Lee et al., 2004). Alternatively, 17β-oestradiol is also a transcriptional factor which can induce the expression COX-2, causing ultimately the upregulation of PGE_2 (Pérez Martínez et al., 2006).

Moreover, there are other pro-inflammatory factors that can induce COX-2 and consequently activate the PGE_2-COX-2 positive feedback loop. For instance, macrophage migration inhibitory factor (MIF) resulted in induction of COX-2 and upregulation of PGE_2, potentially through ERK1/2-MAPK pathway (Carli et al., 2009).

Although these mechanisms have not all been investigated in endometriosis, it is plausible that they upregulate the inducible COX-2 in endometriosis, and ultimately, lead to the upregulation of PGE_2 release via the PGE_2-COX-2 positive feedback loop system.

Alternatively, $PGF_{2\alpha}$ appeared to have the opposite effect to that of PGE_2, since the amount of $PGF_{2\alpha}$ released by the endometrial tissues was significantly blocked ($P=0.001$) when treated with $PGF_{2\alpha}$. Instead, there was an increased intracellular uptake of $PGF_{2\alpha}$ (Fig 5.2.1b).

After subtracting the addition of exogenous $PGF_{2\alpha}$ (1µM) measured from wells without explants tissues (negative control), the values were negative. This represents the amount of $PGF_{2\alpha}$ internalised by the endometrial tissues (Bito and Baroody, 1974). This significant uptake of $PGF_{2\alpha}$ would ultimately lead to intracellular accumulation of $PGF_{2\alpha}$, followed by internal inactivation by 15-hydroxyprostaglandin dehydrogenase as a result of $PGF_{2\alpha}$ overload (Schuster, 1998).

Nonetheless, in-vitro data showed that FP receptor activation can inhibit the uptake of $PGF_{2\alpha}$ for metabolic inactivation, which contradicts *ex vivo* data shown above (Fig 5.2.1b). However, this could be ineffective, or inhibited, when the system is overloaded with $PGF_{2\alpha}$ for a prolonged time, causing desensitisation of FP receptor, and thereby enhancing internalisation of $PGF_{2\alpha}$. (Vezza et al., 2001).

$PGF_{2\alpha}$ seems to lack a positive feedback loop that keeps consistent levels of $PGF_{2\alpha}$ released from endometrial tissues. Even though PGE_2 and $PGF_{2\alpha}$ are internalised

and metabolised, PGE_2 production still outstrips its inactivation (Fig 5.3.1) which makes it a better target for treating endometriosis.

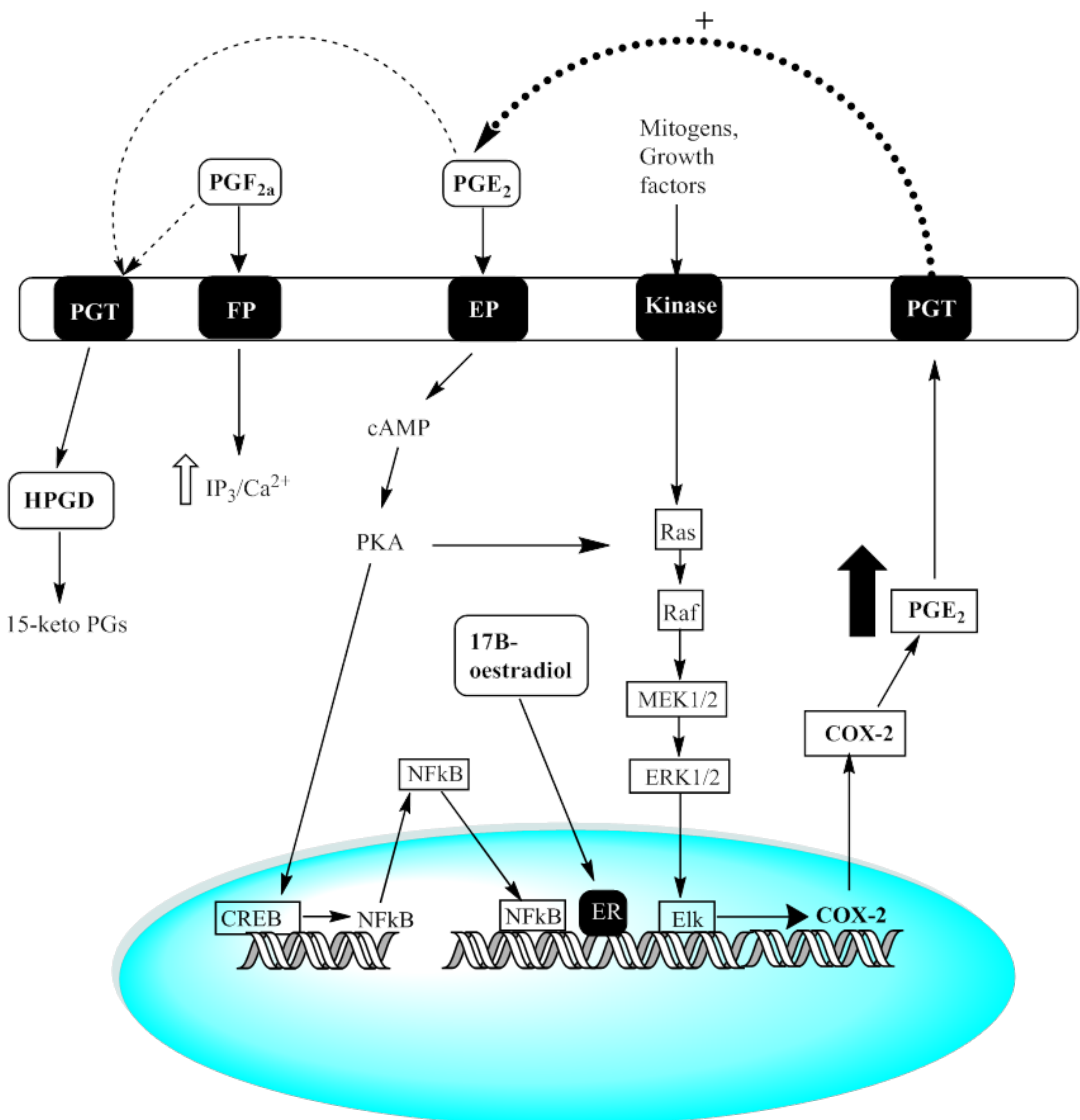


Fig 5.3.1: A diagram illustrating the different mechanisms by which PGE_2 is perpetually produced (positive feedback loop). This feedback loop does not occur with $PGF_{2\alpha}$ which is eventually internalised via PGT and metabolised by HPGD. Abbreviations: **PGT**: prostaglandin transporter; **HPGD**: 15-hydroxyprostaglandin dehydrogenase; **PKA**: protein kinase A; Ras, Raf and ERK: All belong to the mitogen-activated protein kinase (MAPK) pathway.

Neither PGE_2 nor $PGF_{2\alpha}$ treatments had any effect on the level of 17β-oestradiol released from eutopic endometrial tissues (Fig 5.2.1c). This is because eutopic endometrial tissues lack aromatase, which is the rate limiting enzyme for the biosynthesis of oestrone and 17-β-oestradiol (Zeitoun et al., 1999). However, it was expected that AKR1C3 would be upregulated by PGE_2, which in turn reduces basal oestrone to 17β-oestradiol. This did not occur, suggesting that PGE_2 did not have any potential effect on AKR1C3. It is worth noting that the samples used were at different stages of cycle which can affect the results due to changes that occur during the menstrual cycle. Also the samples had different gynaecological diseases that can have overlapping molecular characteristics to endometriosis. Therefore, due to limited sample numbers, it was not possible to stratify the data according to stage of cycle and disease state.

Treatments with PGE_2 and $PGF_{2\alpha}$ were used to investigate whether the gene expression of AKR1C1 and AKR1C3 would be modulated (Fig 5.2.2b). $PGF_{2\alpha}$ did not affect AKR1C1 and AKR1C3 gene expression. This might be because $PGF_{2\alpha}$ is a product of AKR1C1 and AKR1C3 and does not have any regulatory effect on their expression (Dozier et al., 2008). As for PGE_2, AKR1C1 was upregulated by 2-fold and AKR1C3 by 1.4-fold. This gave the impetus to investigate further into the EP receptor, using selective EP receptor agonists, to delineate the effect of each EP receptor subtype on the gene expression of AKR1C1 and AKR1C3 and identify which one(s) may have regulatory effect on these genes.

However, the use of different EP receptor agonists to target the four EP receptor subtypes added another level of complexity to this experiment, as agonists had varied binding affinities towards their respective EP receptor subtypes (Table 5.2.3).

For instance, ONO-D1-004 and butaprost are highly selective to EP_1 and EP_2 receptor subtypes, respectively, but their binding affinities are >100nM, which are not close to the binding affinities of PGE_2. On the other hand, sulprostone is highly selective for the EP_3 receptor subtype, but also activates the EP_1 receptor with a similar binding affinity to that of ONO-D1-004. L-902688 has a binding affinity of <1nM to the EP_4 receptor subtype which is very similar to that of PGE_2. L-902688 is also very selective for the EP_4 receptor subtype, which suggests it is the only agonist that can provide a clearer insight into the mechanism of action of EP_4 receptor subtype in endometrial cells.

The EP_4 agonist, L-902688, had resulted in the significant upregulation of AKR1C1 ($P=0.04$) and AKR1C3 ($P=0.003$) by more than 2 fold. The other EP receptor agonists did not have any significant effect on the expression of AKR1C1 and AKR1C3, which indicates that this is mainly EP_4 receptor mediated effect. Unfortunately, due to limited supply of endometriotic lesions, the experiment did not include any ectopic endometrial cells for measuring AKR1C1 and AKR1C3 expression.

At a functional level, the upregulation of AKR1C3 would result in a direct increase of 17β-oestradiol release, whereas AKR1C1 upregulation has a complementary role through which progesterone is inactivated and consequently results in the downregulation of 17β-HSD type 2, which is responsible for metabolising 17β-oestradiol (Casey et al., 1994). However, PGE_2 and selective EP receptor agonists did not have any effect on 17β-oestradiol release from ectopic endometrial cells. This result was contradictory to Bulun's group which reported that PGE_2 induces *de*

novo biosynthesis of 17β-oestradiol in endometriotic lesions (Noble et al., 1997; Attar et al., 2009).

Unexpectedly, L-902688, EP_4 receptor agonist, which significantly upregulated the gene expression of AKR1C1 and AKR1C3, did not affect 17β-oestradiol release. The release of 17β-oestradiol by eutopic endometrial cells was not shown because 17β-oestradiol levels were below detection limit (9.9 pg/ml).

An explanation for the failure of PGE_2 and selective EP receptor agonists to increase 17β-oestradiol release might be due to unchanged levels of AKR1C1 and AKR1C3 protein expression. This confirms the findings reported by Sinreih et al. (2015), which stated that AKR1C3 protein expression was not upregulated in endometriotic lesions despite the significant upregulation of the mRNA expression.

Moreover, Colette et al. (2009) reported the absence of aromatase mRNA and protein in endometriotic lesions. This means that the hypothesis of 17β-oestradiol stimulating the production of COX-2, which results in elevated levels of PGE_2 and in turn induces aromatase in endometriotic lesions, is controversial.

Metabolic dysregulation in endometriosis has been one of the principal molecular changes that occur in endometriotic lesions. Therefore, mitochondrial respiration and glycolytic activities were investigated in eutopic and ectopic endometrial cells (Fig 5.2.5a - 5.2.5d). However, the results have shown that there was no statistical difference between the eutopic endometrial cells and ectopic endometrial cells.

Endometriotic lesions are hypoxic compared to eutopic endometrium. This was demonstrated by the upregulation of the transcription factor hypoxia-inducible factor

(HIF), in particular, the inducible HIF-1α subunit (Wu et al., 2007). This hypoxic environment causes some metabolic dysregulations in endometriotic lesions. One of which is the decrease of enzymes and transporters involved in oxidative phosphorylation and a display of Warburg's effect, shifting ATP production to glycolysis as the main metabolic pathway for ATP production (Kasvandik et al., 2016). Moreover, the peritoneal fluid of endometriosis patients has significant levels of transforming growth factor (TGF) β1 which elevates the levels of HIF-1α, and hence causes an increased glycolytic activities in endometriotic lesions (Young et al., 2014). Also, pyruvate dehydrogenase kinase 1 (PDK1) was significantly upregulated in endometriotic lesions which results in elevation of lactate production. PDK1 suppresses mitochondrial respiration by inhibiting pyruvate dehydrogenase and the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) needed for the tricarboxylic acid cycle (Young et al., 2014).

The effect of elevated lactate is established in cancer, causing an increase in cell invasion, angiogenesis and immune suppression (Hirschhaeuser et al., 2011). Though, these effects still remain to be established in endometriosis.

Treatments with oestrone and 17β-oestradiol for 24 hours did not affect the mitochondrial respiration and glycolysis. This preliminary experiment was conducted to investigate any metabolic changes as a result of hormonal treatment; however, more experiments are needed to optimise the timing and concentration of treatments.

6. General Discussion

6.1. General discussion

The dynamics of endometriosis and breast cancer are mainly determined by aberrations in the levels of oestrogen and progesterone, which are influenced by the enzymes responsible for their synthesis or metabolism, and their respective receptors for exerting a particular effect.

For instance, the AKR1C isoenzymes have a role in synthesising and metabolising oestrogen and progesterone. These enzymes have a regulatory role that controls the availability of oestrogen and progesterone to their receptors. Indeed, any aberrations to their balance can have a role in the development and progression of the disease.

Furthermore, AKR1C isoenzymes catalyse the synthesis of certain prostaglandins which adds another dimension to the development and progression of these diseases. It also reinforces that female sex steroid hormones and prostaglandins are working in a concerted manner in the endometrium and breast. These overlaps can complicate the understanding of the diseases but, at the same time, it provides novel possibilities to treat these diseases.

Therefore, the interaction between female sex steroid hormones and prostaglandins are investigated to identify any aberrations in their expression and whether targeting the AKR1C isoenzymes can suppress the development and progression of these diseases.

In patients with endometriosis, the gene expression of AKR1C1 was significantly elevated in endometriotic lesions, compared to eutopic endometrial tissues from patients with endometriosis. This was coupled with the upregulation of certain EP

receptor subtypes, namely EP_3 receptor. Gene expression of aromatase was not detected in endometriotic lesions, even though different primers of CYP19A1 gene were investigated. This was consistent with other groups which reported that aromatase gene expression, protein translation and enzymatic activity are absent in endometriotic lesions (Colette et al., 2009; Delvoux et al., 2009). Instead, the hormonal dysregulation actually occurs due to dysfunctional metabolism at a micro-environmental level, or within the ectopic lesions. The impaired metabolism is due to aberrations in AKR1C (1-3) enzymes and other 17βHSDs which control the concentrations of 17β-oestradiol and progesterone in the endometriotic lesions (Huhtinen et al., 2012).

Furthermore, the ratio of ERα:ERβ in endometriosis, especially ovarian, is significantly decreased, meaning that there is upregulation of ERβ and downregulation of ERα (Bukulmez et al., 2008). This is due to the deficiency of methylation of the ERβ promoter, resulting in a pathological upregulation of ERβ and relatively low ERα expression in ectopic endometrial stromal cells (Bulun et al., 2012). The high level of ERβ has been proposed as the reason for low ERα expression which has a knock on effect on progesterone receptors. The decrease in progesterone receptor levels contributes to progesterone resistance and results in the downregulation of 17β-HSD type 2 expression (Trukhacheva et al., 2009; Bulun et al., 2012). This is confirmed by *in vivo* data obtained from ERβ knockdown mice showing a decrease in the proliferation of ectopic endometrial stromal cells (Trukhacheva et al., 2009).

The AKR1C2 enzyme was not detected in the endometrial tissues and endometriotic lesions, but it was detected in the breast and omental adipose tissues. In contrast to AKR1C1 and AKR1C3, AKR1C2 has low 20α-HSD activity, and therefore, progesterone inactivation to 20α-hydroxyprogesterone is not a favourable reaction for AKR1C2 (Beranič et al., 2012). This perhaps renders AKR1C2 insignificant for controlling the microenvironment in the endometrium and hence it is absent or present at very low levels.

It is also worth noting that the therapeutic actions of oral contraceptives in treating endometriosis can be enhanced if progesterone resistance is attenuated to allow the increase in the expression of progesterone receptors, and hence increase their anti-proliferative effect (Kim et al., 2013). This can be achieved by inhibiting the local metabolism of progesterone and decreasing the levels of local 17β-oestradiol.

As stated earlier in Chapter 3 (Section 3.3: Discussion), sex steroid hormones and prostaglandins, PGE_2 and $PGF_{2\alpha}$ in particular, influence each other in a dynamic manner, driving the progression of endometriosis. Firstly, 17β-oestradiol is elevated in endometriotic lesions due to the dysregulation of 17β-HSDs and inactivation of progesterone by AKR1C1 and AKR1C3 (Hevir et al., 2011; Huhtinen et al., 2012). As a result, 17β-oestradiol acts as a transcriptional factor of COX-2, which results in the elevation of PGE_2 release in endometriotic lesions (Pérez Martínez et al., 2006).

The importance of PGE_2 is underlined by the anti-proliferative effect of EP_2 and EP_4 receptor antagonists on eutopic endometrial cells. Moreover, EP_2 and EP_4 receptor antagonists inhibited the migration and invasion of ectopic endometrial cells through the inhibition of several MMPs and upregulation of TIMPs (Lee et al., 2011). Also,

the treatment of ectopic endometrial cells with EP_2 and EP_4 receptor antagonists resulted in a decrease in adhesion to extracellular matrix which would impair the chance of survival in extra-uterine areas (Lee et al., 2013).

On the other hand, AKR1C3 is directly responsible for catalysing the biosynthesis of $PGF_{2\alpha}$ (Watanabe, 2002). The increase in $PGF_{2\alpha}$, as a result of upregulation of AKR1C3, as well as upregulation of FP receptor was reflected in symptoms experienced by endometriosis patients, such as dysmenorrhoea and pelvic pain (Chan and Hill, 1978). Even though the majority of subjects, with or without endometriosis, had pelvic pain, FP receptor and AKR1C3 gene expressions in endometriotic lesions were significantly higher. This suggests that $PGF_{2\alpha}$ and FP receptor have other roles in endometriosis. One of the possible roles could be the activation of MAPK pathway which promotes proliferation and survival of endometriotic lesions (Sales, Milne, et al., 2004).

However, at a functional level, the upregulation of AKR1C3 gene expression was not reflected in the levels of 17β-oestradiol, suggesting that AKR1C3 protein levels were not upregulated. Also, since oestrone is the least preferred substrate for AKR1C3, the active site may have been occupied by more preferable substrates, such as PGD_2, PGH_2 and progesterone.

Nevertheless, targeting AKR1C1 and AKR1C3 can provide a better way of controlling progesterone, 17β-oestradiol, PGE_2 and $PGF_{2\alpha}$ without halting the menstrual cycle or using other undesirable treatments such as NSAIDs. Additionally, targeting the remaining 17β-HSDs which are responsible for the reduction of

oestrone to 17β-oestradiol may drastically improve the management of endometriosis (Jansson, 2009).

In this project, we could not find any significant differences in cell metabolism of ectopic and eutopic endometrial cells. However, other groups found that there was significant shift from mitochondrial respiration to glycolysis in human peritoneal mesothelial cells surrounding endometriotic lesions (Ahmad et al., 2016) . As a result, the peritoneum seems to be providing a suitable environment for endometriotic lesions to survive. It also indicates that it is not only the endometriotic lesions that undergo biochemical changes, but rather the surrounding environment can undergo changes to accommodate these lesions.

Bimatoprost or 17-phenyl trinor prostaglandin $F_{2\alpha}$ ethyl amide was found to inhibit AKR1C3. It inhibited the production of oestrone and 9α,11β PGF_2 in eutopic endometrial cells (Section: 4.2 - 4.3; Chapter 4). Bimatoprost has been approved for the treatment of glaucoma and hypotrichosis of eyelashes which makes it easier to reposition the drug for other conditions.

It is important to develop treatments with an acceptable efficacy and minimal adverse effect profile. As was previously shown, the ratio of local oestrone:17β-oestradiol is not reflected in the serum, which reinforces that molecular changes in endometriosis occur locally, and therefore bimatoprost could potentially be used locally in the peritoneal area (Huhtinen et al., 2012). This can bypass certain systemic side effects, such as osteoporosis, gastro-intestinal and cardiovascular adverse effects caused by traditional hormonal therapies and thereby increasing its bioavailability and reducing its side effect profile.

In the omental fat there were no conclusive findings due to the low number of samples which was not enough for statistical testing. Omental fat has proven it is not merely an inert tissue, but has endocrine functions that can be vital for endometriotic lesions. For instance, leptin which is produced mainly in adipose tissue, as well as other tissues, is a cytokine that has various roles which aids the progression of endometriosis (Considine et al., 1996; Kitawaki et al., 2000). Expression of leptin transcripts and protein were found to be upregulated in endometriotic lesions compared to normal endometrium (Wu et al., 2002). Leptin expression was also found to be elevated secondary to an increase in serum 17β-oestradiol levels (Geber et al., 2012). Additionally, its levels in the peritoneal fluid positively correlated with stage III and IV endometriosis (Wertel et al., 2005).

Leptin could be one of the factors that provide a suitable environment for development and progression of endometriotic lesions. It was reported that leptin promotes angiogenic activity of endometriosis-like lesions in a murine model by upregulating VEGF (Styer et al., 2008). It also increases proliferation of ectopic endometrial cells (Wu et al., 2002). Moreover, leptin increased the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2, as well as increasing vascularisation in a rat corneal model (Park et al., 2001). Furthermore, HIF-1α, which is found to be upregulated in the endometriotic lesions, increases levels of leptin in ectopic endometriotic cells due to hypoxic stress in the peritoneal cavity (Wu et al., 2007).

Owing to the proximity of the adipose tissue to the tumour, it suggests that AKR1C1 and AKR1C3 may have a significant role in the maintenance and proliferation of breast cancer cells (Chapter 3). The upregulation of AKR1C1 and AKR1C3 is responsible for the metabolism of progesterone, thereby limiting the availability of

progesterone reaching the breast tumour. This would lead to an imbalance of the locally synthesised 17β-oestradiol, resulting in higher levels of 17β-oestradiol and lower levels of progesterone. In addition, the ability of synthesising 17β-oestradiol via AKR1C3 can also fuel the growth of breast tumours (Penning and Byrns, 2009). In contrast, AKR1C2 was the least expressed gene, compared to the other AKR1C enzymes. AKR1C2 does not also correlate with tumour size, indicating that it does not have an important role to play in the breast adipose tissue for the maintenance of breast tumour.

Aromatase gene was also investigated in the breast adipose tissues. The aromatase gene was only detected in 3 out of 9 breast adipose tissue samples. This shows that aromatase is weakly expressed in these tissues and underlines the importance of AKR1C (1-3) enzymes in controlling the availability of local 17β-oestradiol and progesterone, since they are strongly expressed in the breast fat tissue and other hormone dependent tissues. The aromatase gene detection via qRT-PCR proved to be challenging in endometrial cells, but was detected in a small number of breast adipose tissue samples, which validates primer design.

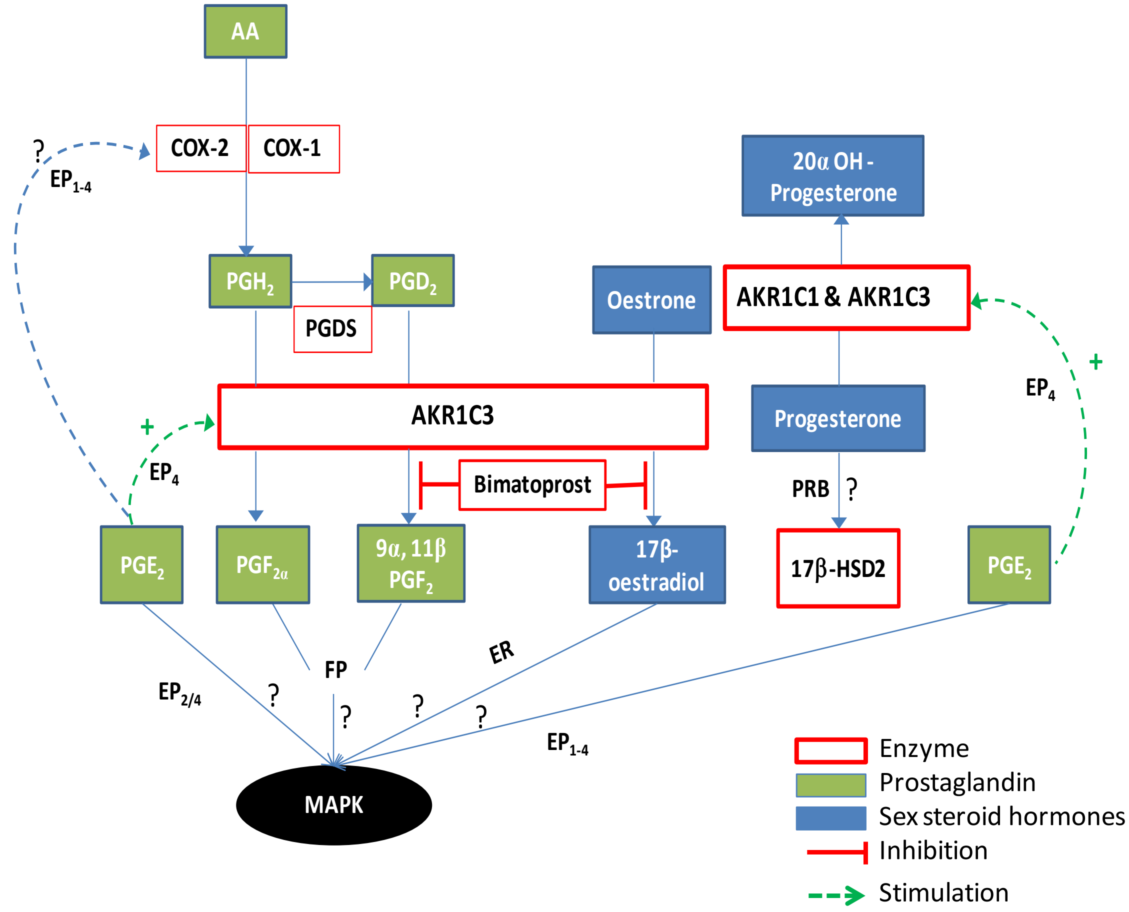


Fig 6.1: A schematic diagram showing the potential relationship between sex steroid hormones and prostaglandins in hormone dependent tissues. This diagram shows what was identified in this project and identifies the gaps needed for future studies. Prostaglandin (PG); mitogen-activated protein kinases (MAPK); oestrogen receptor (ER); progesterone receptor B (PRB); arachidonic acid (AA); prostaglandin receptor E/F (EP/FP); 17β-oestradiol; oestrone; hydroxysteroid dehydrogenase (HSD).

6.2. Limitations of the study

One of the major limitations of the project was the paucity of samples that limited further stratification of the groups. As a result, data was considered in larger, more amorphous groups. For instance, as mentioned in the introduction, there are three different types of endometriosis: peritoneal, ovarian and recto-vaginal endometriosis. The endometriosis groups were not further divided into these categories and instead were combined as one endometriosis group. This would have an impact on comparing the biochemical differences between endometriosis and non-endometriosis groups. Ongoing work in our laboratory suggests that the lipidomic profile varies according to the subtype of the disease.

In addition, an ideal control group with no pathologies was not available. Instead, the control group included an array of samples with benign pathologies not attributed to endometriosis such as fibroids, menorrhagia and dysmenorrhoea. These pathologies overlap with the symptomology of endometriosis and consequently may have some similar biochemical changes.

A much better stratification method would be comparing daily samples from endometriosis and non-endometriosis groups across the whole menstrual cycle to be able to detect any cyclical changes in the expression of AKR1C isoenzymes across the cycle. Unfortunately, the sample number was not high enough to further divide each group according to the menstrual cycle status and it is unlikely that sequential sampling would be possible.

Furthermore, normal breast adipose tissues were not available to allow the comparison of breast adipose tissues from healthy control and breast cancer subjects.

The patient data did not have any information regarding menopausal status of subjects and hence an assumption with respect to the average age of menopause was made.

6.3. Future Studies

Due to time restraints and limited tissue donations, the project does still have gaps that need to be addressed in the future. The main one is to stratify further the endometriotic lesions according to their locations, which would allow delineating any effect of location on endometriotic lesion behaviour, and also detect any differences at a molecular level between different types of endometriosis. Moreover, the mechanism(s) of the PGE_2 positive feedback loop needs to be elucidated by investigating the different mechanisms that were already previously established on different cell types, such as Ishikawa cells. This would need using western blotting technique, in addition to ELISA and qRT-PCR.

In terms of investigating treatments for endometriosis, the development of a battery of *in vitro* tests would be useful for increasing the chance of developing a successful and tailored treatment. For example, investigating the effect of such treatments on certain mitogenic pathways, such as NFκB and MAPK, can be useful for determining the effectiveness of treatments. This can be combined with migration assays and invasion assays which investigate markers such as cadherin molecules, integrins, TIMP, MMPs and uPA. Also, angiogenesis assays, measuring VEGF and VEGFR in the endometrial cells (eutopic and ectopic). Finally, a clinically valid *in*

vivo model of endometriosis needs to be developed to investigate the effectiveness of the treatments such as local delivery devices in the peritoneum.

Temporary *in vitro* knockdown of the AKR1C1 and AKR1C3 in the endometrial cells and adipocytes could be used to further investigate the roles of AKR1C1 and AKR1C3 in the endometrial cells and adipocytes. This could lead to determining the importance of AKR1C1 and AKR1C3 in the production of 17β-oestradiol, 20α-hydroxyprogesterone, $PGF_{2\alpha}$ and 9α,11β PGF_2. It could also be extended to investigate the effects of AKR1C1 and AKR1C3 on NFκB and MAPK pathways.

6.4. Conclusion

The gene expression of AKR1C isoenzymes was investigated in the endometrium, omental adipose tissue and breast adipose tissue. However, the small sample number did not allow the stratification of the data according to the menstrual cycle status or endometriosis type.

The gene expression of AKR1C1 ($p < 0.05$) was significantly higher in endometriotic lesions, compared to eutopic endometrium of patients with endometriosis. In omental adipose tissues, there was no significant difference in AKR1C gene expression between the endometriosis and non-endometriosis groups. The sample number was not high enough for detecting any statistical difference. In breast adipose tissues, there was no significant difference in the gene expression of AKR1C isoenzymes regardless of oestrogen or progesterone receptor status.

The gene expression of EP_3 ($P<0.05$) receptor was significantly higher in endometriotic lesions compared to eutopic endometrium of patients with endometriosis. In omental adipose tissues, there was no significant difference in the gene expression of EP_{1-4} and FP receptors between endometriosis and non-endometriosis groups. The low number of samples was not high enough to detect any statistical difference. In breast adipose tissues, there was no significant difference in the gene expression of EP_{1-4} and FP receptors regardless of oestrogen or progesterone receptor status.

An enzyme kinetic assay was used to determine the inhibitory constant (Ki) of bimatoprost as an AKR1C3 inhibitor using oestrone as a substrate (Ki = 2.9µM and αKi = 0.7µM). Bimatoprost was also assessed *in vitro* using human endometrial cells

to determine whether it can also inhibit the synthesis of 17β-oestradiol and 9α,11β PGF_2. It inhibited the production of 17β-oestradiol ($P<0.05$) and inhibited the production of 9α,11β PGF_2 in a dose dependent manner.

The effect of PGE_2 on the expression of AKR1C1 and AKR1C3 enzymes in human endometrial cells was investigated. PGE_2 did not significantly increase the AKR1C1 or AKR1C3 gene expression. However, L-902688, EP_4 receptor agonist, increased the gene expression of AKR1C1 ($P<0.05$) and AKR1C3 ($P<0.01$) in human endometrial cells. Despite gene expression elevation, L-902688 did not increase the production of 17β-oestradiol, indicating that the protein expression was not upregulated.

Finally, mitochondrial respiration and glycolytic functions of endometrial cells were compared in endometriosis and control groups. There was no significant difference in metabolic activities between endometriosis and control groups. Treatment with oestrone and 17β-oestradiol did not affect mitochondrial respiration or glycolytic activities.

Appendix 1

NRES Committee South West - Cornwall & Plymouth
Bristol Research Ethics Committee Centre
Level 3
Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1328

06 December 2013

Professor Kay Marshall
Head of the School of Pharmacy & Pharmaceutical Sciences
University of Manchester
Stopford Building
Oxford Road
Manchester
M13 9PT

Dear Professor Marshall

Study title:	**Investigation into the role of local hormones and prostaglandins in female reproductive tract disorders_v.1**
REC reference:	**13/SW/0123**
Amendment number:	**1**
Amendment date:	**01 November 2013**
IRAS project ID:	**117359**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	2 (KM.2013_2)	27 November 2013
CV - Sarah Williams	1	27 September 2013
CV - Matthew Rosser	1	27 September 2013
Notice of Substantial Amendment (non-CTIMPs)	1	01 November 2013
CV - Orsolya Kiss	1	27 September 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

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

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

13/SW/0123:	Please quote this number on all correspondence

Yours sincerely

Canon Ian Ainsworth-Smith
Chair

E-mail: nrescommittee.southwest-cornwall-plymouth@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Lynne Webster*
Mrs Lynne Macrae

NRES Committee South West - Cornwall & Plymouth

Attendance at Sub-Committee of the REC meeting

Name	*Profession*	*Capacity*
Canon Ian Ainsworth-Smith	Retired Hospital Chaplain	Lay Plus
Mrs Sheila Bullard	Clinical Research Project Manager	Expert

Also in attendance:

Name	*Position (or reason for attending)*
Ms Julie Closius	REC Assistant

Central Manchester University Hospitals NHS
NHS Foundation Trust

Research Office
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Dr Emma Crosbie
St Marys Hospital
Central Manchester Foundation NHS Trust
Oxford Road
Manchester
M13 9WL

Ref: R03282-Ltr 2-Name Crosbie

Dear Dr Crosbie

PIN: R03282 (Please quote this number in all future correspondence)
REC Reference: 13/SW/0123
Research Study: Investigation into the role of local hormones and prostaglandins in female reproductive tract disorders_v.1

Thank you for submitting the above study for NHS R&D permission. University of Manchester is the Sponsor for this study which ***is not*** on the NIHR portfolio.

I am pleased to confirm that the Research Office has now received all necessary documentation, and the appropriate governance checks have been undertaken. This letter is issued subject to the research team complying with the attached conditions, Trust SOPs, the DH Research Governance Framework, and any other applicable regulatory requirements. This approval is in relation to the documentation listed.

This study is classed by the DH as a clinical trial and as such CMFT are required to report whether the research was initiated within 70 days or provide valid reasons for not doing so. The target date for this study is listed below;

- NIHR 70 Day from Valid Submission to 1st Patient Recruited: **12th September 2013**

Further information regarding the NIHR target can be found on the intranet.

Please update CRIMSON with the date when the first patient was recruited. If you or one of your team requires training on CRIMSON please contact Michael.Horrocks@cmft.nhs.uk

I would like to take this opportunity to wish you well with your research.

Yours sincerely

pp

Lorraine Broadfoot
Research Operations Manager

Date:

Encs. NHS SSI form

cc. Sarah Leo Divisional Research Manager
Professor Kay Marshall University of Manchester
Dr O Zarroug

Documents Acknowledged/Approved

Document	Version	Date
Evidence of insurance or indemnity		19 April 2013
Investigator CV		02 April 2013
Letter from Sponsor		19 April 2013
Other: CV - Mr Osman Zarroug		02 April 2013
Other: Non-pregnant donor info form - Reproduction Research Project	v_1	02 April 2013
Participant Consent Form	v_2	10 May 2013
Participant Information Sheet: Therapies for Reproductive Disorders	v_3	10 May 2013
Protocol	KM.2013_1	02 April 2013
REC application	3.5	29 April 2013

Conditions of Approval:-

- All researchers involved in the study need to have received training appropriate to their role covering aspects of Research Governance or Good Clinical Practice (GCP). Trust policy states GCP training needs to be renewed every 3 years.
- The Research Office must be informed of: (please forward copies of amended documents by email)
    - The actual start date of the project
    - Any changes to the protocol throughout the course of the project
    - Any amendments sent to the MHRA or Research Ethics Committee
    - Any changes to the management of the project
    - Any extensions to the project, and associated additional funding, if applicable.
- The Research Office must be notified immediately of all Serious Adverse Events (SAEs) and Suspected Unexpected Serious Adverse Reactions (SUSARs) via email adverse.events@cmft.nhs.uk or Research Office fax: 276 5766 and/or by copy of official notification to the regulatory authorities (NRES, MHRA as applicable).
- All research taking place on CMFT Trust premises is subject to the Trust monitoring programme, either as part of the annual 10% audit requirement or "triggered" monitoring[1]. The Chief and/or Principal Investigator is required to make him/her self available for any monitoring visit, on a mutually agreed date.
- All Principal Investigators are required to complete and submit an annual self-assessment at the request of the Research Office.
- All Principal Investigators are required to provide recruitment (accrual) data to the Research Office monthly.
- The Research Office must be given a minimum three months' notice, in writing, if the Principal Investigator leaves the employment of CMFT Trust.
- The Research Office must receive immediate notification if the Principal Investigator is unable to continue to fulfil his/her duties as PI for other reason e.g. long-term sickness
- Any evidence of fraud &/or misconduct must be immediately brought to the attention of the Research Office either via the Incident Reporting system, or by direct communication.

Failure to comply with any of the above may result in withdrawal of approval for the project and the immediate cessation of the research. Persistent failure to comply may result in disciplinary action.

The University of Manchester

Faculty of Medical & Human Sciences
The University of Manchester
Oxford Road
Manchester M13 9PT

www.manchester.ac.uk

Friday, 19 April 2013

To whom it may concern

Sponsor Reference:

Role of the Research Sponsor under the Research Governance Framework for Health & Social Care and the Medicines for Human Use (Clinical Trials) Regulations 2004 (SI2004/1031)

I hereby confirm that the University of Manchester would be prepared to accept the role of research sponsor as currently defined in the *Research Governance Framework for Health & Social Care Version 2 (DoH 2005)* and the *Medicines for Human Use (Clinical Trials) Regulations 2004 (SI2004/1031)*, in relation to the study:

Investigation into the role of local hormones and prostaglandins in female reproductive tract disorders

I have been informed that this study will be led by **Professor Kay Marshall** of The University of Manchester.

Sponsorship is subject to the following conditions:

1) The lead investigator for the study must be an employee of the University of Manchester. For student research the academic supervisor is considered to be the lead investigator.
2) An appropriate contract must be agreed between the University and the funding body.
3) The research must be reviewed and approved by appropriate ethics, NHS and regulatory bodies and registered in accordance with University insurance requirements.

To enable the sponsor to meet their responsibilities as listed in section 3.8 of the Research Governance Framework, Chief Investigators are asked to adhere to the responsibilities as outlined in section 3.6 of the Framework (available at: https://www.gov.uk/government/publications). In line with this requirement **Professor Kay Marshall** must ensure that all involved in the research project understand and discharge their responsibilities in accordance with the agreed protocol and any relevant management, ethical and regulatory approvals.

Chief Investigators are also reminded that they must register NHS REC approval with The University of Manchester Research Ethics Office.

If you have any queries about sponsorship of this project then please address them to Professor Nalin Thakker, Associate Vice President for Research Integrity, The University of Manchester, Christie Building, Oxford Road, Manchester M13 9PL, or email research-governance@manchester.ac.uk

Yours Faithfully,

Lynne MacRae

Lynne MacRae Dated: **19.04.2013**
Research Practice Coordinator
Faculty of Medical & Human Sciences

MANCHESTER
1824

The University of Manchester

Consent Form. v_2 10/05/13

Consent Form

Investigation into the role of local hormones and prostaglandins in female reproductive tract disorders

Study number/ Patient ID number ____

Hospital number ____

I agree that the following tissues and/or body fluids may be used for biomedical research (please provide details):

Human eutopic endometrial tissue Human ectopic endometrial tissue Human omental adipose tissue

Please read each item and initial each box to indicate agreement.

1. I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that I will be asked to give tissues and/ or body fluids, which may be stored at the University of Manchester to be used for this study and other future studies. ☐

4. I understand that the clinician/ clinical staff overseeing my care may pass on my medical history in an anonymised form to the research group at the University of Manchester. ☐

5. I agree that my anonymised and relevant clinical data may be kept on the research group's database at the University of Manchester. ☐

6. I agree to take part in the above study. ☐

7. I understand that relevant sections of data collected during the study may be looked at by responsible individuals from the University of Manchester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in the research. I give permission for these individuals to have access to this data ☐

____________________	____________	________________
Name of Participant	**Date**	**Signature**
____________________	____________	________________
Name of Person taking consent	**Date**	**Signature**

Reproduction Research project

Patient Ethical Tissue Number				
Date		Age		
Ethnicity				
Procedure				
Indication				
History of presenting complaint				
Parity				
LMP		K= regular/irregular IMB / PCB / amenorrhoea		
Stage of menstrual cycle	Menstruation	Follicular	Ovulation	Luteal
Contraception/use of hormones				
Past gynae history				
BMI	Smoker yes / no Number / day			
Past medical history				
Medications				
Does the patient have any of the following medical conditions?	Endometriosis	Pelvic pain		
	Fibroids	PID		
	Menorrhagia	Fertility problems		
	Dysmenorrhoea	Diabetes		
Samples obtained	Endometrium Myometrium Ovary	Endometriosis Peritoneal fluid Peritoneal washing	Blood Omental fat Other	
Comments on location, size & number of endometrial lesions				
Any specimen(s) sent to pathology for examination?				

MANCHESTER 1824 The University of Manchester

Reproduction Research Group, School of Pharmacy & Pharmaceutical Sciences, University of Manchester, Oxford Road, Manchester, M13 9PL

Prof Kay Marshall, Dr Debbie Fischer, Mr Osman Zarroug Tel: 0161 275 7678 / 0161 275 7943

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