Investigating the role of tumour derived hydrogen sulphide and its synthases in endometrial cancer tumourigenesis.

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Abstract

Endometrial cancer is the 4th most common gynaecological malignancy in developed countries with a continual rise in incidence of >40% reported between the years of 1993-2007. Though endometrial cancer presents early with abnormal uterine bleeding the therapeutic options are limited with surgery being the hallmark treatment for these women. Recent evidence published has implicated the new gaseous transmitter hydrogen sulphide (H_2S) in the pathogenesis of colorectal carcinoma. The results of this study strongly suggested a role for H_2S synthase inhibitors as a possible therapeutic option. No prior studies have been conducted exploring the role of H_2S in relation to endometrial carcinogenesis. This study aims to produce novel research exploring the role of H_2S in endometrial carcinogenesis.

Immunohistochemical techniques were used to stain a total of 81 human endometrial samples (post-menopausal n=16, complex atypical hyperplasias n=4 and endometrial cancers n=61) with H_2S synthases i.e. Cystathionine-β-synthase (CBS), Cystathionine gamma-lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MPST) to elucidate their differential expression between these groups. *In vitro* experiments were carried out investigating the effects of H_2S donors i.e. GYY4137 and H_2S substrates such as L-cysteine on the proliferative potential of the endometrial cancer cell known as MFE280.

The results of this study concluded that the endometrium positively expresses all 3 enzymes. 3-MPST displayed higher levels of expression in the atrophic post-menopausal endometrium with a significant loss in its expression seen in low grade endometrial cancer cells ($p<0.0001$), suggesting a possible role for 3-MPST as an early disease marker. CSE like 3-MPST displayed a significant reduction in expression in cancerous cells when compared with post-menopausal and hyperplastic endometrium ($p<0.001$) whilst CBS showed no overall difference in expression between all study groups ($p=0.136$). Results of the *in vitro* investigations revealed an overall inhibition of cell viability of endometrial cancer cells when treated with a range of concentrations of H_2S donors and substrates.

Analysis of the above results presents a possible protective role of 3-MPST and CSE within the endometrium and their subsequent loss contributing to the development of endometrial carcinogenesis. Furthermore, the possibility of employing H_2S donors as

therapeutic agents in endometrial carcinomas may be a promising intervention and further functional studies will need to be conducted before conclusions regarding the latter can be determined.

For Mum and Dad

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Abbreviations

AOAA	Aminooxyacetic acid
APES	Aminopropyl triethoxy silane
AR	Androgen receptor
ATP	Adenosine tri phosphate
BSO	Bilateral Salpingo- Oophorectomy
BMI	Body mass index
CAH	Complex atypical hyperplasias
CBS	Cystathionine beta- synthase
CSE	Cystathionine gamma-lyase
COX-2	Cyclooxygenase -2
CO	Carbon monoxide
COCPs	Combined oral contraceptive pills
ER	Oestrogen receptor
FBS	Foetal bovine serum
FIGO	International Federation of Gynecology and Obstetrics
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GYY4137	4-Methoxyphenyl(morpholino)phosphinodithioate morpholinium salt-4-ylsulphanylidenesulphido
GSH	Glutathione
Her2/neu	Human Epidermal Growth Factor Receptor 2

HIER	Heat induced epitope recovery
HRP	Horseradish peroxidase
H_2S	Hydrogen sulphide
H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
K-ras	Kirsten rat sarcoma viral oncogene homologue
LH	Luteinizing hormone
LREC	Local research ethics committee
MCDU	Mercaptolactate-cysteine disulphiduria
MMMT	Malignant mixed Müllerian tumour
MSH2	MutS protein homologue 2
MSH6	MutS protein homologue 6
NaHS	Sodium hydrogen sulphide
Na_2S	Sodium sulphide
NBF	Neutral buffered formalin
NO	Nitric oxide
PAG	DL-Propargylglycine
PCOS	Polycystic ovarian syndrome
PM	Post-menopausal
PR	Progesterone receptor

PTEN	Phosphatase and tensin homologue
P5P	Pyridoxal 5- phosphate
SD	Standard deviation
siRNA	Small interfering RNA
ShRNA	Short hairpin RNA
SEM	Standard error margins
TAH	Total abdominal hysterectomy
TBS	Tris-buffered saline
3-MPST	3-mercaptopyruvate sulphurtransferase

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Chapter 1 Literature review

The endometrium is the internal mucous lining of the uterine cavity. It is a highly sensitive target organ for the action of ovarian hormones such as oestrogen and progesterone. In response to these hormones it is subject to cyclical changes throughout a woman's reproductive life. The endometrium is divided into two functionally different layers, i.e. the stratum functionalis and stratum basalis[3]. The basal cells possess a large capacity for renewal and allow the monthly regeneration of the stratum functionalis to occur. This highly proliferative ability of the endometrium increases the risk of somatic mutations that may cause abnormal replication of endometrial cells. Recent evidence has emerged implicating the role of hydrogen sulphide (H_2S) as a tumour growth factor secondary to its ability to stimulate cellular bioenergetics[4]. The abnormal replication of cells may lead to endometrial hyperplasia or progress to neoplasia. Endometrial carcinoma is the 6th most common malignancy in females worldwide with a continual increase in incidence in the UK by over 40%[5]. A 100% increase in incidence of endometrial cancer is hypothesised by the year 2025 based on trends in incidence seen in Norway[6].

H_2S is an endogenous gaseous transmitter that has both physiological and pathological functions[4, 7]. It is generated from the metabolism of the sulphurous substrate L-cysteine by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), two pyridoxal-5'-phospate–dependent enzymes and the combined action of cysteine aminotransferase with 3-mercaptopyruvate sulphurtransferase (3-MPST)[4, 7]. *In vivo* H_2S was proposed to cause vasorelaxation, promote angiogenesis and stimulate cell proliferation. Angiogenesis and ATP synthesis are both vital for the growth and proliferation of cells. Evidence has shown the selective up- regulation of CBS in both colorectal and ovarian carcinomas.[4] The subsequent increase in H_2S allows the gaseous molecule to exert its effects by causing an increase in cellular mitochondrial function (oxygen consumption, ATP turnover), angiogenesis and stimulation of kinase pathways amongst other mechanisms[4].

The suspected ability of H_2S to initiate and maintain tumour growth is yet to be investigated in endometrial cancer. The most up to date evidence regarding the structure and function of the endometrium, endometrial cancer and hydrogen sulphide's physiological role and its implication in cancer will be reviewed and analysed.

1.1 Anatomy:

1.1.1 Uterus

A pear shaped, thick walled muscular organ, the uterus is 7.5 centimetres long and is mainly contained within the pelvic cavity and perineum[8]. *In vivo* it is mostly anteverted and positioned in the midline amidst the bladder and the rectum, figure 1. A rectovaginal septum isolates the posterior wall of the vagina from the rectum[3]. For illustrative purposes the uterus is divided into 3 major parts; the fundus, body and cervix, as seen in figure 1. Anatomically the uterus is divided into 2 distinct parts: the uterine body and the uterine cervix[3]. Made up of the body and cervix, the uterus communicates with the vagina inferiorly. Superiorly, the fallopian tubes project laterally from the fundus forming a continuum between the endometrial cavity and the peritoneal cavity as they come to rest adjacent to the ovaries[3]. Functionally the uterus exists to receive the fertilised embryo, provide a site for implantation and expand to shelter the foetus during pregnancy before contracting during delivery[3].

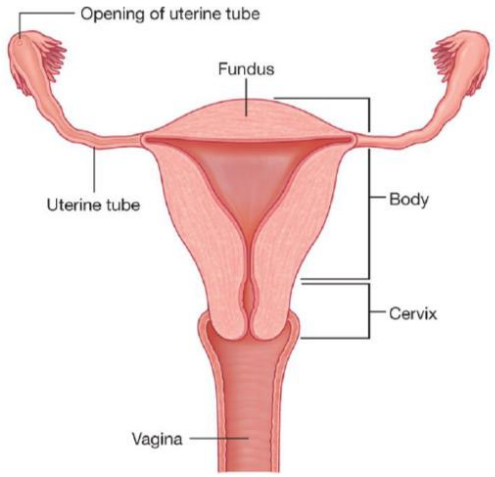


Figure 1 Anatomy of the female reproductive tract. (Adapted from Drake et al.)[1]

The uterus has a triple-layered wall namely: the perimetrium (tunica serosa), myometrium (thick tunica muscularis) and endometrium (tunica mucosa). The myometrium is a smooth muscle layer that is made up of poorly defined muscle fibres arranged in a circular, longitudinal and diagonal direction. It is the thickest layer and accounts for the growth of the uterus during pregnancy and responds to hormones to produce powerful contractions during labour[3].

Uterine ateries are the major source of blood suply for the uterus; they originate bilaterally from the anterior section of the hypogastric artery and runs medially towards the cervix

uteri[9]. Lending a branch to the urethra its reaches the lateral aspect of the uterus and ascends towards the junction between the fallopian tube and uterus[9]. Branches of the uterine atery anastamose with the ovarian artery branches creating an arterial arcade. The uterine veins mirror the path of the uterine arteries and form venous plexuses that drain into the internal iliac vein. They also form 2 plexuses with the vaginal blood vessels inferiorly (utero vaginal venous plexus) and ovarian blood vessels superiorly (utero-ovarian plexus)[9].

The uterine vessels undergo changes in growth through different phases of the menstrual cycle. The endometrium is the sole site of neovascularisation within the body in health[9]. As the endometrium proliferates in the proliferative phase of the menstrual cycle the vasculature undergoes similar growth. The vessels become increasingly coiled as they extend from the basal layer to the functional layer and are known as spiral ateries[9].

1.1.2 Endometrium

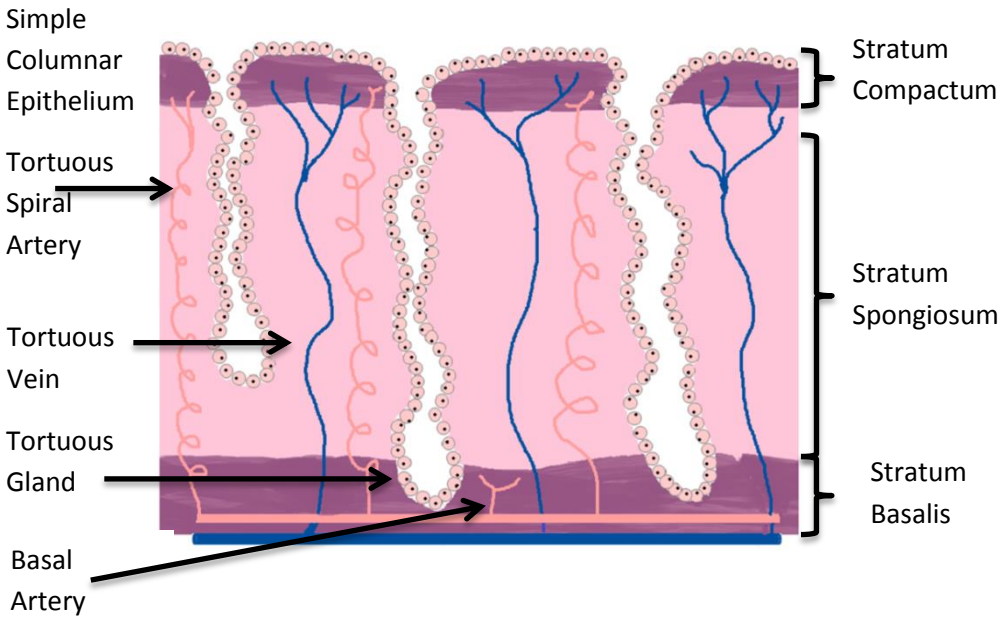


Figure 2 Layers of the endometrium

The endometrium is made up of two functionally different layers: the upper two-thirds known as the stratum functionalis and the lower third, the stratum basalis[3, 10]. The functional layer of the endometrium can be further divided into 2 distinct layers, the most superficial layer known as the stratum compactum and a deeper stratum spongiosum[3]. During the proliferative phase of the menstrual cycle these 2 strata develop into the stratum functionalis. The stratum functionalis contains fewer glands in relation to stroma in the superficial zona compacta, particularly in pre-menopausal endometrium. The deeper zona spongiosa retains abundant number of glands. The stratum functionalis is the location for the implantation of a blastocyst or development of the placenta[3]. The stratum basalis is highly glandular with reduced stroma and consists of endometrial progenitor cells, owing to its role in the monthly regeneration of the stratum functionalis[10]. A single layer of columnar epithelium lines the luminal surface of the endometrium and invaginates into the stroma to form the endometrial glandular epithelium[10]. These glands span the depth of the endometrium through to the myometrial layer. Endometrial proliferative conditions include both stromal and glandular proliferations e.g. hyperplasia or adenocarcinomas arise from the glandular epithelium[10].

Prior to puberty this tissue lies dormant with a constant morphology and thickness. It reverts back to this stage post menopause due to the lack of periodic hormonal change. The features of pre-pubescent endometrium include tubular glands, dense fibroblasts and thin blood vessels. At the onset of puberty the glands, stroma and blood vessels undergo change and under the influence of oestrogens and progesterone the endometrium begins to prepare itself in each menstrual cycle to support a fertilised oocyte[10]. If fertilisation does not occur this leads to degeneration and shedding of the functional layer of the endometrium, resulting in a withdrawal bleed[3].

1.2 Physiology

1.2.1 Ovarian cycle

At birth approximately 500,000 primordial follicles are present in each of the ovaries. An increasing number of ovulatory cycles results in a reduction in the total number of available follicles[3, 11]. Only 480 of the recruited follicles reach the Graafian follicle stage to release an oocyte during ovulation[11, 12]. The initiation of follicular recruitment spans a long duration of time and is autonomous. The hypothalamo- hypophyseal tract controls the monthly recruitment of follicles[13]. The pulsatile release of gonadotropin releasing hormone (GnRH) by the hypothalamus is vital for the release of the two anterior hypophyseal gonadotropins: Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH)[11, 14].

The ovarian cycle is composed of two phases and lasts approximately 28 days[11]. The first phase (14 days) known as the follicular/ proliferative phase is highly variable and involves the recruitment of a cohort of follicles, eventually leading to the maturation of one follicle[14, 15]. This phase ends in ovulation refer to figure 3. The luteal/ secretory phase is fixed and its beginning is marked by ovulation and consists of progesterone production by the corpus luteum[11]. The follicular and luteal phases of the ovarian cycle correspond to the proliferative/ secretory phases of the menstrual cycle respectively. A rise in serum FSH level occurs as oestrogen levels drop secondary to the degeneration of the corpus luteum. FSH sensitive pre-antral follicles are recruited in response to the rising levels of FSH[11, 14]. As the level of FSH peaks a period of time termed the 'FSH window' opens allowing one follicle to mature to the graafian stage and secrete oestradiol secondary to aromatase activity[11, 13]. An increase in LH levels is also required for the sensitisation of theca cells to initiate production of androgens[16]. These androgens are the precursors for the production of oestradiol under FSH stimulation[11, 13]. FSH production is inhibited secondary to the raised serum oestradiol levels, highlighting the long-loop negative feedback mechanisms at the levels of the hypothalamus and

pituitary[11]. This fall in FSH levels limits follicular maturation leading to atresia of remaining follicles, thus preventing multiple follicular ovulations[11, 13, 14].

1.2.2 Menstrual cycle

The hypothalamic-pituitary-ovarian axis marks the three levels at which the hormonal regulation of menstruation takes place via feedback mechanisms[17]. The expression of steroid receptors varies across the phases of the menstrual cycle, thereby regulating the extent of the hormonal regulation[17]. The menstrual cycle is divided into 3 phases: the menstruation phase (1st-4th day), the proliferative/ follicular phase (4th -14th day) and the secretory/ luteal phase (14th-28th days)[17].

1.2.2.1 Menstruation phase

The menstruation phase represents the start of a new menstrual cycle. In the absence of successful implantation, the degradation of the corpus luteum leads to a drop in levels of circulating oestradiol and progesterone[17]. The radial and basal arterioles are not responsive to the drop in serum levels of the sex hormones[18]. The reduction in serum progesterone results in constriction of the spiral arteries that supply the stratum functionalis, thereby causing ischaemia and necrosis[18]. The rupture of the spiral arterioles and rapid blood flow results in shedding of the functional layer of the endometrium. The basal layer obtains its blood supply from the straight arteries and therefore remains undisturbed. Regeneration of the endometrium begins imminently within this phase from the basal glands, with completion of surface epithelial growth within 48 hours post sloughing of the stratum functionalis[8, 9].

1.2.2.2 Proliferative phase

The duration of the proliferative phase is variable lasting between 10-20 days, with any variation in cycle length attributed to this phase. Endometrial cell proliferation, vascularisation and regeneration are stimulated by oestrogen. The tubular glands in the stratum functionalis open into the luminal surface[8]. The thickness of the endometrium increases by >10-fold secondary to the growth of the glands, stroma and spiral arteries in the stratum functionalis[9]. This phase can be divided into an early and late proliferative phase[8]. The early proliferative phase is characterised by a reasonably uniform and thin

endometrium, whereas the late proliferative phase is confirmed by a thickened endometrium that includes a larger number of glands and mitoses, clearly seen in the stroma and epithelium. The stromal cells tend to possess an elongated, spindle like architecture[8]. The maturing follicle causes a peak in oestradiol that causes a surge in luteinising hormone leading to ovulation 35-44 hours after the LH peak[19], thus making the endometrium sensitive to progesterone as it progresses into the secretory phase[17, 18].

1.2.2.3 **Secretory phase**

The secretory phase occurs once ovulation has ensued; the corpus luteum secretes oestrogen and progesterone that influence the endometrium to mature and become receptive for a possible blastocyst[1, 11]. The time period between 20th – and 23rd days is known as the 'implantation window' and represents the most favourable environment of the endometrium to receive a blastocyst for successful implantation. Characteristic features seen in secretory phase include the corkscrew shape of the glands with increased glycogen rich secretions[1, 11].

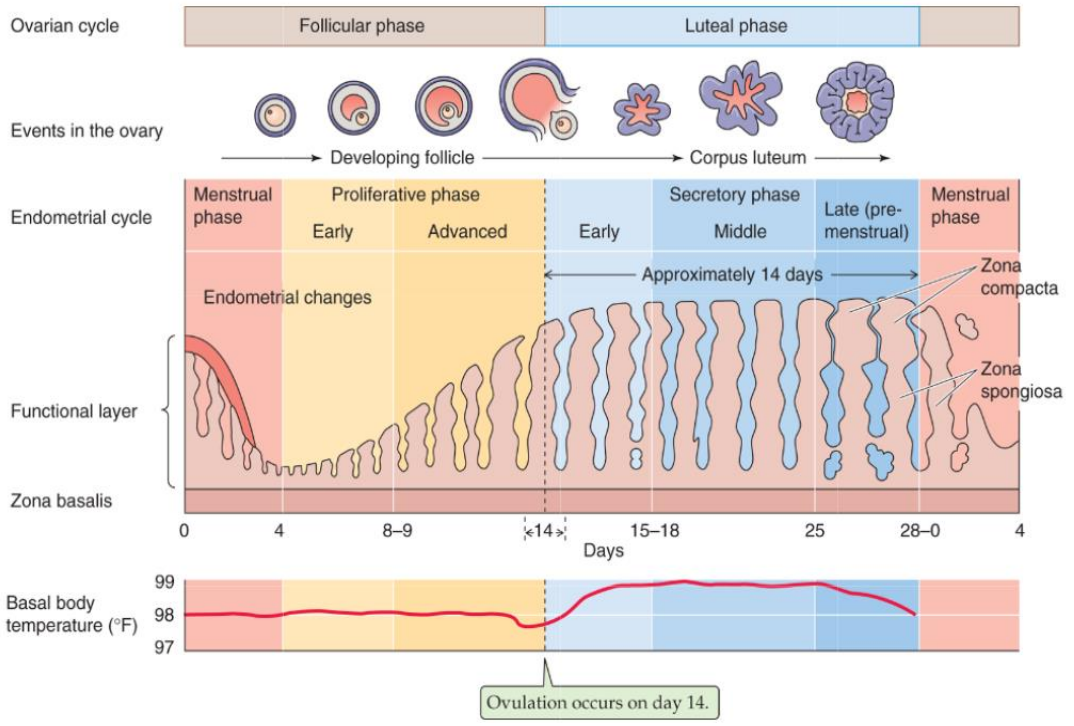


Figure 3 Phases of Ovarian and endometrial cycle[20]

1.2.3 **Menopause**

Once a woman reaches the climacteric phase of her reproductive life she is known to be peri-menopausal. Clinically, menopause is defined as the "the permanent cessation of menstruation and fertility occurring 12 months after your last menstrual period"[21]. In the UK the average age of menopause is 51 years, but some women may undergo menopause through their 30s or 40s. If menopause occurs earlier than 40 years it is deemed as premature menopause.

Menstrual cessation occurs secondary to the failure of the ovaries to respond to hormonal signalling. During the peri-menopausal period the hormone production gradually wanes away leading to irregularity in the menstrual cycles. The non-existence of oogonal stem cells secondary to their exhaustion prior to birth means there is a finite number of follicles available form birth[12]. In addition, with increasing age the loss of a group of follicles monthly results in decreasing numbers of follicles available for recruitment. Monthly recruitment of an ovum no longer takes place and will eventually cease to occur due to the diminishing response of follicles to the pituitary hormones e.g. FSH and LH.

The lack of circulating oestrogen results in the absence of the LH surge that prolongs the proliferative recruitment phase and results in anovulatory cycles[22]. The low serum oestrogen levels within the body can also have physical and emotional effects leading to the common symptoms; hot flushes, night sweats, mood swings and vaginal dryness. The morphology of the uterus was shown to change as the woman goes through menopause[23]. The findings clearly show a reduction in uterine size and endometrial thickness 24 months before compared with after menopause.

Histologically the endometrium undergoes changes during the menopause[24]. Both pre-menopausal and post-menopausal endometrium consists of the luminal epithelium, stroma and glands. Decreasing hormonal stimulation of the endometrium causes structural changes such as: closing of the small tubular glands and reduced stroma, resembling the stratum basalis of menstruating women. 'Cystic atrophy' also termed 'Swiss cheese endometrium' in the past is a characteristic of post- menopausal endometrium where the glands are dilated and matter rich in protein collects in the glands[25]. In the postmenopausal endometrium, atrophy of the stratum functionalis results in difficulty in differentiation between the functional and basal layers, resulting in a thin endometrium. The endometrial

thickness in healthy post-menopausal women ranges from 1 to 5 millimetres (mm) thick, when compared with the normal 15mm thickness seen in the secretory phase of the cycling endometrium[23, 26].

Post-menopausal endometrium is atrophic but retains the ability to respond oestrogen and progesterone if present systemically[27, 28]. Therefore, endogenous (obesity) or exogenous oestrogens (unopposed by progesterone) may cause the endometrium to become proliferative. The post-menopausal endometrium can be further divided into 3 states, depending on its level of proliferation: mixed endometrium, atrophic/ weakly proliferative endometrium, and atrophied inactive endometrium.

1. *Mixed:*

The endometrium is mostly atrophic and inactive, but there are islands of weakly proliferative glands.

2. *Atrophic/ weakly proliferative*

The endometrium is thin measuring 2.2mm with no distinction between the stratum functionalis and stratum basalis. Tortuous endometrial glands are visible and the epithelia appear to be columnar psuedostratified. Minimal mitoses are apparent with oval nuclei surrounded by dense, fibrotic stroma[28].

3. *Inactive endometrium*

The stratum basalis exists alone without a functional layer making the endometrium very thin. A few tubular glands exist within the basal layer with cuboidal epithelium that is inactive. Characteristics of an atrophic endometrium include dilated glands coated by flattened, undifferentiated epithelium[28].

Over 80% of endometrial carcinomas develop in post-menopausal women[28]. The post-menopausal endometrium retains the ability to proliferate in response to oestrogen. Oestrogen receptor regulation is proposed to be lost in post-menopausal endometrium. Type 1 endometrial cancers are known to arise on a background of obesity from post-menopausal endometrium[28].

1.2.4 Steroid hormone receptors

As the endometrium is the target organ for oestrogens and progestins, it expresses the respective receptors. These steroid receptors have many functions, ranging from their role as transcription factors or steroid ligand binding, dimer genesis to communication with other proteins. Genomic and non- genomic signalling controls the expression of these steroid hormones receptors.

1.2.4.1 Oestrogen receptor (-alpha and –beta)

Oestrogen receptors (ERs) control the expression of a variety of genes by either activating or inhibiting them, like transcription factors[29]. The monthly oestrogen induced changes in the endometrium are a result of the presence of the ER in the nuclei of endometrial cells. This allows the prompt conversion of hormonal stimulation into structural re-configuration. Two functionally distinct types of oestrogen receptors exist encoded for by different genes, namely; ER-alpha and ER-beta[30]. Oestrogen can bind to either of these receptors with the resulting cellular response being different and often opposing[30]. Both these receptors are distributed in different tissues, but they both interact with the same oestrogen ligand; oestradiol-17. These oestrogen receptors can be broken down into 3 different sections dependent on their functional capacity; the N terminal is a flexible hinge region where DNA binding occurs, Ligand/ hormone- binding domain and finally a second transactivation domain located within the ligand binding domain termed as the C-terminal[30]. The n-terminal (A/B DOMAIN) consists of only 18% identical amino acids, in contrast to, the hormone binding domain which possesses 56% identical amino acids[30]. This difference seen between these domains justifies the ideology that ER-alpha and ER-beta may exert their carcinogenic function via different methods in endometrial cancer.

ERα and ERβ are expressed in the stratum basalis in both the glands and stroma and remain unchanged throughout the menstrual cycle[31]. The expression of these receptors is variable in the stratum functionalis, post-menopausal and cancerous endometrium. A reduction in glandular expression of both subtypes of ER can be seen in the secretory phase with no change in stromal expression[31]. Furthermore, the expression of these ERs in the stratum basalis stays constant throughout the phases of the menstrual cycle. Oestrogens' proliferative function is recognized in the endometrium, with both ERα and

ERβ functions implicated in endometrial carcinogenesis[32, 33]. Increasing levels of ER-alpha expression have been evidenced to increase expression of progesterone receptor and proliferation of endometrial cells secondary to the effects of oestrogen. ERβ expression was shown in endometrial endothelial cells suggesting that any direct effects of oestrogen in relation to angiogenesis could be regulated by ERβ expression[31].

1.2.4.2 **Progesterone receptor**

The progesterone receptor (PR) exists in the 2 isoforms PR-A and PR-B. PR expression is regulated by the steroid hormone oestrogen. Low levels of PR expression are noticed in the secretory phase as the progesterone receptor is down –regulated by the hormone progesterone (ligand) via a negative feedback loop[32]. Progesterone is also suggested to down regulate the expression of ERα, thus, limiting further action of oestrogen in the luminal and glandular epithelium and stromal cells, thereby allowing cellular differentiation to ensue[32]. A high PR expression was confirmed in stromal cells adjacent to uterine vasculature[34]. PR-positive stromal cells are thereby implicated in mediating the effects of progesterone on vasculature. Studies regarding the endometrial cancer progesterone receptor status have shown improved overall survival rates in those who have PR-positive tumours when compared with PR-negative patients. A reduction in recurrence of cancer has also been highlighted in PR-positive tumours[34].

The expression of PR-A was localised to the nucleus regardless of the presence of progesterone, whereas PR-B is largely expressed in the cytoplasm in the absence of progesterone, but translocates rapidly to the nucleus in the presence of progesterone[35]. Conflicting evidence exists implicating the role of PR status in endometrial cancers. Literature exists proposing an increase in expression of PR-B in advanced endometrial cancer, whilst another study concludes that an increase in PR-A can be seen in poorly differentiated cancers. A study conducted by Arnette-Mansfield et al[36] suggests the loss of both isoforms of PR in endometrial cancer. In contrast, a study conducted by Kumar et al[37] suggests that PR-A and PR-B are associated with low grade tumours and it is the ratio of PR-A: PR-B that is implicated. If the ratio is <1 it is associated with a poor prognosis.

1.2.4.3 **Androgen receptor (AR)**

Androgen receptor is widely expressed in the stromal compartment of the endometrium[38]. The expression of AR is variable and changes in cycling endometrium[38, 39]. Both androgens and AR play a crucial role in the conversion of stromal cells into decidual cells[39]. The importance of androgen signalling for normal reproductive function was evidenced with a deviation from standard expression resulting in reproductive failure[39]. In the proliferating endometrium a fall in progesterone levels secondary to a rise in AR was shown in recent publications[38]. Studies have hypothesised that the up regulation of AR in glandular epithelium secondary to long-standing progesterone administration is a mechanism through which progesterone exerts its anti-proliferative effects.

In the postmenopausal endometrium the circulating androgens are converted to oestrones causing a small reduction in androgen levels[40]. Inconsistent evidence exists attributing either ovarian or adrenal function as the source of androgens in the post-menopausal state. Either way the dominant hormones in the post- menopausal endometrium are androgens and their role in endometrial carcinogenesis was proposed[39, 41]. The mechanisms through which androgens exert their effect are both direct (AR) and indirect (ER- due to increased conversion of androgens to oestrones). The androgens investigated in endometrial carcinogenesis are testosterone, androstenedione (A_4) and dehydroepiandrosterone (DHEA)[42]. A positive correlation was suggested between serum levels of free testosterone and endometrial cancer risk, but no increased risk was noted with A_4 and DHEA. Obesity is known to be a hyperandrogenic state and an increasing level of obesity within the female population was implicated in an increased risk of endometrial carcinogenesis[38].

1.3 Precancerous states and principles of metastasis

Hyperplasia is known as an abnormal increase in cellular number in a tissue or organ, resulting in an increase in the size of the tissue or organ.

Metastasis can be defined as the transfer of cancerous cells from the primary site of origin to one or more distant sited of the body. All malignant tumours have the capability of metastasising. The pathways through which this can occur are discussed later.

1.3.1 Precancerous states

Increased proliferation of the endometrial glands under the influence of oestrogens results in a greater gland: stromal ratio, when compared with normal endometrium. The proliferating glandular epithelium will show irregularities in shape and size and may display cytological atypia. Endometrial hyperplasia can be divided by its ability to progress into neoplasia or remain non-neoplastic[43, 44].

Neoplastic	Non-neoplastic
• Some complex hyperplasias • All complex atypical hyperplasias.	• Simple hyperplasias • Some complex hyperplasias (without atypia)

Neoplastic hyperplasia need not exist as a precursor state for the development of endometrial cancer. However, if present a high risk of progression to endometrial carcinomas exists. This form of hyperplasia tends to progress into the most common form of endometrial carcinoma i.e. endometrioid carcinoma.

World Health Organisation (WHO) classification of endometrial hyperplasia takes 2 specific features into account:

- The architectural pattern of the endometrium: simple/ complex
- Presence of nuclear atypia.

This permits four categories of classification for endometrial hyperplasia[45, 46], as shown below:

- Simple hyperplasia no atypia
- Simple atypical hyperplasia
- Complex hyperplasia no atypia
- Complex atypical hyperplasia

In 2014 WHO devised a new classification method for hyperplasias. The 2 categories include:

- Hyperplasias with atypia
- Hyperplasias without atypia

The occurrence of simple atypical hyperplasia is rare and women with simple hyperplasia without atypia are least at risk of developing endometrial cancer[43]. The most likely cohort that is at an increased risk of developing endometrial cancer is women who are diagnosed with complex atypical hyperplasia[43]. The most insidious finding is nuclear atypia which carries a great risk of incidence of cancer[47].

Women suffering from endometrial hyperplasia tend to present with abnormal uterine bleeding, vaginal discharge or cervical smear abnormalities[46]. Statistically the risk of progression to cancer in complex atypical hyperplasia is around 30% in contrast to no cytological atypia where the risk drops to less than 5%[47] . In addition recent evidence has proposed that the risk of developing well –differentiated endometrial carcinoma from complex atypical hyperplasia has increased to 40-50%[48]. The variance in risk stated above exists due to the difficulty if attributing percentage risk of progression into EC in patients with hyperplasia. Many patients who have a biopsy diagnosis of atypical hyperplasia will also have endometrial cancer in the hysterectomy specimen. In addition an increasing number of patients are developing concurrent carcinomas. The risk of progression will be difficult to assess as most patients will have a hysterectomy. In 2000 the endometrial intraepithelial neoplasia classification was proposed by an international group of pathologists. It suggests two classes of endometrial changes that are clinically relevant such as; endometrial hyperplasia (EH) and endometrial intraepithelial neoplasia (EIN). EH is diagnosed with endometrial morphology that is proliferative and includes a few cysts in and EIN should displace stroma resulting in a stromal volume that is approximately half the tissue volume. The intraobserver reproducibility would not be high to the trained professional and not to a less experienced eye. Furthermore, there is a lack of ability to

differentiate between severity and therefore groups findings that would require individually tailored treatment together[49].

With the increasing rates of obesity, women are exposed to higher levels of endogenous oestrogens which increases the risk of endometrial hyperplasia[46]. Obesity is a hyperandrogenic state, an increased level of aromatase mediated conversion of androgen to oestrones occurs, thereby exposing the endometrium to unopposed oestrogens, thus, increasing risk. In contrast, the use of combined oral contraceptives was shown to decrease the risk of developing endometrial hyperplasia[46].

1.3.2 Principles of metastasis

In health, normal cells retain their architecture with basal to apical differentiation and an intact basement membrane. The primary beginnings of abnormality and loss of normal differentiation can be seen when cells become dysplastic. Dysplastic cells are characterised by their abnormal proliferation and loss of cell shape, size and orientation[50]. Malignant change within cells is apparent when there is a high nuclear/cytoplasmic ratio and clumped chromatin. The lack of invasion into the basement membrane results in the carcinoma being termed as '*in situ*'. When invasion of the basement membrane occurs with the involvement of collagenases and hydrolases, it is termed an invasive carcinoma.

Two theories of metastatic spread have been proposed. Stephen Paget[51] suggested that metastases arose from the replication of a few tumour cells in select organs that provided conditions required for growth. 40 years later James Ewing proposed the 'mechanical entrapment theory' that focused on the anatomical progression of metastases[52]. The notion of this theory explains that the first organ that encounters these cancerous cells will house the largest number of metastatic clusters[52]. Both the theories have some accuracy as some metastases are colonised in the first organ, whereas some are more selective in their spread.

Locally invasive carcinoma has the possibility of metastasising via haematogenous/ lymphatic spread[50]. Many cells can break away from the tumour on a daily basis, but not all of these will metastasise to distant organs within the body. In order to successfully metastasise the neoplastic cells need to survive many steps, primarily the initial separation

from the primary tumour and entry into the blood/ lymph system[50]. Furthermore, in an immune-competent individual the cells need to be able to survive the body's immune response during their travel to their new location. Once in the vascular or lymph system their ability to adhere and invade the vessel walls to arrive at their new site and their ability to thrive there determines their metastatic potential. Due to the vast number of hurdles that the cancerous cells need to overcome, by the time they establish themselves at another location they may no longer resemble the cells of the primary tumour leading to difficulties with treatment[50].

As endometrial cancer generally tends to present early with abnormal uterine bleeding the cancers can be treated with a local surgical procedure and adjuvant pharmacological therapies if required. Cancers that originate in the endometrium can spread locally into the vagina, peritoneum and omentum.[53-55] Metastatic spread to the liver and lungs are common. In some other highly invasive cancers that present late, the cells may have already metastasised and established themselves at other sites at the time of initial diagnosis. The detection of metastasis prior to the primary cancer- is termed 'cancer of unknown origin'[50].

1.4 Endometrial cancer

1.4.1 Epidemiology

Endometrial cancer is the 6th most common malignancy amongst females worldwide and the 4th most common amongst women in developed countries[55]. On average 7400 cases are registered in the UK annually[54]. Over 250+ endometrial cancer patients are diagnosed and treated each year at the Liverpool Women's hospital where this study is being conducted. More than 90% of cases are diagnosed in females over 50 years of age with a median age of diagnosis stated at 63 years[54, 55]. In the UK and EU there was an increase in incidence of endometrial cancer of more than 40% between the years of 1993-2007 with a predicted 100% increase in incidence by the year 2025 as stated in a Norwegian study[6]. In addition the incidence is reported to double by 2015 from the year 2005[6]. Research has shown that the spread of this disease is highest in developed countries such as those of North America and Central and Eastern Europe with lowest incidences occurring in Central and Western Africa. The increase in incidence of this condition is attributed to the continual increase in body mass index and life expectancy[56].

1.4.2 Classification of endometrial cancer

Endometrial cancer generally tends to arise from malignant transformation of epithelial cells, but rarely cancer may be of stromal origin[57]. Endometrial carcinoma can be classified into two major groups dependent on its appearance at light microscopy, clinical behaviour and epidemiology[57]. The majority of endometrial cancers are endometrioid carcinomas. Nevertheless, rare cancers such as carcinosarcomas also arise from the endometrium.

Table 1: Classification of endometrial cancer[57]

Endometrial Cancer	
Type 1 Endometrioid (75-80%)[57]	**Type 2 (15-20%)[57]**
• Ciliated adenocarcinoma • Secretory adenocarcinoma • Papillary/villioglandular • Adenocarcinoma with squamous differentiation	• Uterine papillary serous (<10%) • Mucinous (1%) • Clear cell (4%) • Squamous cell (<1%) • Carcinosarcoma/ MMMT (10%) • Undifferentiated

Type 1 endometrial cancers tend to arise from complex atypical hyperplasias and are linked with prolonged periods of unopposed oestrogen exposure[47]. These cancers are well differentiated and composed of glands that resemble those found in normal endometrium. They tend to be of a lower grade (1/2) and have a favorable prognosis. In contrast, type 2 endometrial cancers develop from atrophic endometrium and are not oestrogen dependent[58]. Grade 3 endometrioid carcinomas are classed as type 2 with the inclusion of tumors of non-endometrioid histology. They tend to be less differentiated, possess an increased risk of metastases and are associated with a poor prognosis[53-55].

The current classification of EC into type 1 and type 2 EC is very simple and difficulties arise in assigning grade 3 endometrioid carcinomas into either of those categories. New molecular/genomic approach of classification of endometrial cancer has arisen combining both genomic and histopathological features to create and integrated classification of clinical and biologically relevant subcategories of EC. The genomic classification divides endometrial cancers into 4 groups as seen in table 2[59].

Table 2 Four genomic classifications of endometrial cancer[59]

	POLE (ultramutated)	**MSI (hypermutated)**	**Copy-number low (endometrioid)**	**Copy-number high (serous-like)**
Copy-number aberrations	Low	Low	Low	High
MSI/*MLH1* methylation	Mixed MSI high, low, stable	MSI high	MSI stable	MSI stable
Mutation rate	Very high (232 × 10^{-6} mutations/Mb)	High (18 × 10^{-6} mutations/Mb)	Low (2·9 × 10^{-6} mutations/Mb)	Low (2·3 × 10^{-6} mutations/Mb)
Genes commonly mutated (prevalence)	*POLE* (100%) *PTEN* (94%) *PIK3CA* (71%) *PIK3R1* (65%) *FBXW7* (82%) *ARID1A* (76%) *KRAS* (53%) *ARID5B* (47%)	*PTEN* (88%) *RPL22* (37%) *KRAS* (35%) *PIK3CA* (54%) *PIK3R1* (40%) *ARID1A* (37%)	*PTEN* (77%) *CTNNB1* (52%) *PIK3CA* (53%) *PIK3R1* (33%) *ARID1A* (42%)	*TP53* (92%) *PPP2R1A* (22%) *PIK3CA* (47%)
Histological type	Endometrioid	Endometrioid	Endometrioid	Serous, endometrioid, and mixed serous and endometrioid
Tumour grade	Mixed (grades 1–3)	Mixed (grades 1–3)	Grades 1 and 2	Grade 3
Progression-free survival	Good	Intermediate	Intermediate	Poor

As we can see in table 2, a simple classification of endometrial cancer into type1 and type 2 is insufficient due to the diversity of endometrial cancers. Type I EC according to the genomic classification encompass tumour with microsatellite instability, copy- number low and POLE characteristics. In contrast, type 2 EC may include grade 3 and serous carcinomas[59]. Genomic classification of EC will have application beyond therapeutic aid and

could possibly allow for the development of accurate screening tests and methods for disease monitoring[59].

1.4.3 Histopathology and grading

The most common site of origin of endometrial cancer is within the corpus proper; it may also develop in the lower uterine segment[60]. On a macroscopic level no distinction can be made in regards to the cell type of the cancer. The appearance may be grossly described as a lush, polypoid expansile mass with yellow necrotic regions, located within the endometrial cavity[60]. The cancer can be visible as a single, large dominant mass or widespread involvement of the endometrium can show a sunken appearance with no visible exophytic mass. In the presence of myometrial involvement, islands of well-defined grey-white areas are seen when compared to the surrounding unaffected myometrium.

Microscopic assessment of the cancer tissue allows the cell type to be differentiated, guiding the appropriate management. Characteristic microscopic changes that occur within endometrial cancer cells include: 1) stromal invasion – cribriform pattern of well differentiated endometrial cancer, 2) presence of cytological atypia (prominent = high grade, minimal= low grade), 3) myometrial invasion[61].

The current most validated classification of staging and grading for endometrial carcinoma was developed by The International Federation of Gynaecology and Obstetrics (FIGO), refer to table 3. This ever evolving classification provides a common language between professionals when diagnosing and managing patients. In 1998 FIGO classification of endometrial carcinoma was based mostly upon the architecture of the cancer as seen in table 2.

Table 3: 1998 FIGO classification of grading endometrial cancer[62, 63].

Grade 1	< 5% of solid areas
Grade 2	6-50% solid areas
Grade 3	> 50 % solid areas

Table 4: Revised FIGO 2009 staging for endometrial cancer (excluding uterine sarcomas[55, 62])

ENDOMETRIAL CANCER STAGING[63]	
IA	Tumour confined to the corpus uteri, +no involvement of myometrium OR +< ½ myometrial invasion
IB	Tumour confined to the corpus uteri, +> ½ myometrial invasion
II	Tumour invades cervical stroma but does not extend beyond the uterus
III	Local and / or regional spread of tumour
IIIA	Tumor invades the serosa of the corpus uteri and/or adnexae
IIIB	Vaginal and/or parametrial involvement.
IIIC	Metastases to pelvic and/or para-aortic lymph nodes
IIIC1	Positive pelvic nodes.
IIIC2	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
IV	Tumor invades bladder and/or bowel mucosa, and/or distant metastases
IVA	Tumor invasion of bladder and/or bowel mucosa
IVB	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes.

1.4.4 Associated risk factors

A risk factor is defined as any reason that alters a person's risk of developing morbidities. The risk factors that lead to the development of endometrial cancer are mainly secondary to excessive unopposed oestrogens, either endogenous or exogenous[53-55]. High levels of oestrogen have a proliferative effect on endometrial cells, thereby increasing mitoses and the likelihood of mutations.

Obesity (BMI 30-39.9 kg/m^2) and morbid obesity (BMI>40kg/m^2), increasing age, nulliparity and longer reproductive years of a woman are vital risks that increase the possibility of females developing endometrial cancer[64] as highlighted in table 4. In pre-menopausal women an imbalance between the 2 main regulatory hormones; oestrogen and progesterone can cause a disruption in the monthly menstrual cycles. In females who have polycystic ovarian syndrome (PCOS) higher levels of oestrogen and androgens exist systemically when compared to progesterone, contributing to an increased risk of neoplastic change[65].

Extensive research into the correlation between obesity and endometrial cancer has shown that with increasing BMI there is a 3.5 fold rise in risk of developing endometrial cancer. Within the UK 50% of women diagnosed with endometrial cancer have a BMI >30kg/m^2. A high BMI (>30kg/m^2) is associated with an overall increased risk in developing endometrial cancer. Furthermore, a positive correlation was revealed between obesity and the development of endometrioid cancers when compared to their non-endometrioid counterparts[66]. In the post-menopausal state there are low levels of circulating oestrogens. In obese women the peripheral adipose tissue allows the enzyme aromatase to convert adrenal androgens and existing androgens into oestrogen[67, 68] refer to figure 3. This creates an unopposed (by progesterone) hyper-oestrogenic state in these post-menopausal females. This continual stimulation of oestrogen receptors allows the endometrium to retain its proliferative functions, rather than becoming atrophic as expected post menopause. This persistent endometrial cell proliferation creates an increased risk of neoplastic change secondary to an increase in probability of mutations occurring in these cells[46, 66].

Obesity not only poses a threat via alteration of physiological function[56] but also affects management of women suspected of or diagnosed with endometrial cancer. Obese

women are at an increased risk of developing co-morbidities, such as: hypertension and secondary cardiac events or insulin resistance[69]. A more intensive peri-operative assessment is required to minimise the problems due to co-morbidities. This intensive care in turn places pressure on medical resources. Therefore reassessment of treatment for endometrial cancer in the rising BMI population is necessary.

The use of combined oral contraceptives was proven to be a protective factor against the development of endometrial cancer[70]. The protection lasts for up to ten years after cessation of the pill. In contrast, other drugs such as: Tamoxifen (selective oestrogen receptor modulator) is thought to have pro- oestrogenic effects in the endometrium, thereby- contributes to increasing the risk of developing endometrial cancer.

Table 5: Endometrial cancer risk factors

Endometrial cancer risk factors[53-55]			
Factors implicated in **increased risk**	Factors associated with **decreased risk**	Interventions associated with **decreased risk**	**No effect on risk**
• Endogenous or exogenous oestrogens • SERMs: Tamoxifen and Raloxifen • Anovulatory cycles • Obesity • Increasing age Genetic predisposition: • PCOS[65, 71] • Lynch syndrome[72]	• Increasing parity • Increasing lactation	• COCPs • Physical activity	• Weight loss • Increased intake of fruits, vegetables and vitamins

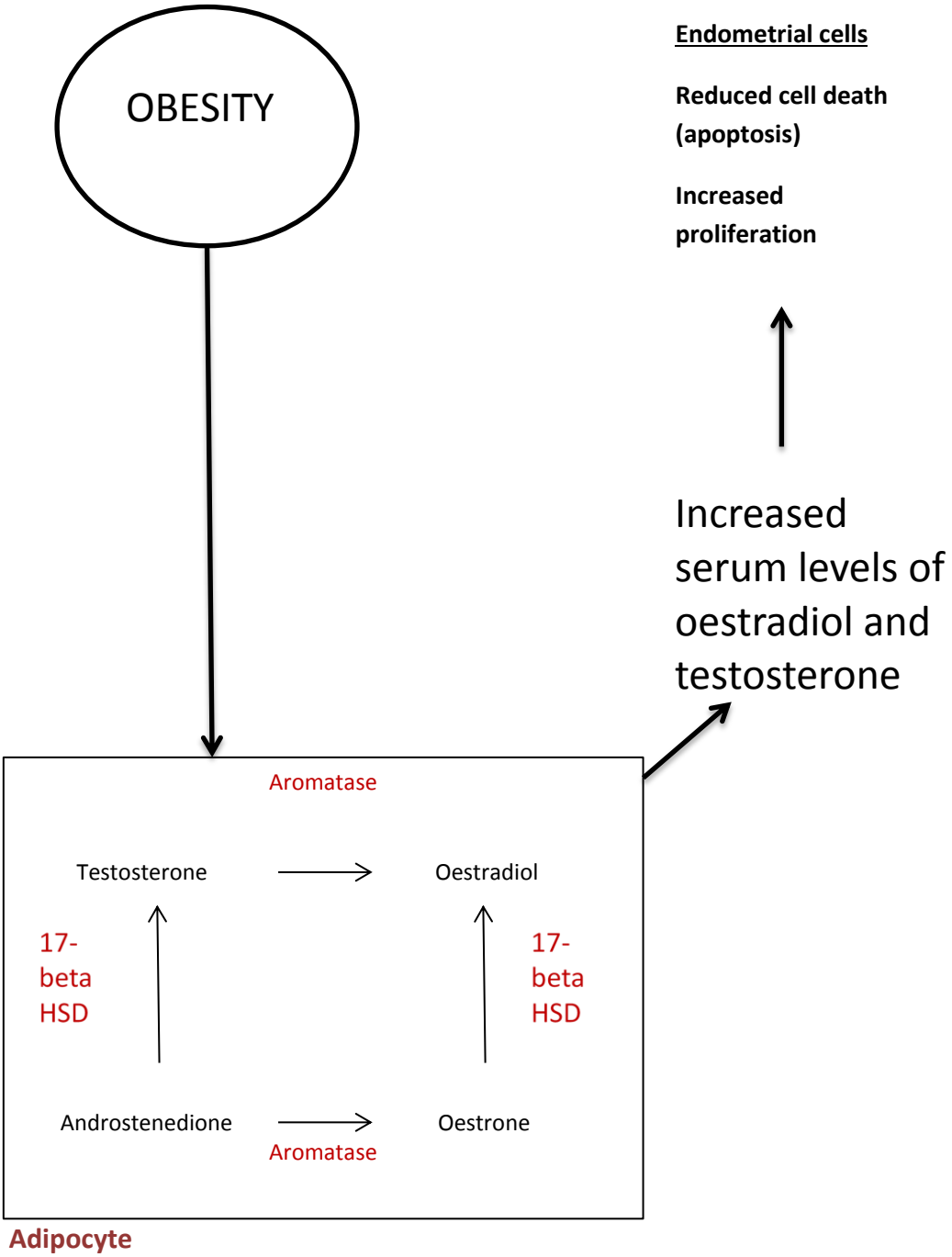


Figure 4 Conversion of excess androgens resulting in elevated plasma oestradiol and testosterone in obesity contributing to increased risk of endometrial carcinogenesis.

1.4.5 Clinical presentation and management of patients

Endometrial cancer presents early with abnormal uterine bleeding (post-menopausal bleeding or intermenstrual bleeding). Blood soaked discharge and pelvic pain are other ominous ways in which the cancer may present, as they are also privy to menopausal women and are not specific to cancer. Peak incidence occurs in women aged mid to late 50s or above, with 1:100 females being affected. Once a female presents with symptoms suggestive of cancer they are assessed rapidly and managed accordingly[53-55].

The National Institute of Clinical Excellence (NICE) has set gold standard guidelines for the management of patients with suspected endometrial cancer. The primary assessment involves a thorough clerking of the presenting complaint followed by a bimanual examination[55]. Once an appropriate referral to the gynaecology outpatient department is done, a transvaginal ultrasound scan is the first investigation of choice. Samples of endometrium obtained from a pipelle biopsy or dilatation and curettage[73] at hysteroscopy are analysed. If pathology suggestive of neoplasia is detected further management of the patient is reviewed by the gynaecologic oncology MDT[55].

Management is highly dependent upon the stage of cancer at diagnosis[62]. Diagnostic procedures can be invasive or non-invasive. Invasive methods involve the use of dilatation and curettage (D&C), endometrial biopsies or hysteroscopy and directed biopsy. Non-invasive methods include ultrasonography or endometrial cytology[74].

With endometrial cancer the hallmark of treatment is surgery. Procedures such as total abdominal hysterectomy with bilateral salpingo-oophorectomy and peritoneal washings are most common treatment measures. In the presence of advanced stage disease local lymphadenectomy and omentectomy can be carried out to analyse metastatic potential of the cancer[62, 74, 75].

Previous inconsistencies exist with the diagnosis of non- atypical endometrial hyperplasias generally being phenotypically poly-/ mono- clonal[61, 76]. In contrast, clear evidence has emerged defining the architectural diagnostic criteria required, thereby standardising the diagnosis of endometrial hyperplasias. Management of hyperplastic endometrium is varied depending on the presence of cytological atypia. Simple and complex hyperplasias with no cytological atypia are managed conservatively with use of progesterone therapy[77]. The

most commonly used progesterones are medroxyprogesterone acetate or megestrol acetate[78]. They may be administered in a cyclical or continuous fashion. In addition to oral progestins many patients are now treated with Mirena (levonorgestrel) which provides local progesterone and proposed to be more effective. In the presence of cytological atypia no role exists for conservative pharmacological management due to the strong association (19% to 62%) between invasive carcinoma and cytological atypia. In the presence of simple or complex atypical hyperplasias, hysterectomy is adopted as the choice of treatment.

In contrast, literature validating the role of progesterone therapy leading to regression of atypical hyperplasias in 60% to 95% of patients was published. Nevertheless the strong relationship between invasive EC and atypical hyperplasia showing a high risk of progression results in hysterectomies being the standard treatment, reserving progesterone therapy for women who require preservation of fertility.

Generally low grade well differentiated adenocarcinomas without myometrial invasion are managed with a TAH+BSO alone without the necessity for node sampling or adjuvant therapies, refer to table 5. Poorly differentiated high grade cancers (stage 2 and above) are given post-operative radiation[62].

Radiotherapy and chemotherapy are adjuvant therapies that can be employed in the management of endometrial cancer. Radiotherapy was useful to shrink cancers that are inoperable to allow surgical management. Current research is being carried out analysing the efficacy of combined chemotherapy and radiotherapy or the effect of radiotherapy alone in palliation[55].

Table 6: Management options for endometrial cancer[55]

Treatment options for endometrial cancer			
Surgery	**Radiation therapy**	**Chemotherapy**	**Palliation**
Total hysterectomy TAH+BSO, peritoneal washings. +/- Lymphadenectomy +/- Omentectomy	External beam/ vaginal brachytherapy	Systemic / regional chemotherapy Carboplatin+/- Doxorubicin+/- Taxol	Radiotherapy Hormone therapy Supportive interventions

For prognosis the FIGO staging criteria holds much importance and weight. 5 year survival rates are generally promising when the disease is picked up at an early stage. More than 50% of recurrences are secondary to type 2 endometrial carcinomas i.e. clear cell or serous carcinomas. A delay in diagnosis leads to a rapid drop in the survival rates of these women, as highlighted in table 6:

Table 7 5 year survival rates for endometrial cancer[55]

STAGE	**5 – YEAR SURVIVAL RATE**
1	92%
2	75%
3	50%
4	20%

Although endometrial carcinoma is a rising problem with the western population, screening for early endometrial cancer is only likely to marginally reduce mortality. Furthermore, current minimally investigative methods i.e. histology from endometrial biopsies or transvaginal ultrasonography (TVU) are highly accurate for diagnosis and the early presentation of endometrial cancer with postmenopausal bleeding means that disease can be caught in its early stages. Emphasis should be placed on health education of the current population to the importance of investigating abnormal uterine especially in post-menopausal women or those undergoing Tamoxifen therapy.

1.4.6 Pathogenesis of endometrial cancer

Several molecular pathways have been implicated in the initiation and maintenance of endometrial carcinoma[28]. The exact biological interactions that occur are not clearly defined. The architectural changes seen in endometrial cancer cause a disruption in the normal hormonal interaction between the glands and extra cellular matrix/ stroma[28]. Some of the pathways that are postulated to contribute to the development of endometrial cancer have been highlighted.

1.4.6.1 Embryology

Stem cells can be defined as unspecialised cells that possess the capability to replicate via mitosis to produce more undifferentiated cells or become tissue specific cells that carry out specific functions. They have the ability to regenerate on a long-term basis retaining their undifferentiated status with a high proliferative potential[79]. The role of endometrial somatic stem cells present in the stratum basalis is to regenerate the endometrial stratum functionalis on a cyclical basis. Therefore the malfunctioning of these stem/ progenitor cells and/or their surrounding cells can contribute to the incidence of gynaecological disease, such as; endometrial hyperplasia, endometrial cancer or even the formation of endometriosis[80].

1.4.6.2 Hormonal imbalance

Prolonged, unopposed oestrogen exposure was strongly linked as a risk factor in endometrial cancer. In the normal cycling endometrium the presence of progesterone counter-acts the increased level of oestrogen secretion by the corpus luteum. The endometrium proliferates under the effects of oestrogen, whereas progesterone has an anti-proliferative effect on the endometrial cells, thereby inhibiting the epithelial to mesenchymal cell transition[77]. In advanced disease a loss of progesterone signalling or an increase in systemic levels of oestrogen encourages endometrial proliferation. Although elevated oestrogen levels pose a risk, development of cancer is multifactorial and ensues in response to genomic mutations[77].

1.4.6.3 **Genetic Mutations**

Multiple genetic mutations are responsible for the development of endometrial cancer. Different pathways are implicated in endometrial carcinomas, dependent on their oestrogen sensitivity[81]. In addition to the microsatellite instability seen in endometrioid carcinomas, the most frequently altered gene is phosphatase and tensin homologue (*PTEN)* and a mutation in the β-catenin gene is also seen in 20% of these cancers. Kirsten rat sarcoma viral oncogene homologue (*K-ras)* mutations are detected in 15-30% of these cancers, and show no association with the existence of endometrial hyperplasia[81]. Dualistic model of carcinogenesis can be seen with specific genetic mutations being attributed to the 2 types of endometrial carcinomas. Type 1 endometrial carcinomas are associated with mutations in: PTEN[82] as aforementioned, KRAS2 oncogene[82], faults in DNA mismatch repair and near- diploid karyotype. In contrast, most type 2 endometrial carcinomas (non-endometrioid) are most are non-diploid and associated with TP53[83] and *Her2/neu* pathways[82, 84].

79% of endometrioid endometrial cancers have reported mutations in the PTEN tumour suppressor genes. The loss of this gene is mainly localised to the tumour epithelia. The somatically mutated cells that failed to express the PTEN protein secondary to mutation and /or deletion of the gene were found to form high-density clusters[85]. Conflicting evidence exists describing the effect of progesterone therapy on these tumours. However, recent evidence has shown the high sensitivity of these tumours to progesterone therapy and described PTEN as a possible biomarker of response to progesterone therapy in endometrial cancer patients.

Inheritable mutations e.g. Lynch syndrome characterised by a MSH2/MSH6 protein complex deficiency is associated with [72] endometrial cancer. The development of endometrial cancer is localised to the lower uterine segment and endometrioid carcinomas of a woman of any age whose tumour displays peritumoural lymphocytes and tumour heterogeneity is associated with Lynch syndrome. Particularly in dedifferentiated EC[86].

1.4.6.4 **Trans-sulphuration pathway**

Hydrogen sulphide is one of the by-products of sulphur metabolism within the body. A gaseous transmitter within the body, H_2S was implicated in the pathogenesis of colon and ovarian carcinomas. The angiogenic and cellular proliferative functions of H_2S have based the mechanisms through which it initiates and maintains neoplasia. Comprehensive investigations carried out on cancer cell lines have affirmed the possibility of using enzyme inhibitors to limit the production of H_2S and thereby halt tumour progression. This role of H_2S has not yet been investigated in the endometrium and its prospective pathway that may contribute to endometrial carcinogenesis.

1.5 Hydrogen sulphide

Hydrogen sulphide (H_2S) is a colourless, flammable gas, famously known by its rotten egg odour. It is a highly water soluble and lipophilic gas[4, 7, 32, 87]. H_2S is an upcoming novel gaseous transmitter implicated in tumourigenesis behind nitric oxide (NO) and carbon monoxide (CO)[4]. H_2S is detected at levels of 0.0047 ppm and at a higher concentration of approximately 500 ppm it is known to be fatal. Like the other noxious gases, in the body it is converted to a less toxic form. *In vivo* H_2S is synthesised by 2 possible methods e.g. the local sulphate- reducing bacteria present in the colon or metabolism of L-cysteine.

Initial studies conducted by Kimura in 1996[88] defined the role of H_2S as a neuromodulator in the brain, thus propelling research associated with biological effects of H_2S. The functions of hydrogen sulphide have been suggested in an increasing number of tissues including: anti-nociception[89], blood pressure[90] control mediated by vascular dilatation and neuromodulation[91] by shielding the neuron from oxidative stress and stimulating glutathione (GSH) production[92]. Increasing the concentrations of H_2S in cancer may be reversed by the use of H_2S synthase inhibitors (CBS/CSE inhibitors)[93], thus, implicating the successful role of H_2S synthase inhibitors in modulating H_2S production.

Studies investigating the role of H_2S as a pro-/ anti-apoptotic agent have emerged[94]. H_2S was implicated in modulating the proliferation of various cells within the body, displaying a tissue specific action[94]. Its highly soluble and lipophilic nature allows the rapid diffusion of H_2S through cell membranes without the need for transport proteins, allowing it to have rapid effects on cells[95]. Once H_2S has entered the cytoplasm, intracellular signaling is activated resulting in communication between signaling and target proteins. Under the influence of H_2S the target proteins alter cellular programming to bring about apoptosis or DNA repair. Mitotic division of cells is a highly regulated process and regulated by DNA expression. H_2S was proposed to alter cellular replication by interrupting the cell cycle phases[96]. However, its definite role is still under debate.

The pharmacological functions of H_2S on cell growth and its uses as an alternate therapy for inflammation and pain have been described[97]. Peculiarly a rise in endogenous H_2S synthesis is seen at sites of inflammation, associated with exponential proliferation of cells[97]. Its anti-inflammatory function has not been clearly defined. Further to this a study conducted in septic mice has shown that H_2S exerts its effects by restoring neutrophil migration (via an ATP-dependent K^+ channel- dependent pathway) to sites of infection[98].

The cytoprotective and therapeutic nature of H_2S was described in studies investigating its roles in vascular diseases e.g. atherosclerosis[99], hypertension[99] and ischaemic- reperfusion injury[100] or in inflammation as previously described. In addition the scavenging role of H_2S was described, thus regulating the production of reactive oxygen species (ROS) and conserving tissue function[101]. In contrast, it has also been described to potentiate diseases such as asthma[102] and diabetes[103].

1.5.1 Endogenous H_2S synthesis

The source of sulphide within our bodies comes from the essential amino acid; methionine. Methionine is not synthesised in the body and therefore needs to be ingested. The production of endogenous H_2S occurs by desulphuration of cysteine (derived from methionine) via the trans-sulphuration pathway, the only pathway capable of removing sulphur-containing amino acids when in excess[4, 104-106]. The three enzymes involved in the conversion of cysteine to H_2S include CBS a homotetramer, CSE a tetramer and 3-MPST a disulphide-linked dimeric protein or a monomer. Although the expression of all three enzymes is tissue specific, they all possess the same role; conversion of cysteine and its derivatives to H_2S[4, 7, 87].

In adults CBS is expressed predominantly in the brain, nervous system and liver. CBS is a sulphur metabolising enzyme coded for by the CBS gene and is mapped onto the gene locus on the long (q) arm of chromosome 21 at position 22.3[107]. The presence of this gene enables the production of CBS *in vivo*. CBS has a molecular weight of approximately 530 kDa and consists of 3 functional domains; the C-terminal, N-terminal and middle domain. Composed of haem, the N terminal domain regulates the enzyme in response to redox reactions. The C- terminal domain controls the allosteric activation of the enzyme by S-adenosylmethionine. Finally, the middle domain account for the catalytic activity that is responsible for the production of hydrogen sulphide via the pyridoxal 5 phosphate prosthetic group[107, 108]. This enzyme occupies a pivotal position in mammalian sulphur homeostasis at the homocysteine junction[109]. At this point in the chemical pathway CBS catalyses the conversion of homocysteine to cystathionine via the use of vitamin B6 to enable the β-replacement of the sulphhydryl group of homocysteine and the hydroxyl group derived from serine to form cystathionine[7]. In the presence of a mutation of this

gene, homocystinuria develops. 150 mutations of this gene have been implicated in the development of this disease. These mutations lead to a disruption in the standard functioning of this enzyme causing a build-up of homocysteine and other toxic compounds systemically, resulting in its excretion via the urinary system. Hyperhomocysteinaemia has also been implicated as a risk factor for co-morbidities such as preeclampsia, congenital birth defects e.g. spina bifida and miscarriages in humans[110]. However, no literature was published looking at CBS expression in cycling, atrophic or cancerous endometrium. To date its expression and role in the human endometrium has not been defined.

Human CSE is a protein made up of four subunits, 2 dimers and each monomer binds to pyridoxal 5- phosphate (P5P), the prosthetic group of this enzyme. CSE is mapped onto the gene locus on the short arm of chromosome 1, made up of 13 exons and 12 introns. Secondary to a variation merging of the protein, 3 different isoforms of CSE exist. CSE is a key enzyme in glutathione synthesis and enters the chemical pathway at the cystathionine junction where cystathionine is converted to cysteine and alpha-ketobutyrate. The enzyme CSE converts cystathionine by catalysing the elimination of α, γ-carbon to produce cysteine, ammonia and α-oxobutyrate. In addition CSE was described to potentiate the β-elimination of cysteine to form the intermediate thiocysteine, which in turn degrades to form H_2S[111]. The α, β-elimination of cysteine was described as the predominant reaction for H_2S synthesis. A reduction in expression of CSE leads to a reduction in levels of cysteine and a systemic increase in cystathionine causing cystathioninuria[112]. This is an autosomal recessive condition that results in an excess of cystathionine excretion in the urine, secondary to the high levels in the blood due to dysfunctional conversion to cysteine. In vascular endothelium H_2S produced by CSE was implicated in neovascularisation. Therefore CSE expression is expected to be strong in the endothelial cells[113]. The mechanism through which CSE regulates angiogenesis will be discussed later[114].

3-mercaptopyruvate sulphurtransferase, also known as 3-MPST, is part of the chemical family of transferases. This class of enzymes regulates the transfer of functional groups from a donor molecule to the acceptor molecules. This family of enzymes throughout the body oversees many biochemical reactions and therefore they play an integral role in physiological functioning[115]. 3-MPST occupies a key role in 2 major reactions such as cysteine degradation and cyanide detoxification[116]. The role of 3-MPST under investigation is its ability to convert cysteine into H_2S as part of the trans-sulphuration pathway. A

deficiency of this enzyme has reported to play a part in a rare inheritable disorder known as mercaptolactate-cysteine disulphiduria (MCDU)[117]. A defective MPST gene has not been identified and associated with this disorder.

3-MPST is now well recognised as a H_2S generator. It is expressed in most cells and is distributed between the mitochondrial and cytosolic compartments of most tissues[118]. H_2S produced via the 3-MPST pathways was evidenced to play a role in vasodilation or as a neurological signalling molecule. Experiments conducted in hepatocytes have shown the down regulation of intramitochondrial 3-MPST mediated production of H_2S secondary to exposure to increased levels of H_2O_2[119]. Therefore under conditions of increased oxidative stress H_2S production via the 3-MPST pathways is proposed to be reduced.

Oxidative stress occupies a vital role in mammalian bioenergetics. The reactive oxygen species (ROS) e.g. superoxide (O_2^-) are produced under physiological and pathological conditions[120]. Mitochondrial respiration produces oxidants that are successfully counteracted by endogenous antioxidants. However, in conditions of increased oxidative stress this clearance of radicals does not happen optimally. In endometrial adenocarcinoma and hyperplastic endometrium the activity of the antioxidant superoxide dismustase is significantly down regulated by 50%, when compared to benign endometrial disease[120]. This reduction in anti-oxidative activity leads to an accumulation of free radicals, thereby increasing oxidative stress on cells[120]. No evidence exists looking at the regulation of 3-MPST in endometrial cancer and with above the evidence a reduction in 3-MPST mediated production of H_2S should be seen.

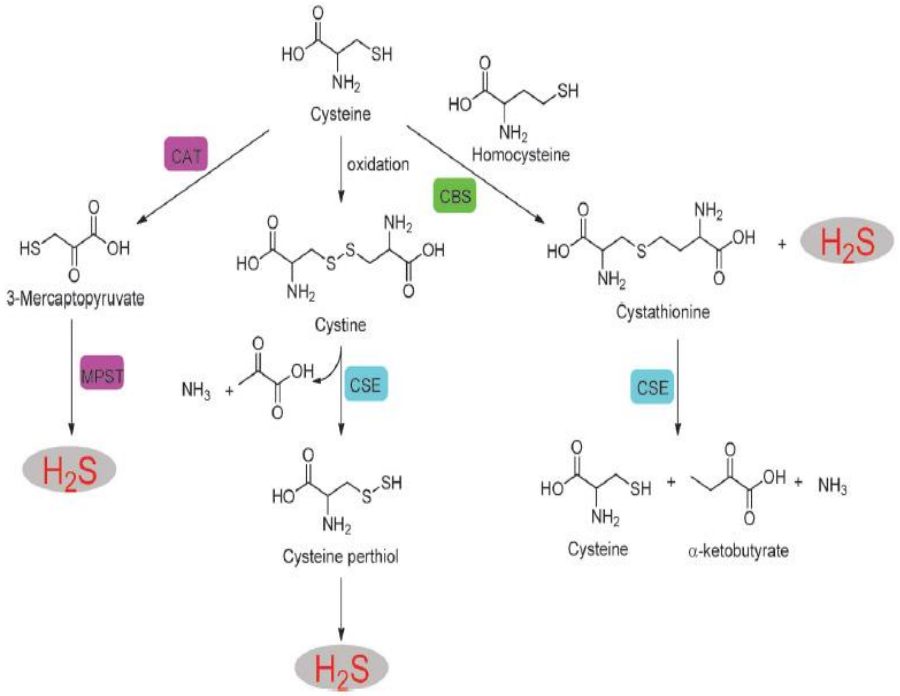


Figure 5 Conversion of L-cysteine into hydrogen sulphide via the trans-sulphuration pathway. (Adapted from: H2S releasing agents: chemistry and biological applications)[2]

1.5.2 Commercial compounds used in the investigation of H_2S in tissues

H_2S is a sulphur analogue of water with a lower polarity allowing it to dissociate freely in a medium of any polarity[121]. As aforementioned the highly lipophilic nature of H_2S allows it to dissolve and cross cell membranes without the need for transporter proteins. It is weakly acidic in solution with a pH 7.4 at 37°C as found *in vivo.* At 37°C 80% of H_2S dissociates to form HS^- ions. It is not clearly defined which form of H_2S contributes to its physiological effects. *In vivo* the predominant HS^- species binds to haemoglobin voluntarily or reacts with other metallic compounds[121].

The role of H_2S in redox reactions was explored and its strong 'reducing' nature was confirmed[122]. It readily oxidizes to different forms of sulphur i.e. sulphur or sulphur dioxide and these reactions are catalysed by the presence of metals including Fe^{2+} and blocked by the addition of metal ion chelators such as diethylene triamine pentacetic acid (DTPA)[123].

Besides directly measuring H_2S levels drugs can also be used to indirectly observe the effects in biological samples. Drugs related to hydrogen sulphide can be classified as H_2S administrators or inhibitors of H_2S synthesising enzymes. The H_2S administrators augment the systemic and local production of H_2S and the inhibitors have the opposite effect. H_2S donors can be organised into 3 groups; inorganic, organic and agonists of H_2S synthesising enzymes[124].

1.5.2.1 Inorganic sulphide salts[2]

The inorganic compounds such as sodium hydrogen sulphide (NaHS) and sodium sulphide (Na_2S) have a rapid reactivity upon contact with aqueous solutions, with a half-life of 5 minutes[2]. The use of inorganic compounds in research is limited as a rapid peak in H_2S, as seen in a pulse cure, can be toxic to local tissues and errors in long term investigations leading to divergent results. In contrast, inorganic sulphide salts have been employed to treat various diseased cellular states, with resultant protective effects being exposed. Protective effects of sulphide salts were noticed against myocardial injury and inflammatory states such as osteoarthritis. Nevertheless these effects were highly sensitive to concentrations and duration of treatment, and showed pro-inflammatory effects when exceeding therapeutic parameters.

This highly unstable release of H_2S by these inorganic donors impedes tight control over H_2S release *in vitro* and can be damaging *in vivo*. Notably commercial inorganic sulphides consist of impurities leading to the formation of polysulphides in NaHS solutions[2].

1.5.2.2 Lawesson's reagent 2

2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulphide or also known as Lawesson's reagent is produced by combining an anisole with phosphorus pentasulphide over heat. This reagent expressed H_2S donor properties as an anti-inflammatory. Its role as a H_2S donor is limited secondary to its poor solubility and rapid release of H_2S aqueous solutions.

Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphino-dithioate (GYY4137) is a water soluble derivative of Lawesson's reagent, produced by the chemical reaction between morpholine and Lawesson's reagent at room temperature. For the current study

experiments have been conducted over a period between 24-72 hours. Therefore a synthetic slow release organic compound GYY4137 was employed. This organic compound has a sustained and controlled release of H_2S in aqueous media. GYY4137 was identified to cause a concentration dependent apoptosis of cancer cells (MCF-7) with no obvious detriment to normal cells, such as IMR90. As H_2S is toxic to tissues at high doses it was imperative that pilot experiments were conducted to determine the concentrations at which the endometrial cancer cell lines could tolerate being treated with GYY4137. H_2S release from GYY4137 is dependent on experimental parameters such as pH and temperature of solutions. An increased release of H_2S is seen in acidic solutions and reduced at low temperature[2].

A comparative study looking at the function of H_2S and GYY4137 in culture media has shown the differences in H_2S release from these two donors[125]. The release of H_2S from GYY4137 was 10% of that released from NaHS but the release of H_2S was controlled and sustained over a period of 7 days. NaHS released H_2S rapidly with peak values reached between 30-50 minutes with a resultant decline in H_2S levels[125].

To observe the effect of suppressing the activity of H_2S enzyme inhibitors have been used successfully. *In vivo* aminooxyacetic acid (AOAA) acts an inhibitor of aminobutyrate aminotransferase activity (GABA-T), thereby raising the levels of gamma- aminobutyric acid in tissues[126]. Further to this it is a known general inhibitor of pyridoxal phosphate dependent enzymes. It acts at the level of Schiff base links by attacking the interaction between pyridoxal phosphate (prosthetic group) and the enzyme. This prevents the substrate-enzyme complex from forming, thus impeding the catalytic activity of the enzyme[126].

DL-Propargylglycine (PAG) is a selective irreversible inhibitor of cystathionine gamma-lyase. Due to its high lipophilic properties it is able to easily permeate the cell membrane with little to no damage and exert its effects.

1.5.3 Chemical reactions involving H_2S

H_2S was described to exert its effects by 3 mechanisms including methylation, oxidation and scavenging. The methylation process is divided into primary and secondary stages. Primary methylation occurs in the cytoplasm. H_2S is methylated into methanethiol and this reaction is catalysed using S methyltransferase. Methanethiol has a distinct odour and is subsequently converted to dimethylsulphide , secondary methylation. H_2S methylation is a much slower process than oxidation[127].

Oxidation is the main process of H_2S catabolism and occurs 10,000 times faster than methylation of H_2S. Oxidation is the process through which the atoms in an element lose electrons with a resultant increase in the valence of the element. Mitochondrial oxidation of H_2S produces thiosulphate, which is converted to sulphite and sulphate[128]. This conversion is catalysed sulphide-detoxifying enzymes such as Rhodanese. The oxidation process of H_2S is catalysed by the presence of metal compounds i.e. Fe^{2+}[129]. H_2S is a strong reducing compound and is used by the endogenous oxidation species present in vasculature such as superoxide and hydrogen peroxide.

The scavenging role of H_2S has described under conditions of acute oxidative stress. H_2S was shown to elicit vasoprotection by both scavenging free radicals and by reducing radicals derived by the oxidation of NADPH. Furthermore, methaemoglobin can scavenge H_2S to form sulphhaemoglobin. In the presence of methaemoglobin the production of H_2S is suggested to be reduced[128].

1.5.4 Measuring tissue and blood levels of H_2S

Much deliberation and controversy exist around the most suitable method for monitoring the levels of H_2S production and blood levels[130]. This is owed partially to the difficulties that arise during attempts to measure free sulphide in biological tissues. H_2S as a gas is highly volatile oxidative and reactive[130]. In addition inconsistencies arise in measurement of H_2S due to the presence of bound labile sulphur in tissues. Hydrogen sulphide has a pKa of 6.84 at a temperature of 37°C and hence, at body temperature approximately 30% of H_2S is present in that form and the rest dissociates to form HS^- anions. Uncertainty exists regarding whether one or both species of hydrogen sulphide contribute to its physiological function. In order to accurately measure free sulphide levels in samples the technique needs to be able to differentiate between free and bound labile sulphur, address the volatility and reactivity of H_2S and distinguish between H_2S and HS^{2-}.

Several methods of measuring H_2S are suggested and they can be direct or indirect methods. The direct methods measure H_2S itself by trapping the gas; these include: gas chromatography and HPLC, methylene blue assay. Methods using fluorescence have been described where liberated H_2S is trapped and the resulting product (sulphide dibimane) measured by reverse phase HPLC[131, 132]. A modification of the methylene blue assay consists of a zinc precipitate phase. As the methylene blue assay takes place in acidic conditions the modified zinc precipitation method eliminates the acidic conditions and an extra protein solubilisation and wash step to remove artefacts that may produce inaccurate results[131, 132]. H_2S serum levels in healthy adults have been measured using the methylene blue assay and sulphide sensitive electrodes.

1.5.5 Role of H_2S in vasculature

Endogenous H_2S was shown to play a role in both physiological and pathological functions[133-136]. The physiological roles of H_2S include vasorelaxation, cytoprotection against oxidative stress and promotion of angiogenesis[4]. These functions of H_2S and its increased production in cancers have directly implicated it in the progression of many tumours. Angiogenesis is a prerequisite for the development and maintenance of tumour growth. It is defined as neovascularisation from existing blood vessels[137]. In adults angiogenesis is tightly regulated, with the endometrium being the site of cyclical neovascularisation. The endometrium expresses both pro- and anti-angiogenic factors. 2 of the main angiogenesis regulatory factors in the endometrium include the growth factors families {vascular endothelial growth factor (VEGF) and fibroblast growth factor family (FGFs)} and molecules involved in mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signalling[138]. The VEGF family is made up of six members (VEGF –A, -B, -C, -D and –E) with 3 receptors to bind to: VEGFR-1, -2 and -3. The ligands are dimeric cysteine – linked glycoproteins. In the endometrium VEGF-A is mainly up regulated in hypoxic conditions but is also found to be oestrogen responsive. Oncogenes are also known to stimulate the production of VEGFs[137]. VEGF-C/ uNK cells have been implicated in angiogenesis in the endometrium and its expression is noted to vary with menstrual cycle phase. Moreover the receptors VEGFR-2 and VEGFR-1 are expressed in the endometrium, with up regulation of VEGFR-2 in the proliferative phase of the endometrium and its subsequent down regulation after ovulation with accompanied increased expression of VEGFR-1. VEGFR-3 is not expressed in the endometrium. The fibroblast growth factors (FGF) 1, 2 and 4 are expressed in the endometrium and play a synergistic role with the VEGF family. The FGF family up regulates the expression of VEGF-2 which in turn increases the release of FGF from the extra cellular matrix (ECM)[87, 136, 137, 139].

In many tumours VEGF-A is proposed to be the most effective angiogenic factor and its secretion is crucial in tumour maintenance. Epithelial membrane protein – 2 belongs to the GAS-3/PMP22 family and was shown to exert angiogenic effects via the up regulation of VEGF-A expression in endometrial vascular endothelial cells[136]. It is therefore apparent that the VEGF family play an important role in the tumourigenesis. In this study the role of hydrogen sulphide is being investigated. H_2S is a known promoter of angiogenesis via stimulatory and inhibitory mechanisms[136]. In vascular tissue H_2S production is mainly

regulated by CSE. Pathways implicated in H_2S mediated angiogenesis include: the mitogen activated protein kinase pathway, PI-3K/Akt pathway and ATP- sensitive potassium channels. H_2S is also proposed to inhibit phosphodiesterase, thereby promoting angiogenesis[87].

The role of H_2S in the regulation of smooth muscle activity was proposed as one of its major functions. The endogenous levels of H_2S in aortic smooth muscle have shown to be up to 100-fold more when compared with other organs such as liver, brain or heart, thereby suggesting an importance of H_2S in smooth muscle regulation[140]. H_2S was shown to cause relaxation of smooth muscle in the GI tract, bronchial passages and bladder[141]. *In vitro* studies investigating the effect of H_2S on rat thoracic aorta by H_2S and NaHS and L-cysteine resulted in relaxation of the arteries. This relaxation was counteracted with the addition of the CSE inhibitor PAG; thereby showing that L-cysteine exerted its effects via H_2S production. *In vivo* studies showed the intravenous injection of H_2S reduced mean arterial pressure[142].

1.5.6 Pathways involved in exerting effect of H_2S

H_2S exerts its effects through a variety of mechanisms. The most implicated pathway in H_2S signaling is the K_{ATP} channels. Hyperpolarisation of the vascular smooth muscle cells results in the opening of these channels. Research studies where smooth muscle cells have been isolated from the rat mesenteric artery and incubated with H_2S has shown the increased recruitment of open K_{ATP} channels with no alteration in their conductance. Furthermore, the use of K_{ATP} channels antagonist, glibenclamide opposed the vascular relaxation effect of H_2S both *in vivo* and *in vitro*. The incubation of smooth muscle cells in high K^+ conditions also counteracted the effects of H_2S. In contrast, studies have also found that vascular endothelial relaxation is not dependent on K_{ATP} channels[143].

Further mechanisms involved include L-Type calcium channels, activation of myosin light chain phosphatase and stimulation of Cl^-/HCO^{-3} in smooth muscle cells[143]. Sulphhydration of the reactive cys residue in target proteins was linked to H_2S signaling. It was proposed that H_2S exert its effect on ion channels via this mechanism. Sulphhydration is the addition of sulphur to cysteine from H_2S to result in the conversion of the –SH group to –S-SH[144].

1.5.7 H_2S signalling in neoplasia

H_2S may have an indirect approach in initiating neoplasia via interactions with other biological agents. H_2S is known to act as a strong reducing agent and can be utilised endogenously in oxidative reactions. H_2S is a highly reactive substance and can interact with S-nitrosothiol, producing metabolites such as NO, NO^-, NO^+. Nitric oxide (NO) was implicated in the endometrial cancer tumorigenesis via a variety of mechanisms. The expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are up regulated in endometrial cancer[145]. The role of COX-2 as an angiogenic agent exerting its effect via the release of growth factors was implicated in endometrial tumorigenesis. NO was associated with carcinogenesis through its ability to assist genetic changes occurring in early phase disease, thus initiating tumorigenesis and the maintenance of tumour cells through angiogenesis[145]. A synergistic relationship between COX-2 and iNOS was described secondary to evidence suggesting that NO produced by iNOS activity enhances the activity of COX-2[145].

Recent evidence published by Csaba Szabo confirming the role of tumor derived H_2S produced by CBS in colon cancer has lent a skeleton upon which experiments can be modified to apply in endometrial cancer. Human colon cancer biopsies and patient matched normal marginal mucosa were analysed to reveal a selective up regulation of CBS with a simultaneous increase in H_2S, which was also true of colon cancer cell derived epithelial cell lines (HCT116, HT29, LoVo) when compared to benign colonic mucosal cells (NCM356.). As a secondary method ShRNA- mediated silencing or pharmacological inhibition using the CBS inhibitor aminooxyacetic acid (AOAA) resulted in a reduction of proliferation, migration and invasion of the HCT116 cell line[4]. Animal studies were conducted prior to which ethical approval was sought and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Nude mice were treated with AOAA resulting in attenuation of the growth of patient derived colon cancer xenograft and reduced tumour blood flow. Expression of CSE levels remained unchanged in colon cancer, thereby silencing of CSE using its inhibitor DL-Propargylglycine (PAG) did not affect tumour growth or bioenergetics. The overall concluding point highlighted by this study was the identification of CBS derived H_2S as a tumour growth factor and anticancer drug target[4].

The up regulation of CBS has also been described and its role attributed towards the progression of advanced ovarian cancer and drug resistance. Using patient tissue microarray (TMA), *in vitro* and *in vivo* studies were conducted during which it was shown that the expression of CBS was common in primary serous ovarian carcinoma. TMA consists of many small tissue samples derived from a variety of numerous cases on a single slide, thereby allowing analysis of many samples at one time. Results of this study concluded that *in vitro* the effects of pharmacological CBS silencing can be reversed by exogenous supplementation with the H_2S donor sodium hydrogen sulphide[146].

Conflicting evidence was published investigating the use of slow release H_2S donors as anti-cancer drug targets. The alternating role of H_2S in exerting pro-apoptotic or anti-apoptotic effects was reportedly previously in cultured cells, though the exact mechanisms still remain undefined. *In vitro* and *in vivo* treatments of neoplastic and normal cell lines with the slow release GYY4137 revealed an anti-proliferative effect on neoplastic cells with no effect on normal cell line proliferation[125]. The use of a rapid releasing H_2S donor i.e. sodium hydrogen sulphide (NaH_2S) showed no effect on either neoplastic or normal cell lines. A study was conducted investigating the role of various slow release H_2S donors (GYY4137 analogues) to elicit their anticancer potential. A dose- dependent cellular apoptosis of neoplastic cells of seven differing human cancer cell lines was seen, with no effect on normal human cells. This conflicting evidence could be a result of the complex and ambiguous role of H_2S and its synthases in tumorigenesis[125]. The above articles are proof to the tissue specific roles of H_2S and its effect in the endometrium is yet to be described.

This study presents novel evidence looking at the expression of the three H_2S synthesising enzymes CBS, CSE and 3-MPST in endometrial cancer and their correlation with expression of steroid receptors such as ER- alpha and –beta, PR and AR. Further cell culture investigations using the H_2S donor GYY4137 and the H_2S substrate L-cysteine have been used to treat an endometrial cancer cell line (MFE280s) to elicit any effects on proliferation if present. These are preliminary experiments and aim to show an association between H_2S and endometrial cancer if present.

Chapter 2 Summary of project aims and hypotheses

Growing evidence exists defining the role of endogenous hydrogen sulphide as tumour growth factor. Its effects include maintenance of cellular bioenergetics, angiogenesis and vasorelaxation, thereby supporting tumour growth and maintenance. This effect of hydrogen sulphide was confirmed in recent publications. Szabo et al[4] compared patient matched normal colonic mucosa with colon cancer epithelial cells and cell lines to conclude the positive selective up regulation CBS. A similar selective up regulation of CBS and a subsequent rise in H_2S was shown in primary ovarian carcinoma by Bhattacharya et al[146] Owing to the established evidence this project explores the possibility of an existing relationship between H_2S and endometrial cancer within a large cohort of women.

Proof of principle for this study came from a small scale investigation conducted at The Women's Hospital in Liverpool. Samples from a small sample size of n= 31 women were used for this study. 25 of these females had been diagnosed with endometrial carcinoma and n=6 were a postmenopausal, control group. Results of this study confirmed the presence of both CBS and CSE in human endometrium. The positive staining was confined to the endothelial and epithelial cells. There was weak diffuse cytoplasmic staining and strongly stained cells for CSE. CBS staining appeared solely cytoplasmic. A grade dependent increase in expression was also found with CBS ($P<0.007$). This pilot study concluded that CBS was a possible novel therapeutic drug target in endometrial cancer.

2.1 Project objectives and hypotheses

2.1.1 Main objectives

1. **Do hydrogen sulphide and its synthases (CBS, CSE and 3-MPST) play a role in endometrial cancer tumourigenesis?**

   Hypothesis: There will be an up regulation of all the hydrogen sulphide synthesising enzymes, evidenced as increased cytoplasmic staining within the endometrial cancer cells when compared to their post-menopausal controls.

2. **Does a grade dependant expression of enzymes exist?**

Hypothesis: *The intensity of enzymatic cytoplasmic staining of cells will increase with an increase in grade of endometrial cancer..*

2.1.2 Secondary objectives

1. **Does the addition of a H_2S donor have a direct effect on the proliferative potential of the MFE280 endometrial cancer cell line?**

   Hypothesis: *The addition of the organic H_2S donor, GYY4137, will show an increase in proliferation of the MFE280s, thereby displaying the tumorigenic effects of H_2S.*

2. **Does addition of the substrate L-cysteine have an effect on the proliferation of MFE280s cell line?**

   Hypothesis: *The addition of L-cysteine will indirectly have a positive effect on proliferation of the endometrial cancer cell line. The H_2S synthesising enzymes will metabolise the L-cysteine to produce H_2S, with subsequent increase in proliferation.*

Chapter 3 General materials and methods

3.1 Ethical approval

Human endometrial samples were collected from women undergoing surgery at the Liverpool Women's hospital. The approval to do so was granted by the Liverpool Adult Local Ethics Committee **(Appendix 1)**. The endometrial cancer and post-menopausal samples were collected under the terms stated in LREC references 11/H1005/4. Prior to sampling the endometrium, written informed consent was gained from every patient on the day of surgery.

3.2 Patient recruitment

3.2.1 Patient identification and enlistment

Planned theatre lists at the Liverpool Women's Hospital were studied to identify women who were undergoing suitable gynaecological surgeries. Once recognised, these women were approached by selected GCP trained recruits within the research team to be consented. At the time of gaining informed voluntary consent, further details were attained with regards to patient demographics. This included information concerning their: BMI, age, menstrual history, parity, previous gynaecological conditions, current or previous hormonal treatments and smoking history. During the consultation, a detailed information sheet (refer to appendix 2) covering the content of the research project was specified. Furthermore, the rights of the patient to refuse participation were stressed with reassurance that their decision would not affect their management.

3.3 Study groups involved

In this study non- pregnant endometrial cancer samples were compared against normal post-menopausal tissue. Patients were only recruited into the study if they met all the inclusion criteria and excluded if they met any of the absolute exclusion criterions. The absolute exclusion criteria included: pregnancy, breastfeeding or women undergoing hormonal therapy within 3 months of surgery.

3.3.1 Endometrial cancer patients

61 endometrial cancer biopsies were obtained from women diagnosed with either type 1 (n=43) {FIGO grade 1 (n=24), grade 2 (n=11) or grade 3 (n=8)} or type 2 (n=18) endometrial cancer.

3.3.2 **Post- Menopausal patients**

The post-menopausal group was included as a comparative/ control group for the endometrial cancer group. All of the patients diagnosed with endometrial cancer were post-menopausal in this study (age range 51-87). The women included in the post-menopausal group (n=16) were undergoing surgery for pathologies distinct from neoplastic endometrium.

PM endometrium is atrophic and the use of pre-menopausal proliferating endometrium may also be valid as endometrial cancers develop with oestrogen stimulation. However some endometrial cancers develop without oestrogen stimulation. PM endometrium retains the ability to respond to oestrogen and progesterone if present systemically and therefore PM endometrium has been used for this study.

3.3.3 Inclusion and Exclusion criteria

Highlighted below are the inclusion and exclusion criteria for the recruitment of patients for this study.

Table 8 Inclusion criteria for both endometrial cancer and post-menopausal groups within the study

Group	***Inclusion criterion***
Endometrial cancer	1- Patients must have the capacity to give written informed consent 2- Undergoing surgical hysterectomy and pipelle biopsies at the Liverpool Women's hospital for endometrial cancer
Post-menopausal	1- 12 months since LMP 2- Operated upon at the Liverpool Women's Hospital for a benign, non-endometrial pathology. *3*- Patients must be competent and thereby have the capacity to give written informed consent

Table 9: Exclusion criteria for both endometrial cancer and post-menopausal groups within the study

Group	***Exclusion criterion***
Endometrial cancer	1- Pregnancy, breast-feeding or hormone therapy up to 3 months before surgery.
Post-menopausal	1. History of post-menopausal bleeding / endometrial pathology 2. Hormone replacement therapy (HRT)

3.4 Tissue collection techniques

Either full thickness or pipelle sampling can obtain endometrial biopsies. The process of endometrial sampling is dependent on the type of surgical technique employed. Trained individuals of the research team held the responsibility of collecting tissue samples in theatre once the surgical procedure had been completed. Tissue samples used for this study were collected over the past 3 years.

3.4.1 Endometrial biopsy

- *Full thickness samples:*

Women undergoing hysterectomies were the group from whom full thickness endometrial biopsy samples were taken. Full thickness samples comprise of the endometrial lining extending through to the myometrium. In order to collect a full thickness sample, once the uterus was excavated from its anatomical position it was transferred onto a surgery trolley and placed face down. An incision was made with the use of a size 22 carbon steel surgical blade (Swann- Morton, UK) on the posterior aspect of the uterus spanning the length, from the fundus down to, but not including the cervical canal. The exposed endometrium was sampled by making a 25 mm lateral incision and dissecting 10 mm into the uterine wall. To ensure that the endometrium, endo-myometrial junction and myometrial layers were acquired the uterine tissue was held by Bonney tissue forceps (Phoenix Surgical instrument, Ltd, UK) whilst a medial cut was made to free the tissue.

- *Pipelle Samples:*

In cases where a hysterectomy was not performed a pipelle sample was taken at commencement of surgery. The patients' external genitalia and surrounding area were thoroughly washed using a sterile aqueous solution (Sterets Unisept, Medlock Medical Ltd, UK). Once the area had been cleaned a Sims speculum was inserted into the vagina, allowing the cervix to be visualised. Vulsellum uterine forceps (Phoenix Surgical instrument, Ltd, UK) were used to draw the anterior lip of the cervix into the vagina. A pipelle was then passed through the cervix up to the fundus of the uterus. Once the total length from the external cervical os to the uterine fundus was noted, the plunger was

pulled back to create suction, drawing the endometrial tissue into the pipelle as it was slowly rotated and withdrawn from the uterus. This slow rotatory movement ensured maximal sampling of the endometrium.

3.5 Tissue processing

Once the endometrial tissue samples for immunostaining were obtained they were placed directly into 15 ml of neutral buffered formalin (NBF; Sigma-Aldrich, UK) contained within a universal tube. NBF consists of approximately 3.7-4% formaldehyde. This is an organic compound that allows the formation of cross links between primary amino groups in proteins to adjacent nitrogen atoms in proteins or DNA. By placing the samples into NBF, the tissue sample is preserved as close to its original biological state as possible. Prior to processing the samples were incubated for at least 24 hours in NBF. The Shandon Citadel 1000 machine was then used to dehydrate and impregnate these tissue samples with paraffin. For a detailed explanation of tissue processing please refer to appendix 3a.

3.5.1 Tissue processing protocol

The incubation times and solution in which the tissue was processed is highlighted below in table 9.

Table 10 Tissue processing protocol

Solutions	Time (Minutes)
10% NBF	45
Ethanol 60%	60
Ethanol 70%	60
Ethanol 90%	60
Ethanol 100%	60
Ethanol 100%	90
Ethanol 100%	120
Xylene 1	60
Xylene 2	90
Xylene 3	120
Parrafin wax 1	150
Paraffin Wax 2	210

3.5.2 **Sectioning**

Once processed the samples were embedded in paraffin wax, after which sectioning took place. Care was taken whilst embedding to ensure the accurate orientation of the tissue to depict its morphology accurately. The paraffin embedded tissue samples were sectioned into 4µm thick slices using the Microm HM355 rotary microtome (Microm Ltds, UK). Once the samples were cut they were floated onto a waterbath kept at a temperature of ~40°C (refer to appendix 3b 'Cutting paraffin sections'). Slides coated with aminopropyl triethoxy silane (APES) were dipped into the water bath collecting the tissue onto them (refer to appendix 3c for 'APES coating procedure'). The slides were labelled and allowed to air-dry prior to use. Once dry, the slides were filed away into labelled plastic slide cases.

3.6 Preparation of slides prior to IHC

Once the appropriate tissue samples had been selected, they were labelled with the experiment criterion: date, incubation time, antigen retrieval specifics and concentration of antibody used. The slides were transferred to a metal staining rack and later placed into a heated chamber at 60°C for one hour or at 37°C overnight for 'baking'. Heating the slides ensures the removal of residual moisture between the tissue section and slide that may impede adhesion. At this point care was taken not to 'over bake' the slides leading to degradation of proteins within the tissue leading to divergent staining results.

3.7 Immunohistochemistry (IHC)

IHC is a procedure that shows specific antigens in tissues by the use of enzyme markers. The application of primary antibodies allows specific antibody –antigen complexes to form and horse radish peroxidase labelled secondary antibody reacts with the enzyme substrate to produce a brown coloured product, thereby allowing the visualisation of antibody localisation. Therefore it can be concluded that a crucial component of immunostaining is the antibody.

Antibodies are proteins that are also known as immunoglobulins or IgG molecules. These immunoglobulins are synthesised by the plasma cells and there are 5 major groups within the body, namely: Immunoglobulin A (IgA), IgD, IgE, IgG, IgM. They are present in the blood and are used by the immune system to attack foreign molecules (antigens) that enter the body. Antigens are also protein compounds, often polysaccharides that are present on the surface of cells. IgG antibodies are found in most body fluids and are present in antiserum once the cells and fibrin are removed. This property can be used to generate antibodies for clinical use. An antigen from a human can be injected into a host animal, such as a mouse. This host species creates antibodies against the antigen. These antibodies are extracted and purified, to be applied to human tissue to identify the location of antigen within a cell.

Monoclonal antibodies are a homogenous group of immunoglobulins that can bind only to a single epitope and are most commonly developed in mice. Monoclonal antibodies were used in this study and are made by combining myeloma cells with splenic cells from a mouse that was injected with the antigen of choice. Though monoclonal antibodies are expensive to produced when compared with polyclonal antibodies, the advantages of

using monoclonal antibodies include; homogeneity, highly specific, unlimited quantitates of a single, well- defined reagent can be produced. Although relatively inexpensive to produce polyclonal antibodies tend to recognise many epitopes on a single antigen and display batch-to-batch variability.

3.8 Immunohistochemistry Methodology

3.8.1 Deparaffinisation regime

In order to successfully stain the tissue, the sections were submerged in xylene and progressively rehydrated by stepping the slides down an alcohol gradient. This ensures the successful adherence and tissue penetration. After de waxing samples the metal racks were transferred to a staining dish filled with tap water (See appendix 3d 'prepping paraffin section for immunostaining').

Table 11 Deparaffinisation regime

Solution	Minimum time in solutions (minutes)
Xylene 1	10
Xylene 2	10
Ethanol 100%- 1	5
Ethanol 100%- 2	5
Ethanol 90%	1
Ethanol 70%	1

3.8.2 Epitope (antigen) recovery

Formalin fixation prevented tissue autolysis and immobilised antigens. During fixation antigens undergo chemical alterations of their primary, secondary and tertiary structures. This can result in masking of antigenic sites due to changes to the epitopes or adjacent proteins, thus preventing the formation of antigen-antibody complexes. Resultant divergent results would be seen as false negative staining / weakly stained tissue samples. The epitopes were unmasked by the use of a heated solution such as citric acid or Tris - EDTA known as heat induced epitope retrieval (HIER). The table below represent the HIER parameter for all 3 antibodies used.

Table 12 Antigen retrieval conditions

	CBS	**CSE**	**3-MPST**	**ER-alpha**
Antigen retrieval	Tris- EDTA 3 minutes	Citrate 4 minutes	Citrate 4 minutes	Citrate 2minutes

The samples were heated at full pressure in a pressure cooker. The buffer solutions used include a 0.1M concentration citrate based buffer (pH 6) for CSE, 3-MPST and ER-alpha 10mM Tris in 1mM –EDTA concentration Tris-EDTA buffer (pH 9) for CBS. Both buffer solutions were diluted with distilled water immediately prior to use. Citrate (150mls buffer+1350mls distilled water), Tris-EDTA (300mls buffer+ 1200mls distilled water). The heated environment causes protein links to unfold whilst the buffer solution maintained the unravelled structure of the proteins. The duration of treatment varied for all 4 antibodies that were stained for and are stated in the table above. Once the slides underwent antigen retrieval they were returned to the staining dishes filled with water, following which they were transferred into Tris buffered saline (TBS) for 5 minutes.

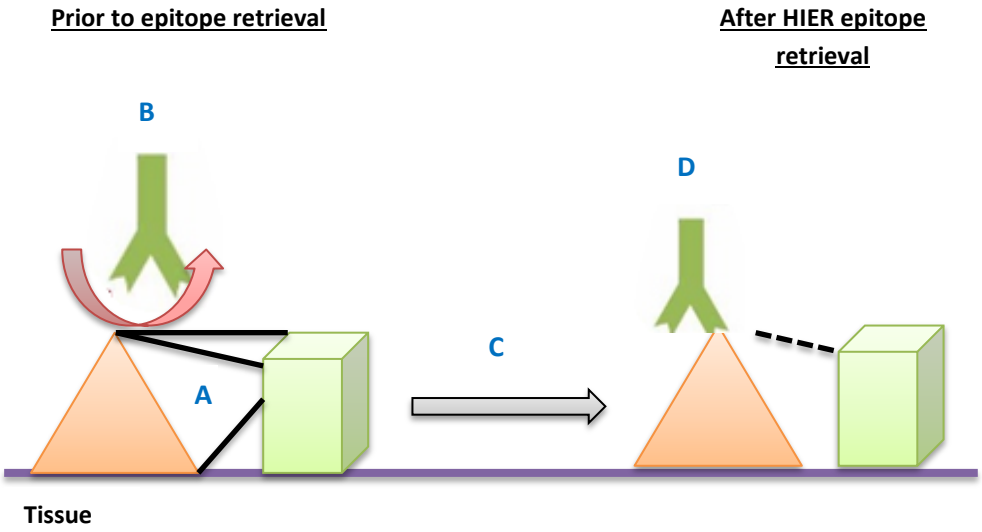


Figure 6 HIER: A) protein bonds masking antigen binding sites, B) Primary antibody, C) buffer solution + heat and D) formation of primary antigen- antibody complex.

3.8.3 Blocking endogenous peroxidase activity

Red blood cells and eosinophils may contain reactive sites that are similar to the binding sites on target antigens, resulting in false positives staining, illustrated in figure 6. Reduction of background staining can be achieved by incubating the tissue samples with a buffer solution known as hydrogen peroxide (Sigma, lot# S2BC3560V, H1009-500 mls, USA) 30% (w/w) in water for 10 minutes. This buffer blocks the reactive sites at which the antibodies may bind, thus limiting background staining.

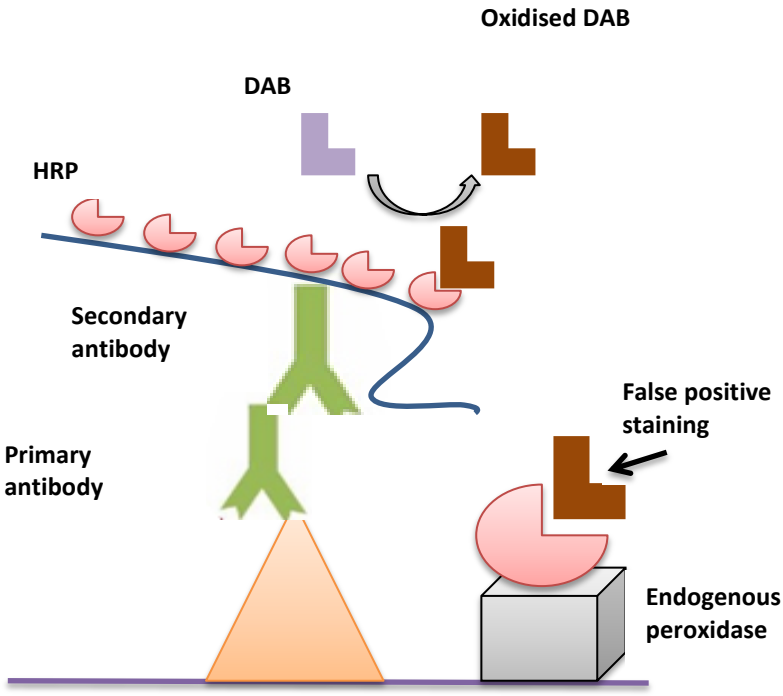


Figure 7 Illustration showing the false positive staining prior to blocking endogenous peroxidase activity

Incubating tissue samples with normal horse serum prevents non-specific antibody binding refer to figure 7. Preliminary experiments conducted using the mouse monoclonal antibodies CBS, CSE and 3-MPST revealed no significant change in staining with or without a horse serum block. Hence this step was omitted from the IHC protocol for these antibodies in this study. However whilst staining for ER-alpha a rabbit polyclonal antibody the tissue sections were incubated with normal horse serum for 30 minutes.

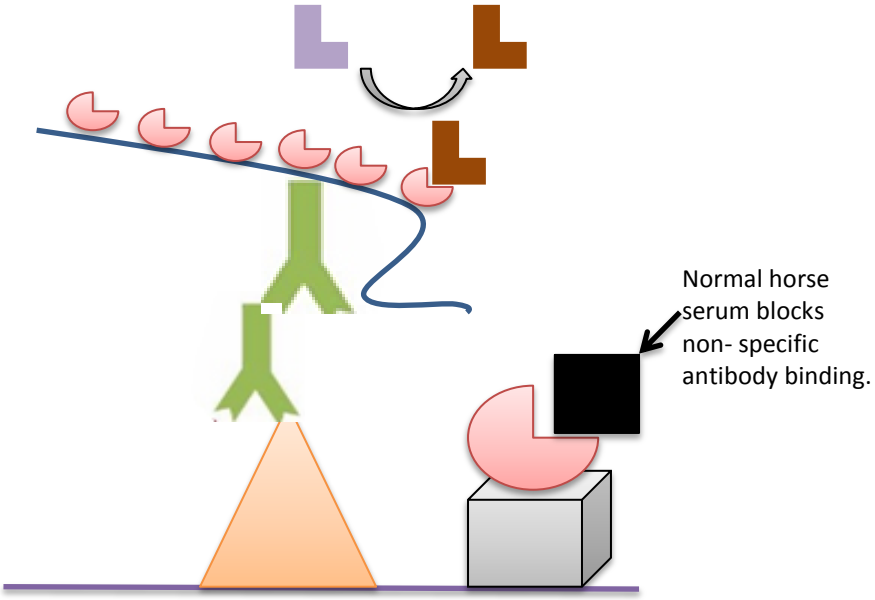


Figure 8 Illustration displaying results of blocking non- specific antibody staining.

3.8.4 Primary and Secondary antibody staining

Prior to the application of primary antibody to the tissue sections a DAKO hydrophobic marker pen (DAKO, Cambridge, UK) was utilised to encircle the sections, after which the slides were returned to the humidifying chamber.

The IHC protocol can be split into 2 stages. The first stage focused on incubation of the tissue sections with a primary antibody, to allow the formation of an antibody-antigen complex, as shown below.

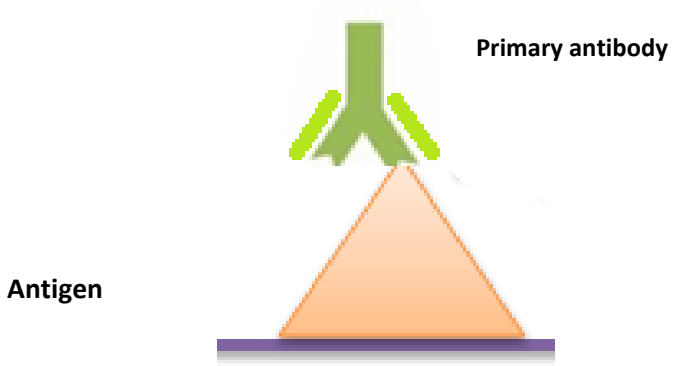


Figure 9 Illustration of a primary antibody-antigen complex

Two different CSE antibodies have been used to stain the tissue sections in this project, owing to the first batch running out partway through the experiments. The first run (7 sections out of 81) was stained with CSE 1:500 dilution, Novus biologicals, 0.1mg/ml. Thereafter all sections have been stained using the CSE antibody sourced from Abnova following its optimisation as will be discussed in chapter 4.

A table containing the details of the primary antibodies used in the study is listed below.

Table 13 Immunohistochemistry (IHC) – Antibody details and conditions

Conditions	**Enzymes stained for**		
	CBS	**CSE**	**3-MPST**
Company	Abcam	Abnova	Santa Cruz
Concentration	1:300	1:3000	1:400
External control	Liver	Liver	Human colon
Antigen retrieval	Tris-EDTA 3 minutes	Citrate 4 minutes	Citrate 4 minutes
Negative control	Mouse IgG 1:500	Mouse IgG 1:500	Mouse IgG 1:500
Incubation period	4°C overnight	4°C overnight	4°C overnight
Mono/poly clonal	Monoclonal	Monoclonal	Monoclonal
Clone	3E1 (ab 124276)	4E1-1B7	H-11
Suggested dilutions	5µg/ml.	0.5 mg/ml	200 µg/ml

For correlation of CBS, CSE and 3-MPST expression with steroid receptor expression, a similar sample dataset were stained and score by Dr. Areege Kamal and Miss Eve Bunni. The antibody details and experimental parameters are detailed in table 13.

Table 14 IHC: Antibody details for steroid receptors stained and scored by Dr. Areege Kamal and Miss Eve Bunni.

	Androgen receptor	**Progesterone receptor**	**Oestrogen Receptor-beta**
Company	Dako, Glostrup, Denmark	Dako, Glostrup, Denmark	Serotec
Concentration	1:75	1:1000	1:50
Antigen retrieval	Citrate 3 minutes	Citrate 2minutes	Citrate 2 minutes
Internal control	Endocancer 74-2	SPCN 118 tube	Endocancer 72
Negative control	Mouse IgG	Mouse IgG	Mouse IgG
Incubation period	4°C overnight	30 minutes, room temperature	4°C overnight
Clone	M3562	M3569	MCA1974S

Table 15 IHC antibody details for oestrogen receptor-alpha

	Oestrogen receptor- alpha
Company	Abcam, Cambridge, UK
Concentration	1:50
Antigen retrieval	Citrate 2 minutes
Internal control	Endocancer 72
Negative control	Rabbit IgG
Incubation period	2 hours, room temperature
Clone	Ab137738, Rabbit polyclonal

For CBS, CSE and 3-MPST the tissue sections were incubated with 70 µl of primary antibody, overnight at a temperature of 4˚C. The primary antibody was diluted in phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumen (BSA). For ER-alpha the section were incubated with 70 µl of primary antibody for 2 hours at room temperature. Once the primary antibody incubation was completed it was removed by tapping the slide on its side before the slides were washed twice for 5 minutes in TBS to remove any excess antibody. The tissue sections were incubated for 30 minutes at room temperature with 1-2 drops of Horseradish peroxidase (HRP) labelled secondary antibodies derived from mouse hosts (Vector ImmPRESS, Cat #: MP-7402, Vector, Peterborough, UK).

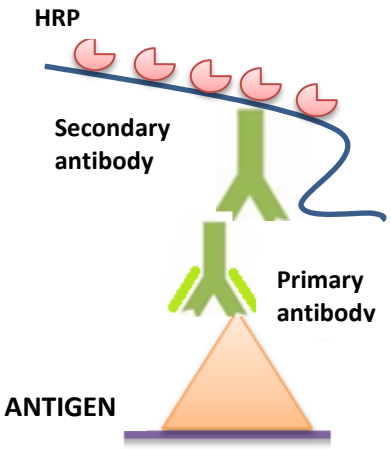


Figure 10 Formation of the secondary antibody-antigen complex after the administration of HRP labelled secondary

HRP is a common enzyme found in tissues and is often used to label secondary antibodies to allow the detection of target antigens. The use of DAB to detect these complexes is based upon enzymatic action. The addition of DAB chromogen to the HRP labelled secondary antibody initiates an instantaneous formation of coloured insoluble precipitate, seen as a dark brown colour.

DAB chromogen solution (30 µl of DAB concentrate in 1ml OF ImmPACT diluent) (ImmPACT DAB, Vector, Peterborough, UK) was applied to the tissue section for 10 minutes at room temperature. Upon removal of DAB the slides were transferred immediately into water, to halt the reaction. Gills-2 hematoxylin (Thermoscientific, Loughborough, UK) was used to counterstain the slides for 2 minutes and the slides were placed under running tap water for 1 minute. The slides were momentarily immersed in 1% acid alcohol removing excess hematoxylin staining. This is a regressive method of staining where tissue samples are deliberately over stained then differentiated until acceptable staining is reached. Following acid alcohol treatment the slides were placed under running tap water. The tissue sections were dehydrated up a concentration gradient of alcohols, ending in xylene and mounted with cover slips using Consul Mount (Thermoscientific, Loughborough, UK). The slides were allowed to dry within the fume hood overnight before they were imaged. For a detailed description of the IHC procedure please refer to Appendix 3E.

3.8.5 Controls

Ordered sections of one positively staining sample was cut and included in every run. These positive internal controls were paralleled against one another to compare the intensity of staining in each run. Provided the intensity of staining was consistent throughout all runs, the samples were scored. In addition to the positive controls, each run also included a negative IgG control for each specimen in one slide at the very least to ensure specificity of staining in each run. External tissue positive control sections were also included in a run at commencement of staining for each antibody and were consistent throughout. This allows an accurate assessment of staining to be made compared to a tissue in which the staining pattern was established. A negative control was included for each antibody in every run as highlighted in table 15.

Table 16 IHC controls for all antibodies

Antibody	Internal intensity of staining control	External positive control	Negative control
CBS	Endocancer 14-2	Liver	Endocancer 14-2 Mouse IgG
CSE	Endocancer 15-1	Liver	Endocancer 15-1 Mouse IgG
3-MPST	Endocancer 3	Human colon	Endocancer 3 Mouse IgG
ER- alpha	Endocancer 72-4	Endometrium	Endocancer 72-4 Rabbit IgG
ER-beta	Endocancer 72-4	Endometrium	Endocancer 72-4 Mouse IgG
PR	SPCN 118- tube	Endometrium	SPCN 118- tube Mouse IgG
AR	Endocancer 72-4	Endometrium	Endocancer 72-4 Rabbit IgG

3.9 Image capture

In order to assess the differential staining patterns seen with all 3 antibodies it was necessary to assess the location and intensity of staining.

3.9.1 Image capture

High-resolution images of the stained tissue sections were captured using the Nikon Eclipse microscope and camera head (Nikon, Tokyo, Japan). 10 consecutive detailed micrographs containing the malignant cells of the endometrial cancer tissue sections were taken. With regards to the post-menopausal and other full thickness samples, only the glandular epithelium was imaged and scored as it is the epithelial cells that undergo malignant transformation. By ensuring that only the functional layer of the endometrium is imaged and scored, a more accurate direct comparison of these samples can be made with those obtained by a pipelle. The images were taken at x400 magnification (x40 objective and x10 eyepiece).

3.9.2 Modified QuickScore

The fundamental principles of this technique focus on combining the intensity and proportion of staining. Prior to the commencement of scoring these samples, images were taken to define the weak, moderate and strong staining for each enzyme. The samples were thoroughly examined on a lower power at x4 or x10 and once an overall picture of staining was attained the samples were visualised at a higher magnification x20 and imaged at x40. A standardised set of values was used to keep scoring consistent between users, as listed in the table below.

Table 17 Standardised criteria for Quickscore

Percentage of stained tissue (%)		**Intensity of staining**	
1	Less than 25%stained	0	No staining
2	25-50 % stained	1	Weak staining
3	50-75% stained	2	Moderate staining
4	More than 75%	3	Strong staining

Before the final scores were used to create graphs all the micrographs were scored 3 times to ensure accuracy of scoring. Further to this 16 slides from the 81 cohort were blinded and scored by one other person. The scores were then compared and only 2 scores were found to be no more than 2 points off one another with the rest being identical.

Once the score was determined the values were inserted into a Microsoft Excel spreadsheet and a total value for each section was calculated as seen in table.

A formula to calculate the total score for each tissue section is as follows:

Total quick score = (0xa) + (1xb) + (2xc) + (3xd)

The maximum score achievable by the use of this method is 12 (sum of % x intensity of score). Furthermore, a maximum value of 4 can be attributed for percentage of tissue stained. If 100% of the tissue section is strongly stained then the scores would be as follows.

Grade	Cancer Sample	Intensity				Total	Total
		0	1	2	3		
Example: 1	**Endocancer**	**0**	**0**	**0**	**4**	**0+0+0+12**	**12/12**

Table: Example score sheet.

Grade	Cancer Sample	Intensity				Total	Total
		0	1	2	3		
1	Endocancer 2	0	2	1	1	2+2+3	**7**
Serous	Endocancer 6	0	1	3	0	1+6	**7**
MMMT	Endocancer 7	0	2	2	0	0+2+4	**6**

3.9.3 Statistical analysis

Prism 6, GraphPad (GraphPad Software, Fay Avenue, USA) was used for the analysis of data obtained in this study. SPSS database 20 (IBM, PORTSMOUTH UK) was used for the correlational studies conducted with the steroid receptors. Non-parametric tests were used for the analysis as the data were not normally distributed. Mann-Whitney test was used to compare pairs of data and the Kruskal-Wallis test was used for comparison of groups of data. P values <0.05 were considered to be significant and post hoc tests were used at the second stage of analysis of variance (ANOVA). Once expression of CBS, CSE and 3-MPST between the cancer and control cohort had been determined, correlational studies using the Spearman's rank correlations test were conducted.

3.10 Cell culture

The essence of cell culture is the removal of cells from their hosts and their consequent growth in an artificial environment under favourable conditions. Cells can be cultured in an adherent/ monolayer culture or a suspension culture. In this study the former method was used.

Endometrial cancer cell lines were cultured *in vitro* in order to treat them with hydrogen sulphide donors and enzyme substrates to evaluate the subsequent effect on their proliferative potential. 2 endometrial cancer cell lines were chosen to undergo the above investigations.

Initial experiments were carried out using the Ishikawa cell-line which was derived from a grade 1 well-differentiated human endometrial adenocarcinoma (Public Health England, Salisbury, UK). 10 millilitres of Dulbecco's Modified Eagle Medium (DMEM/F12) (Sigma, UK), FBS 10% (BioSera, UK) to which the antibiotic, Primocin^{TM} (1:500 in media, InvivoGen) and L-glutamine (2mM, Sigma) were added was used as the culture medium to support the growth of the cells. The same medium was used to culture another endometrial cancer cell line known as MFE280.

Later experiments were conducted using the MFE280s alone. These cells were cultured and maintained in 10mls of DMEM/F12 (phenol free, Life Technologies, UK) with 5% charcoal stripped FBS (Sigma, UK). In contrast to the Ishikawa cell line, the MFE280s are slow growing and require a long incubation period ranging between 4 and 6 days to achieve an appropriate confluence (80-90%) before cells can be seeded. In light of using reduced serum media to maintain cells the cells required a change in media 3 days post commencement of culturing. This is to ensure that cells did not enter a state of starvation and consequently their survival compromised.

3.10.1 **Cell culture methodology**

The human endometrial MFE280 cell line (#98050131) was from Public Health England, Salisbury, UK. The cells were derived from a recurrent, poorly differentiated endometrial carcinoma from an elderly patient. The cell lines were stored at -70°C in a freezer in solution (90% FBS+ 10%DMSO). Dimethyl sulphoxide (DMSO) stops intracellular crystallisation of cells. Prior to placing them onto cell culture plates they were defrosted rapidly in a waterbath at 37°C. Once defrosted, they were handled in the class II microbiological safety cabinet (MSC). 25 centilitre tissue culture flasks were filled with 10 ml of pre warmed DMEM (Sigma, UK) containing 10% FBS (BioSera, UK), Primocin™ (50 mg/ml, 1:500 in media, InvivoGen) and L-glutamine (2mM, Sigma) to which 1ml of MFE280s were added using a 1000 µl pipette and left to adhere to the surface of the flask over-night in an incubator maintained at a temperature of 37°C. Ensuring the cells had adhered to the culture plate, the media was changed 24 hours after plating to ensure all DMSO was cleared. The cells were monitored daily to assess the confluence.

Once the cultured cells reached an appropriate confluence (80-90%) they were 'split'. The cell culture plate (100mmx 20mm treated and non-pyogenic) was transferred to the MSC where the resting media was removed using a Pasteur pipette. The cells were washed thrice using phosphate buffered saline (PBS). 1 ml of trypsin was added to the cells for 5 minutes and returned to the incubator to allow protein bond degradation between the cells and the tissue culture plates. Once a 'mucus' consistency was seen in the plate 2 ml of media was added to the plate to inactivate the trypsin. Cells were collected into a 15 ml tube and centrifuged to allow the formation of a cell pellet. The centrifuge was set to run for 5 minutes at 500 RCF. The supernatant was tipped into Virkon, and 3 ml of fresh media was added to the cells and thoroughly mixed to create the stock solution. 3 consecutive 15 ml tubes were arranged into which the serial cell dilutions were placed. 1 ml of stock solution was mixed into 10 ml of media. Once the 2 solutions had been combined, 10µl of this dilution was placed onto a cell grid to enable analysis of number of cells/100 µl. Further dilutions were calculated dependent on number of cells present.

100 µl of cellular media was pipetted into each well and seeded in triplicates at various densities ranging from $18.5x10^4$ cells/ml to $4.5x10^4$ cells/ml in 96-well tissue culture microtiter plates (Nunc, Fisher Scientific, UK). More than one plate was set up at once with

respect to different treatment times. The 96 well microtiter plates were returned to the incubator overnight to allow adhesion of cells to the surface of wells prior to treatment of cells with various reagents.

3.10.2 Substances used to alter the proliferation rate of endometrial cancer cells lines

GYY4137 is a small molecule releaser of H_2S and is used in investigations where slow and controlled release of H_2S is required over a long period of time. An 80 mM stock was produced by dissolving 30 mg of GYY4137 in 1 ml of water. Further dilutions of 50, 100, 200 and 400 µM solutions were used for the treatment of Ishikawa cells in initial experiments. However, concentrations over 100 µM were observed to be toxic and so weaker concentrations of 25, 50, 75 and 100 µM were used for further investigations.

L-cysteine (25mg/ml in water, Sigma, UK) is a known sulphur containing compound that is used as a substrate by CBS, CSE and 3-MPST in H_2S synthesis. MFE280s were treated with 100 µl of 1 mM and 3 mM concentrations of L-cysteine and any effect on proliferative potential of the cell line was measured reading the absorbance of the solubilised solution.

After the overnight incubation of the 96 well microtiter plates, they were removed from the incubator and transferred to the MSC. The existing media was removed using a pipette and disposed into Virkon, after which the cells were treated with 100 µl of various concentrations of GYY4137and L-cysteine. In addition to treated cells a row of control cells were left untreated to which 100 µl fresh media was added. These cells were returned to the incubator for the times stated below.

Table 18 Experimental solvents and criteria for investigating cell viability of the MFE280 endometrial cancer cell line

	GYY4137	**L-cysteine**
Final Concentrations	25, 50, 75 and 100 µM	1, 3 mM
Final Times	24 hours	48 hours

3.11 Monitoring cell proliferation using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Determination of cellular proliferation is an essential technique in cell based research. Proliferation is a useful marker to evaluate cytokine or hormonal activity. Techniques to stain cells are varied and can include tryphan blue staining that uses cell membrane integrity to approximate cell proliferation or death. However, this method is not very sensitive and cannot be moulded for high throughput screening. Radioactive material such as tritium-labelled thymidine is accurate but there are hazards associated with working with radioactivity.

The technique used to evaluate the number of viable cells in this study was a colorimetric assay known as the MTT assay. This method of cell determination is useful in the measurement of cell growth in response to mitogens and growth factors, cytotoxic studies and production of cell growth curves. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazole. This yellow, soluble substance is reduced to the insoluble purple formazan only in the presence of reductase enzymes found within the mitochondria of living cells.

When the mitochondrial reductase enzymes come into contact with the MTT they cleave the tetrazolium ring which results in the production of formazan crystals which are insoluble in aqueous solutions and can only be solubilised in acidified isopropanol. Once the insoluble formazan crystals were solubilised the absorbance of this solution can be measured using a spectrophotometer at a wavelength of 570nm. The absorption max tends to rely on the solvent used. The cells were harvested in triplicates and were treated with varying concentrations of the substance under investigation, in this case the organic H_2S donor GYY4137. The treated cells were then directly compared with cells that were left untreated, the controls. This allows the deduction of any relationship between the cells and H_2S and provides results to evaluate whether a dose response relationship exists.

If an increase in absorbance is detected, this assumes an increase in the amount of formazan produced representing a rise in the number of viable cells. Although accurate, the MTT assay does have certain drawbacks, such as the physiological conditions and variability of the activity of the reductase enzymes in different cells. Furthermore, there are potential sources of error whilst conducting this assay. Appropriate storage of MTT at -

20 °C away from light should be ensured to avoid degradation. Possible contamination with microbes leading to an increase in cleavage of tetrazolium rings causing divergent results. Finally irregular evaporation of media from wells in multi-well plates could produce erroneous results.

3.11.1 Cell proliferation assay

Once the treatment time with solutions was completed the plates were returned to MSC where the existing media was removed and replaced with 100µl of fresh media and 20µl of 3(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (5 mg/ml) (MTT; Sigma, USA) dissolved in PBS. Once the MTT was applied to the cells they were returned to the incubator for 3.5 hours in 5% CO_2/air at 37°C. The 120 µl of solution was removed from the cells and disposed into Virkon. The cells were solubilised using 100 µl of a MTT solubilising solution made up of 4mM HCl 0.1% Triton X-100 in isopropanol. The microtiter plate was covered with foil and placed on the varishaker (DYNATECH) for 15 minutes. The absorbance of the solution was recorded using MultiSkan Ascent (Thermo Electron Corporation) with the Ascent software version 2.6 (Thermo Lab systems).

Absorbance readings were averaged and corrected for blank (media alone with MTT reagent). The ratio relative to control (value of 1) was calculated. GraphPad Prism 6 was used to analyse the standard error margins and create graphs of the numerical data. All graphs were plotted relative to the control value.

Chapter 4 Optimisation Chapter

4.1 Immunohistochemistry (IHC) optimisation

IHC is a highly sensitive staining technique that requires all components, such as: antibody concentrations, antigen retrieval, antibody incubation times and DAB to be optimal for effective staining. Prior to the commencement of immunostaining, various concentrations of antibodies were used to stain specific tissue sections. Different times of antigen retrieval were also employed. The optimal concentrations and times were chosen for staining. CBS antibody was optimised prior to the commencement of this project and therefore will not be discussed further in this chapter.

4.1.1 Optimisation of cystathionine gamma-lyase (CSE) antibody

A new batch of CSE antibody was received partway through the project and gave much stronger staining than the previous batch due to different stock concentrations (CSE 1:500 dilution, Novus biologicals, 0.1mg/ml). Therefore titration of this antibody was required to match the staining intensity for the 2 batches on the same tissue before continuing with staining the remaining samples. The new batch of CSE antibody was sourced form Abnova (0.5 mg/ml) and was used to stain the endometrial tissue with various concentrations. This is an unpurified mouse monoclonal antibody raised against a full length recombinant CSE. Initially the new CSE antibody from Abnova was trialed at 1:100, 1:200, 1:400 and 1:500 dilutions. Published evidence on the use of CSE antibodies for immunostaining revealed the appropriate conditions for antigen retrieval. The CSE (0.5 mg/ml) antibody was sourced form Abnova (clone number: 4E1-1B7). IHC staining was carried out using a horse-radish peroxidase (HRP) labelled polymer technique with (3,3-diaminobenzidine, DAB) (DAKO Ltd). A hydrogen peroxide (0.3% hydrogen peroxide in TBS) block was applied to sections for 10 minutes to inactivate all endogenous peroxidase activity. The sections were incubated in a humidifying chamber, overnight at 4°C. 70 microliters (µl) of the CSE antibody diluted in Tris-buffered saline (TBS) containing 0.5% (w/v) bovine serum albumen (BSA) with concentrations ranging from 1:100 to 1:500 were applied to the sections. These concentrations produced very dark and intense staining of the antibody throughout the tissue section, as can be seen in figure 12.

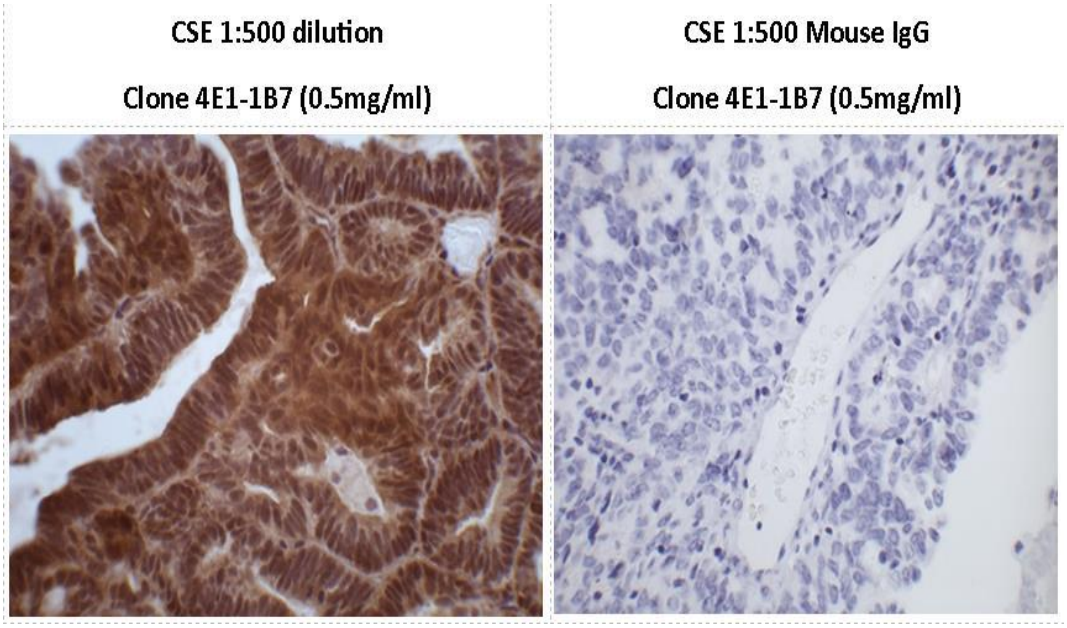


Figure 11 Comparison between the 2 clones of CSE antibodies

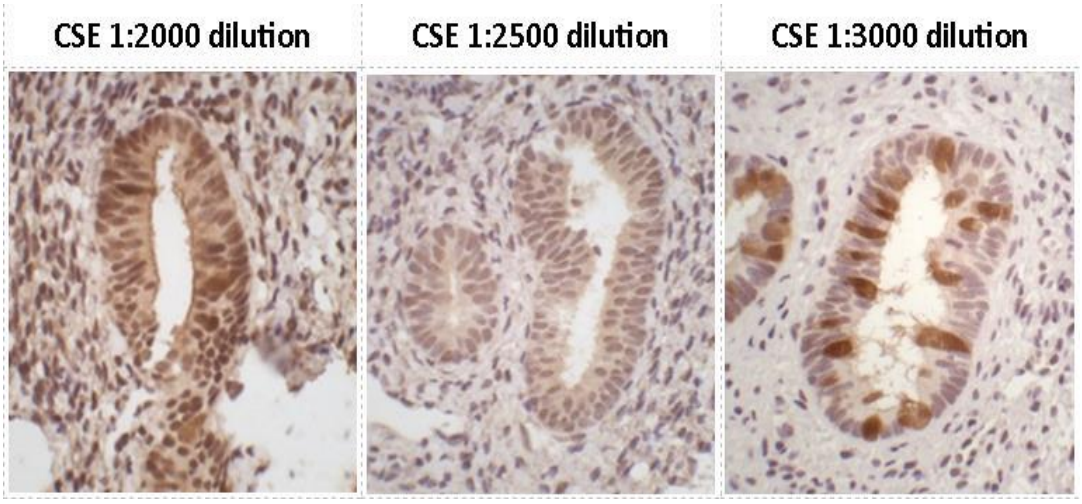


Figure 12 Serial dilutions of new clone of CSE antibody at 1:2000, 1:2500, 1:3000 dilutions

The increased staining seen at concentrations of 1:500 resulted in further dilutions being applied. The CSE antibody was further diluted to produce concentrations of 1:1000, 1:2000, 1:2500 and 1:3000. Dilutions of 1:2000 and 1:2500 still appeared very strong, as seen in figure 11.

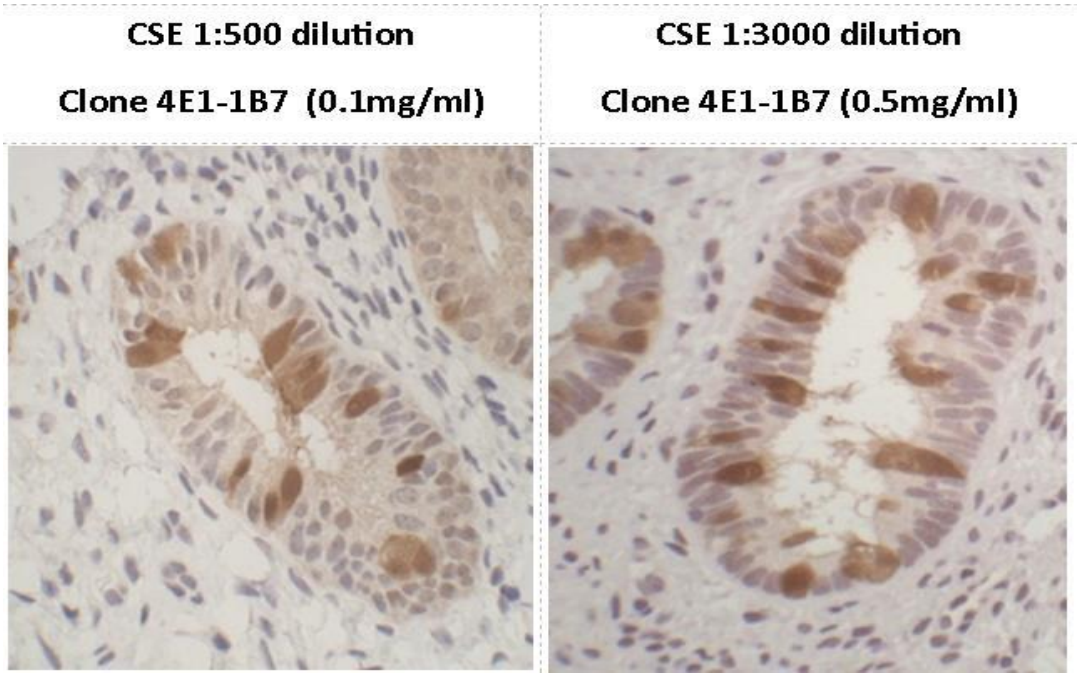


Figure 13 similar staining seen between 1:500 dilution of the previous CSE antibody and 1:3000 dilution of the new batch of CSE antibody

High magnification x400 {x40 objective and x10 eyepiece} micrographs displaying similar staining intensities at 1: 500 and 1:3000 CSE antibody dilutions as seen in figure 12.

As the antibodies have been supplied at 2 different concentrations the dilutions were bound to differ. The difference in stock concentrations would suggest a 1:500 dilution of the previous antibody to contain a similar protein concentration in a 1:5000 dilution of the new CSE antibody sourced from Abnova. At 1:3000 dilutions the staining between the two antibodies was similar resulting in a 0.3 µg/ml staining with the new antibody and 0.2µg/ml staining with the previous CSE antibody. A 1:3000 concentration was therefore decided upon for staining with the new CSE antibody.

4.1.2 Optimisation of 3-mercaptopyruvate sulphurtransferase (3-MPST) antibody

Little evidence exists regarding the expression of 3-MPST in the endometrium and any associated pattern of change in its expression in endometrial disease. The suggested dilution for optimal 3-MPST antibody staining is 200 µg/ml (Santa Cruz Biotechnology, USA).

An initial 1:400 dilution was used to stain specimens with 'human colon' being included as the external positive tissue control. Myometrial and endometrial samples were prepared with HIER in a citric acid buffer solution for 4 minutes and incubated overnight at 4°C.

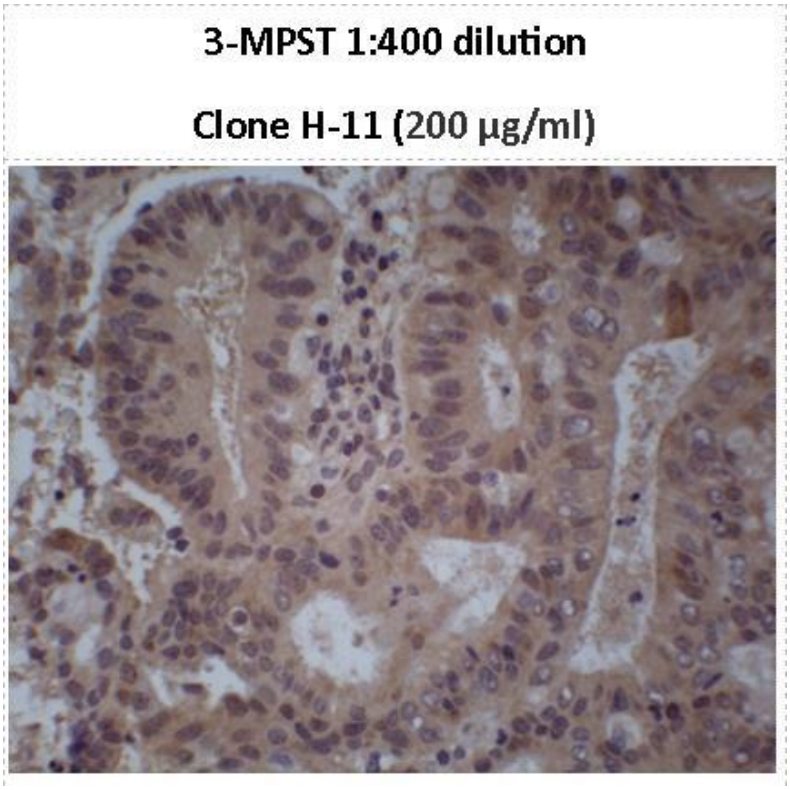


Figure 14 Faint staining of 3-MPST seen at 1:400 dilutions in citrate for 2 minutes on an endometrial sample

The human colon was used as the external positive control for 3-MPST. When comparing the intensity of staining between human colon and the type 1 EC samples stained in figure 13 it is apparent that the staining is weaker. The antigen retrieval time was increased from 2 minutes to 4 minutes. This produced similar intensity of staining between the external human colon control and endometrial sample.

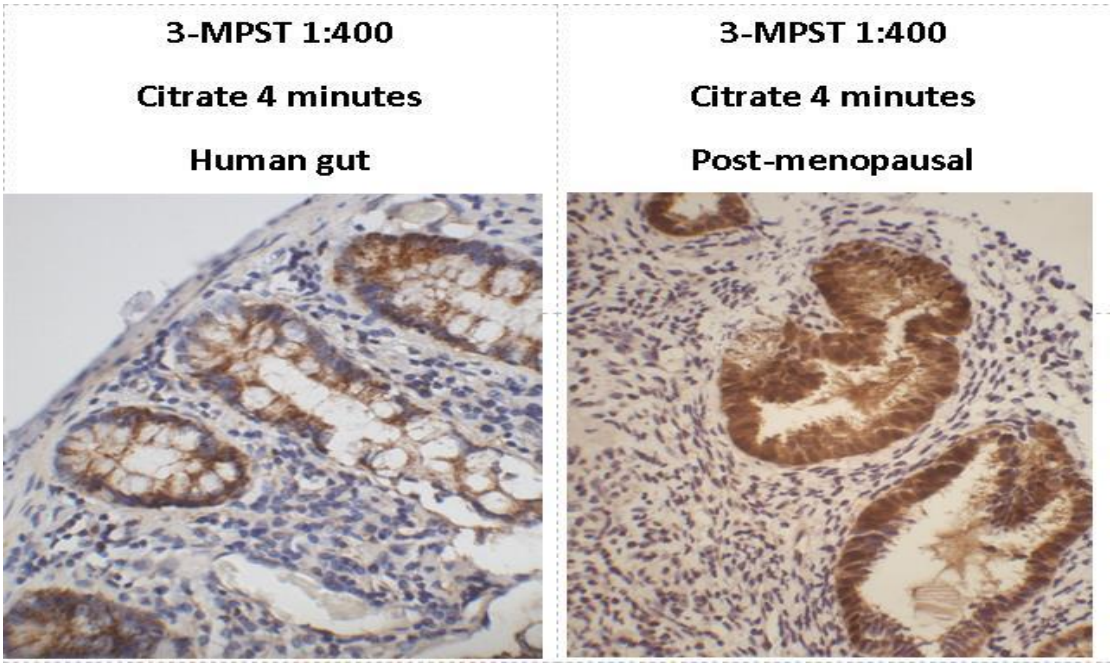


Figure 15 Similar staining seen between human colon and PM controls

4.2 Optimisation of GYY4137 concentrations

In vitro functional studies have been conducted with the use of GYY4137, an organic H_2S donor derived from Lawesson's reagent. The Ishikawa cell line (ISK) derived from a grade 1, well differentiated, endometrial adenocarcinoma was used. Once cells were cultured and seeded at appropriate densities, subsequent treatment of cells seeded in triplicates with GYY4137 over long periods of time, ranging from 12-72 hours was commenced. Concentrations ranging from 0 to 400 micromolar (µM) were investigated. Analysis of MTT assay results showed a lack of a dose dependent relationship, illustrated in figure 15.

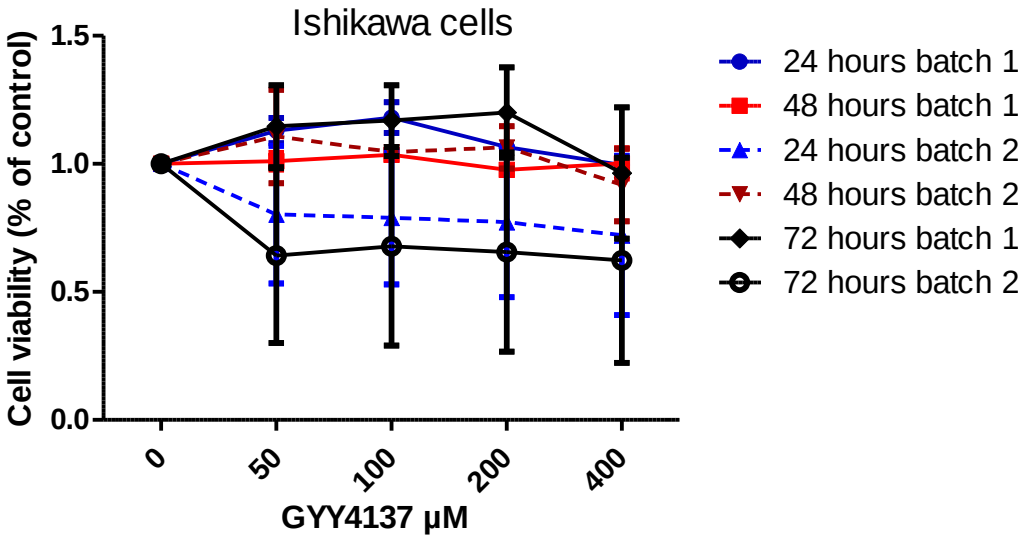


Figure 16 No change in cell viability of the Ishikawa cells could be seen after the administration of GYY4137

The lack of proliferation of the ISK cells in response to GYY4137 was thought to be due to its lower expression of CBS when compared to the MFE280s relative to GAPDH. In order to find the cell line that expressed CBS the most, all available cell lines were normalised to the enzyme glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The results of normalisation can be seen below.

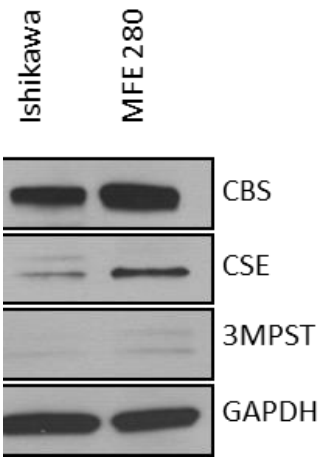


Figure 17 Western blot illustrating normalisation of all available endometrial carcinoma cell lines to GAPDH

The MFE280 cell line expressed CBS the most and therefore was used to conduct all further *in vitro* experiments. The MFE280s have been derived from recurrent, poorly differentiated endometrial carcinomas. The cells required a longer incubation time in DMEM (Sigma, UK) containing 10% FBS (BioSera, UK), Primocin^{TM} (50 mg/ml, 1:500 in media, InvivoGen) and L-glutamine (2mM, Sigma). More than 7 days were needed to allow the cell to form a confluent monolayer.

The cells were 'split' and seeded at densities ranging from $18.5x10^4$ cells per well to $4.5x10^4$ cells per well in triplicates. From an 80mM GYY4137 30mg / 1ml of water (Santa Cruz biotechnology, USA) stock solution, serial dilutions of 50, 100, 200 and 400 µM were created. The cells were treated over a 24, 48 and 72 hour period.

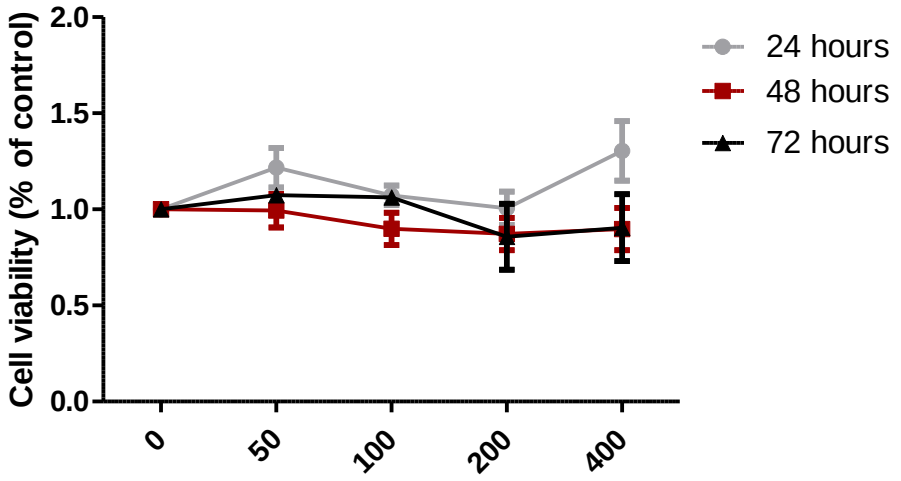


Figure 18 Graphical representation of mean and standard error margins (SEM) for viability of MFE280s in response to GYY4137 concentrations of 0, 50, 100, 200 and 400 µM at 24, 48 and 72 hours.

An increase in proliferation can be seen between GYY4137 concentrations of 0 and 50 µM within a 24 hour time period as seen in figure 17. To investigate further the GYY4137 concentrations were further broken down into 0, 25, 50, 75 and 100 micromolar and treated for 24 and 36 hours.

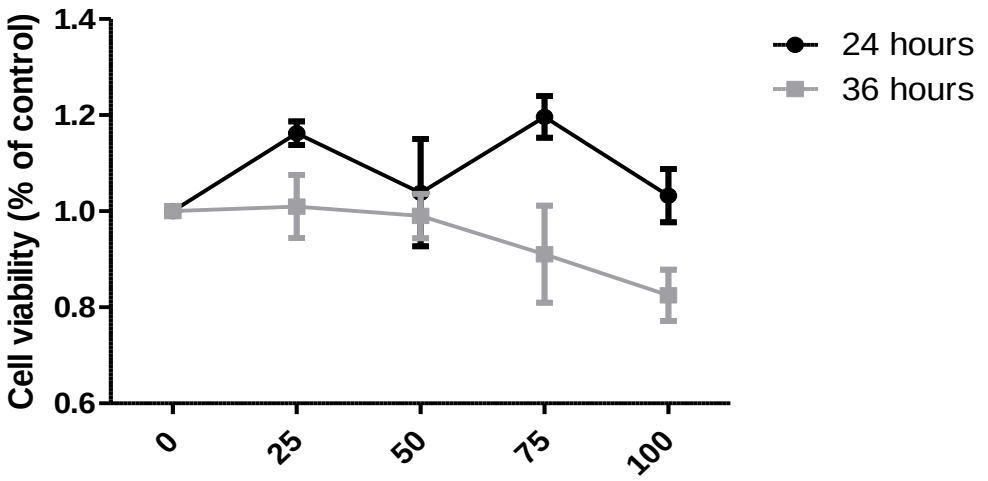


Figure 19 Graphical representation of mean and standard error margins (SEM) for proliferation of MFE280s in response to GYY4137 concentrations of 0, 25, 50, 75 and 100 µM at 24 and 36 hours.

Comprehensive numerical analysis of the data has shown a clear increase in proliferation of cells at 24 hours between concentrations of 0 and 25 µM. The biphasic appearance of the graph and the large SEMs does not make the values at 50 µM concentrations highly reliable. All available values for GYY4137 concentration of 50 and 100 µM at 24 hours were added to the current data set. This resulted in 15 replicate values being plotted at this concentration.

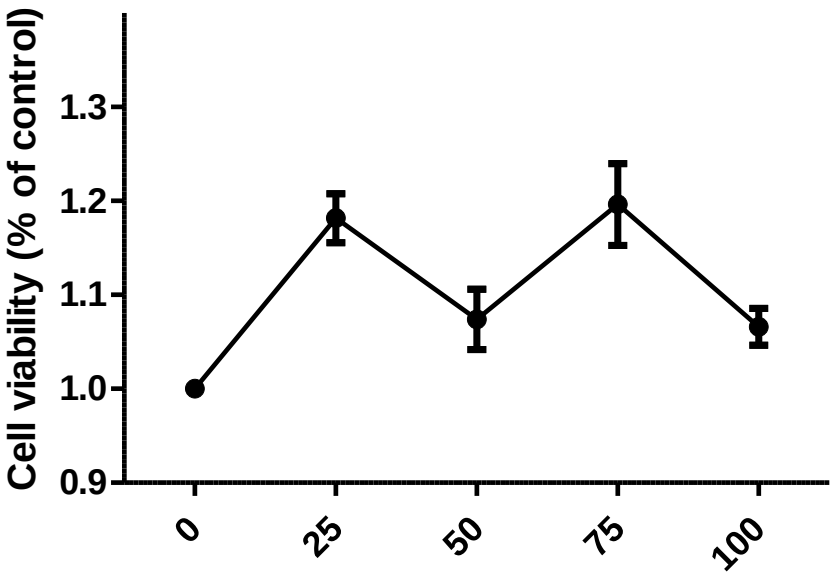


Figure 20 Graphical representation of mean and standard error margins (SEM) for proliferation of MFE280s in response to GYY4137 concentrations of 0, 25, 50, 75 and 100 µM at 24 hours

The standard error margins have tightened with the addition of 15 replicate values with no change seen in the biphasic nature of this graph. Final concentrations for further experiments included 25, 50, and 75 µM at 24 hours.

Chapter 5 The expression of H_2S synthases is altered in endometrial carcinomas

5.1 Introduction

Endometrial cancer is the 4th most common malignancy seen in women residing in UK and EU countries[5, 53, 54]. 7400 new cases are diagnosed annually with a continual rise in incidence[54]. Endometrial carcinoma was classified into two sub groups. Type 1 endometrioid carcinomas, whose development was associated with an unopposed hyper-oestrogenic environment, can be preceded by atypical hyperplasia. Type 2 endometrial carcinomas or also known as non-endometrioid carcinomas hold a poor prognosis, are oestrogen independent and known to arise from an atrophic endometrium. The general presentation of endometrial cancer regardless of type is irregular and continuous bleeding secondary to invasion of vessels by malignant cells[5, 53-55]. Majority of women are post-menopausal at presentation with a median age of diagnosis of 63 years. It is an unlikely diagnosis in women less than 40 years, although not unknown. Due to this post-menopausal endometrium was used as the control group in this study.

Current evidence implicates the much verified role of unopposed oestrogens both endogenous and exogenous in the development of endometrial carcinoma. Dysregulation of hormonal balance between oestrogen and progesterone in conditions such as diabetes, PCOS[65, 71] or obesity[56, 68] are known risk factors for the development of endometrial carcinomas. Increasing literature was published implicating various molecular pathways in carcinogenesis. Pathways such as PTEN, KRAS, and beta-catenin are associated with type 1 carcinomas and TP53 typically seen in type 2 carcinomas have all been implicated as cancer drivers of the genome[147]. Driver mutations are those that encourage the growth and spread of neoplasia whereas other mutations, namely, passenger mutations tend not to contribute to cancer development. Discerning between these mutations can further current understanding of ominous pathways contributing to development and mortality of cancers[147].

Besides the much verified mitotic role of unopposed oestrogen in the development of endometrial cancer[53-55], this project studies the prospective role of H_2S as a biological signalling molecule in endometrial cancer. Experimental studies conducted in various organic tissues have defined some tissue specific roles of H_2S. Furthermore, the presence of CBS, CSE and 3-MPST was described in various carcinomas[4, 118].

No consensus of opinion exists regarding the best method of measuring H_2S[132]. This presents difficulty in direct measurement of H_2S and requires the adoption of alternate method of detecting H_2S. Methods such as IHC and western blot analysis allow the detection of H_2S synthases in human tissues. Therefore, attempts were made to employ IHC staining for the localisation and quantification of the expression of H_2S synthases in healthy PM endometrium and endometrial carcinoma tissue samples.

CBS as aforementioned was shown to be up regulated in colorectal and primary serous ovarian carcinoma and the subsequent rise in H_2S production was implicated in tumorigenesis[4, 146]. To ensure that the changes in proliferation seen are secondary to CBS derived H_2S they explored the proliferative potential of another by product of CBS metabolism i.e. l-Cystathionine. The administration of l-Cystathionine failed to affect the proliferation rate of the cells. The release of H_2S was directly quantified using the methylene blue assay. In addition the pharmacological inhibition of CBS using AOAA and shRNA mediated silencing of CBS revealed a reduction in tumour growth *in vitro* and *in vivo* thereby implicating the rise in H_2S levels in carcinogenesis[4]. No change in expression of CSE and 3-MPST was observed in colorectal carcinomas. The neovascularisation potential of CSE as a protective agent in cardiovascular disease and in tumour maintenance was evidenced by studies reporting the protective function of H_2S against progression of cardiovascular disease through vasorelaxation, inhibition of cardiovascular remodelling and resistance to the formation of foam cells[99].

The release of free radicals and other reactive oxygen species was proposed in endometrial carcinogenesis with alteration in the function of the antioxidant superoxide dismustase[148]. This interference between free radical and anti-oxidant activity leading to increased oxidative stress has shown a down regulation of 3-MPST/ H_2S pathway[119]. 3-MPST has not been implicated nor its role clearly defined in carcinogenesis. Its cytoprotective role was proposed in a variety of tissues[149, 150].

Furthermore, an extensive literature search has shown no previous description of the expression of these enzymes or the role of H_2S in the endometrium. This following chapter describes the examination of differential expression of H_2S synthases at a cellular level in endometrial tissue acquired from women diagnosed with endometrial cancer and healthy PM endometrium with no endometrial pathology, in order to explore the possibility of the involvement of H_2S or its synthases in endometrial carcinogenesis.

5.2 Method: Immunohistochemistry

5.2.1 Patient and specimen recruitment

The approval for this study was granted by Liverpool Adult Local Research Ethics committee (LREC references 11/H1005/4) and written informed consent was gained from all patients by GCP trained research personnel. The absolute exclusion criteria for all patients included: pregnancy, breastfeeding and women who have had hormone therapy within 3 months of surgery. The inclusion criteria required the patients to be competent and retain capacity to understand the procedure which was had to take place at the Liverpool Women's Hospital. Further to this the post-menopausal patients did not have menstrual bleeding for a minimum of 12 months since their last menstrual period. Human endometrial tissue was obtained from 81 women undergoing gynaecological procedures (mean age 67.5 years, mean BMI 29.9kg/m^2) at the Liverpool Women's Hospital. 61 women had a prior diagnosis of endometrial cancer along with 4 women who were diagnosed with complex atypical hyperplasia and underwent surgical treatment in the form of hysterectomies. A further 16 women who were post-menopausal with no history of abnormal vaginal bleeding or known endometrial pathology undergoing hysterectomies for uterine prolapse were recruited into the study.

Pipelle or full thickness endometrial samples were collected and fixed in NBF. Tissue processing, embedding and subsequent IHC staining was completed following the protocols listed in the appendix 3. 4µm histological sections were cut and dried onto APES coated slides, deparaffinised using xylene and rehydrated through decreasing concentrations of alcohol. IHC staining was carried out using a readily available horse-radish peroxidase (HRP) labelled polymer technique (Vector ImmPRESS, Cat #: MP-7402, Vector, Peterborough, UK) with (3,3-diaminobenzidine, DAB) (DAKO Ltd). A hydrogen peroxidase (30% in water) block was applied to sections for 10 minutes to inactivate all endogenous peroxidase activity. The sections were incubated in a humidifying chamber, overnight at 4°C. 70 microliters (µl) of the following antibodies were used for the incubation; CBS 5 µg/ml 1:300 (clone 3E1, Abcam, Cambridge, UK), CSE 1:3000 (clone 4E1-1B7, Abnova, Taiwan) and 3-MPST (SC-376168, clone: H-11, Heidelberg, Germany) 1:400 dilutions in phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumin (BSA). For all 3 antibodies the anti-mouse immunoglobulin G (IgG) was applied at a dilution 1:500 as a negative control. After an overnight incubation the sections were washed in

TBS twice for 5 minutes. 2 drops of the HRP labelled secondary mouse antibody was applied to the samples for 30 minutes at room temperature. After a final wash in TBS for 10 minutes, DAB chromogen was applied to the samples for 10 minutes and the antigen-antibody complexes visualised as a brown reaction product of tissue. All sections were placed into water to immediately stop enzymatic reactions and counterstained for 2 minutes using Gills-2 hematoxylin. They were washed under running tap water before being immersed in 1% acid alcohol to remove excess hematoxylin staining and washed again under tap water. The samples were then gradually dehydrated by increasing alcohol % gradient and mounted in Consul mount. Owing to the large sample number the slides were divided into runs and a positive internal control were used to ensure that the staining runs were all consistent. The positive external controls for all 3 antibodies differed and were stained at the commencement of staining for each antibody.

Antibody	**External positive control**
CBS	Liver
CSE	Liver
3-MPST	Human colon

The collected endometrial samples were assigned into 4 groups according to clinic-pathological features and histological characterisation by qualified clinical pathologists at Royal Liverpool University Hospital (RLUH). Two groups of endometrial cancer; type 1 (n=43) and type2 (n=18) and the healthy control group PM (n=16). The type 1 samples were further categorised based on grade (grade1 (n=24), grade 2 (n=11) and grade 3 (n=8)). The type 2 carcinomas were divided into clear cell (n=4), serous (n=4) and MMMT (n=9). Finally the n=4 complex atypical hyperplasias were included.

5.2.2 Analysis of IHC staining

10 consecutive micrographs (taken with Nikon Eclipse microscope, Nikon, Japan) were taken and assessed using the modified quick score method. A variation in staining was seen within the sample and these areas were accounted for and scored accordingly.

5.2.3 Statistical analysis

Non – parametric tests have been used to analyse data which were not normally distributed. Mann- Whiney test was used to assess pairs of data and the differential

expression between all groups of data was analysed with the Kruskal- Wallis test. These tests enabled the calculation of a P value, allowing any statistical significance ($p<0.05$) if present to be highlighted.

The quick score values for all three antibodies were used to correlate the expression of antibodies with steroid receptors e.g. AR, ER-alpha, ER-beta and PR. Spearman Rank correlation test was used. The software package IBM SPSS statistics 20 (IBM, PORTSMOUTH UK) was used to run the correlation tests.

5.3 Results

5.3.1 Patient demographics

Table 19 Patient Demographics

Patient cohort (sample number)		Age (years)	Height (m)	Weight (kg)	BMI (kg/m^2)	Parity	Disease stage
Complex atypical hyperplasia / CAH (4)	*Mean(*± SD) *Median* *Range*	52 ± 5 54 44- 55	1.59 ± 0.03 1.60 1.55 – 1.63	102 ± 41 98 50- 154	38 ± 14 39 23- 57	1 1 1	
Grade 1 (24)	*Mean* *Median* *Range*	62 ± 10 62.5 36-84	1.59 ± 0.076 1.59 1.38-1.74	86 ± 23 76.5 52-138	33.9 ± 8 30.5 23.1-53.2	1± 1 2 0-6+	2 ± 1 1 1-3
Grade2 (11)	*Mean* *Median* *Range*	65 ± 13 64 37-83	1.59 ± 0.09 1.62 1.47-1.73	84 ± 32 81 48-163	32 ± 9 31 22-54	2 ± 0 0 0-3	1 ± 1 1 1-3
Grade3 (8)	*Mean* *Median* *Range*	67 ± 9 69 52-80	Missing datum	Missing datum	29 ± 7 27 24- 43	2 ± 2 2 0-6	2 2 1-3
MMMT/ Carcinosarcoma (9)	*Mean* *Median* *Range*	73 ± 10 78 59-87	1.57 ± 0.04 1.57 1.52-1.65	70 ± 17 66 48-93	28 ± 7 24 20- 37	2 ± 2 2-4	1 1 1-3
Serous (5)	*Mean* *Median* *Range*	68 ± 3 67 64-73	1.59 ± 0.06 1.58 1.52- 1.67	71 ± 11 71 57 -84	28 ± 2 28 25- 30	2 ± 3 2-3	2 2 1-3
Clear cell (4)	*Mean* *Median* *Range*	68 ± 14 70 52-82	1.59 ± 0.09 1.63 1.46-1.65	75 ± 11 75 63-86	29 ± 2.0 30 26.6-31.5	6 ± 6 1-6	2 1 1-3
Post-menopausal (16)	*Mean* *Median* *Range*	56 ±15 56 51-78	1.60 ± 0.08 1.6 1.45- 1.76	71 ± 13 73 56- 93	28 ± 4 27 20- 38	3 ± 1 2 1-5	

5.3.2 Grouped demographics

Table 20 Grouped demographics

Demographics	***Mean(*± SD)** ***Median*** ***Range***	**PM (n=16)**	**Complex atypical hyperplasia (n=14)**	**Type 1 (n=43)**	**Type 2 (n=18)**
Age (years)	*Mean(*± SD) *Median* *Range*	56 ±15 56 34-78	52 ± 5 54 44- 55	64 ± 11 64 36- 84	71 ± 10 67 52- 87
Height (m)	*Mean(*± SD) *Median* *Range*	1.60 ± 0.08 1.6 1.45- 1.76	1.59 ± 0.03 1.60 1.55 – 1.63	1.59 ± 0.08 1.59 1.38- 1.74	1.58 ± 0.06 1.58 1.46- 1.67
Weight (kg)	*Mean(*± SD) *Median* *Range*	71 ± 13 73 56- 93	102 ± 41 98 50- 154	83 ± 24 78 48- 163	71 ± 14 69 48-93
BMI (kg/m^2)	*Mean(*± SD) *Median* *Range*	28 ± 4 27 20- 38	38 ± 14 39 23- 57	32 ± 8 30.3 22- 54	28 ± 5 29 20-37
Parity	*Mean(*± SD) *Median* *Range*	3 ± 1 2 1-5	1 1 1	3 ± 2 2 0 - 6	2 2 1-3

The individual values for height and weight in some grade 3 endometrioid carcinomas were not recorded at time of consenting. These samples were gained prior to the commencement of this study and therefore the true values could not be found. However due to the presence of BMI values, this missing evidence did not produce bias.

5.3.3 Overview of staining patterns of 3-MPST, CSE and CBS

Stained IHC micrographs of the stained sections were taken and the expression of all 3 enzymes is described below

5.3.3.1 3-MPST staining

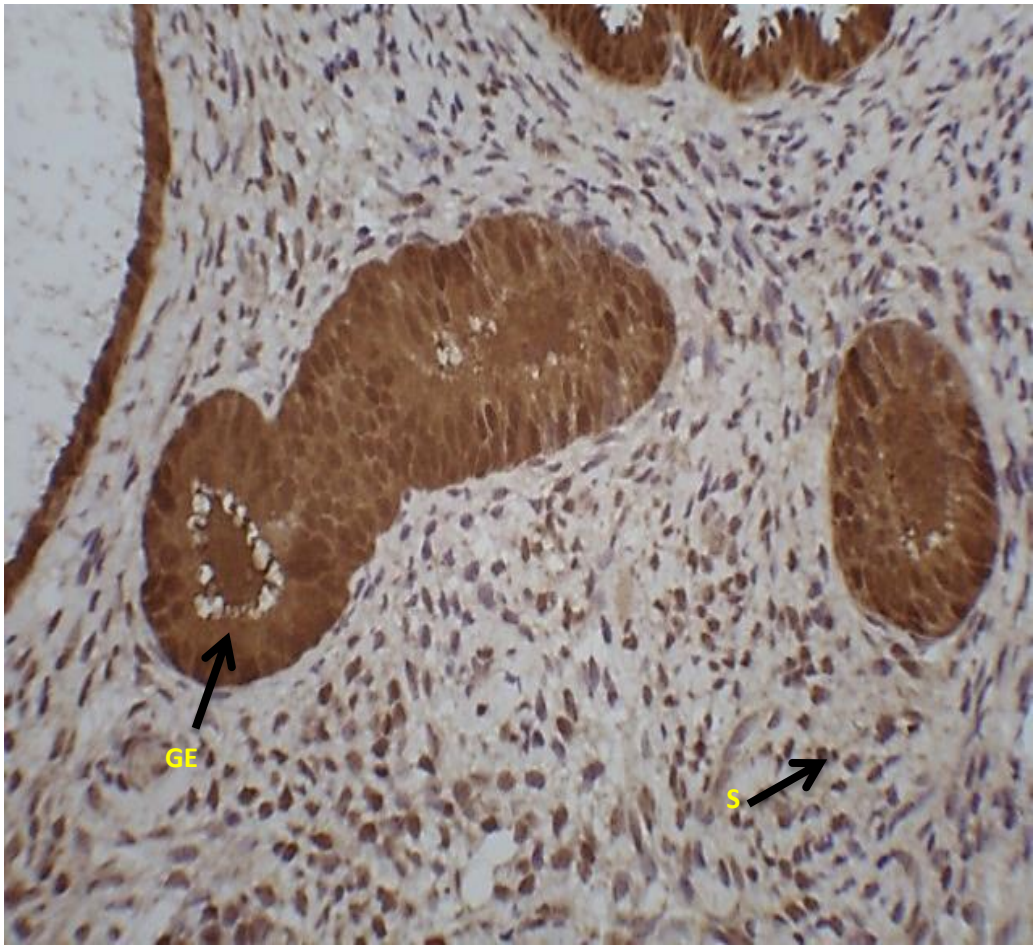


Figure 21 (x400) {x40 objective and x10 eyepiece} micrograph illustrating 3-MPST staining in glandular epithelium (GE) and stromal (S) compartment in a PM sample

3-MPST expression was localised to the glandular and stromal compartment of the endometrium.

5.3.3.2 **CSE staining**

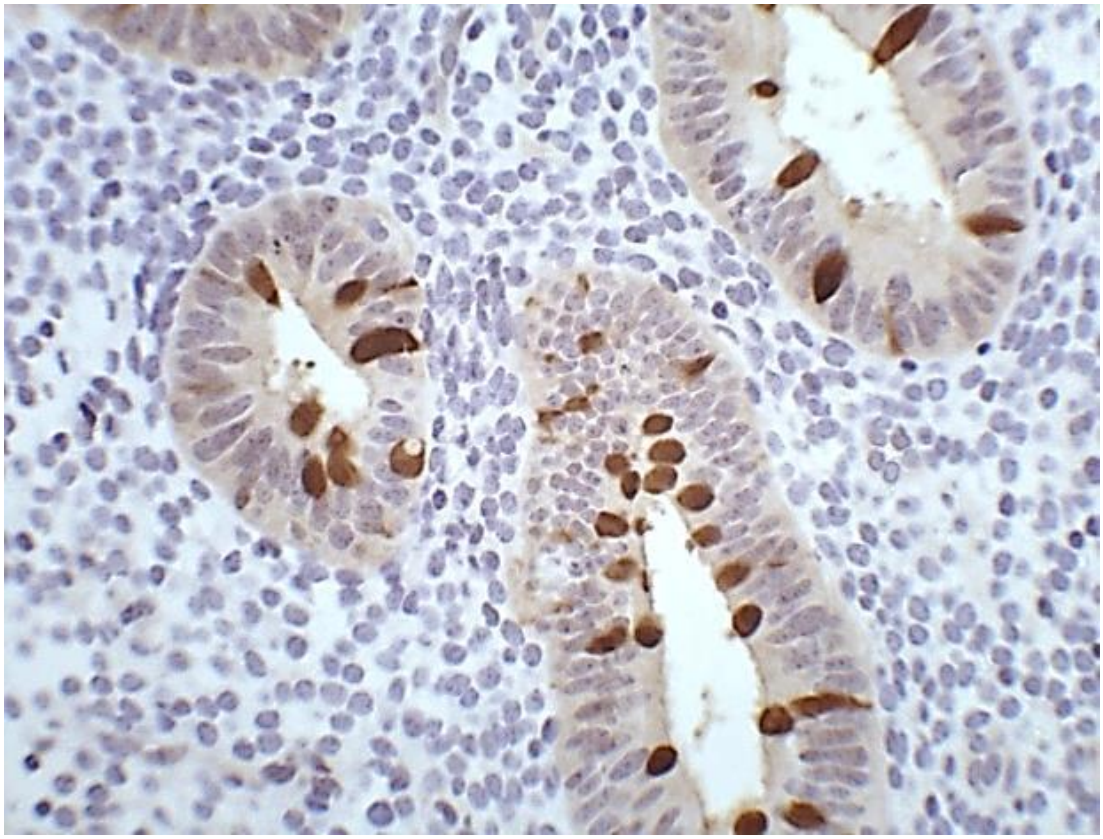


Figure 22 (x400) {x40 objective and x10 eyepiece} micrograph illustrating CSE staining in glandular epithelium in PM sample

Two distinct patterns of staining were noted for CSE, e.g. diffuse weakly stained cytoplasm (A) and distinct strongly stained cells (B). CSE expression was confined to the endothelial and epithelial cells. As CSE was extensively found to expressed in vascular endothelium, micrographs highlighting IHC staining between PM, CAH, type 1 and type 2 endometrial carcinomas have been shown in figure 22.

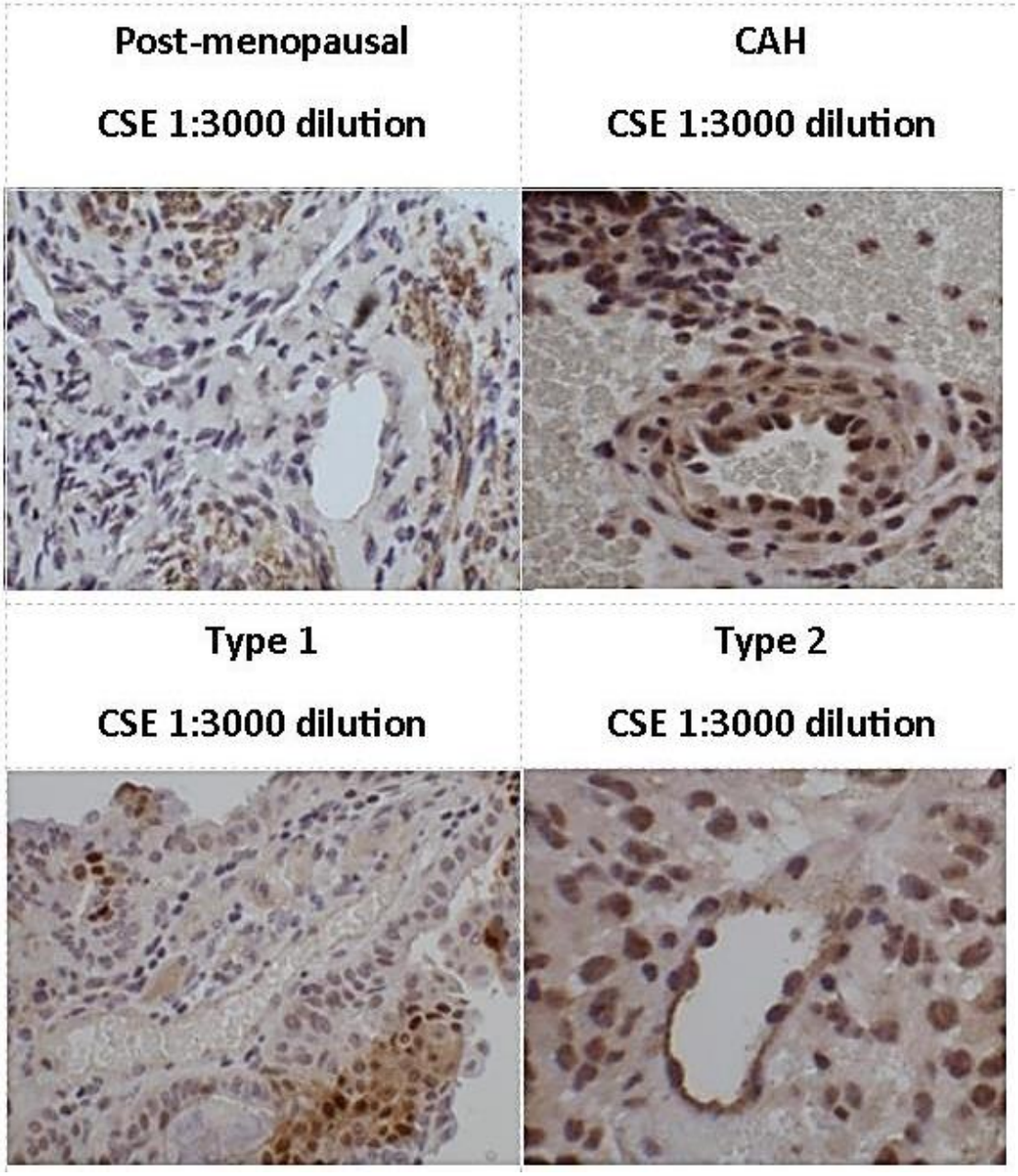


Figure 23 (x400) {x40 objective and x10 eyepiece} micrograph illustrating CSE staining in the endothelium

5.3.3.3 **CBS staining**

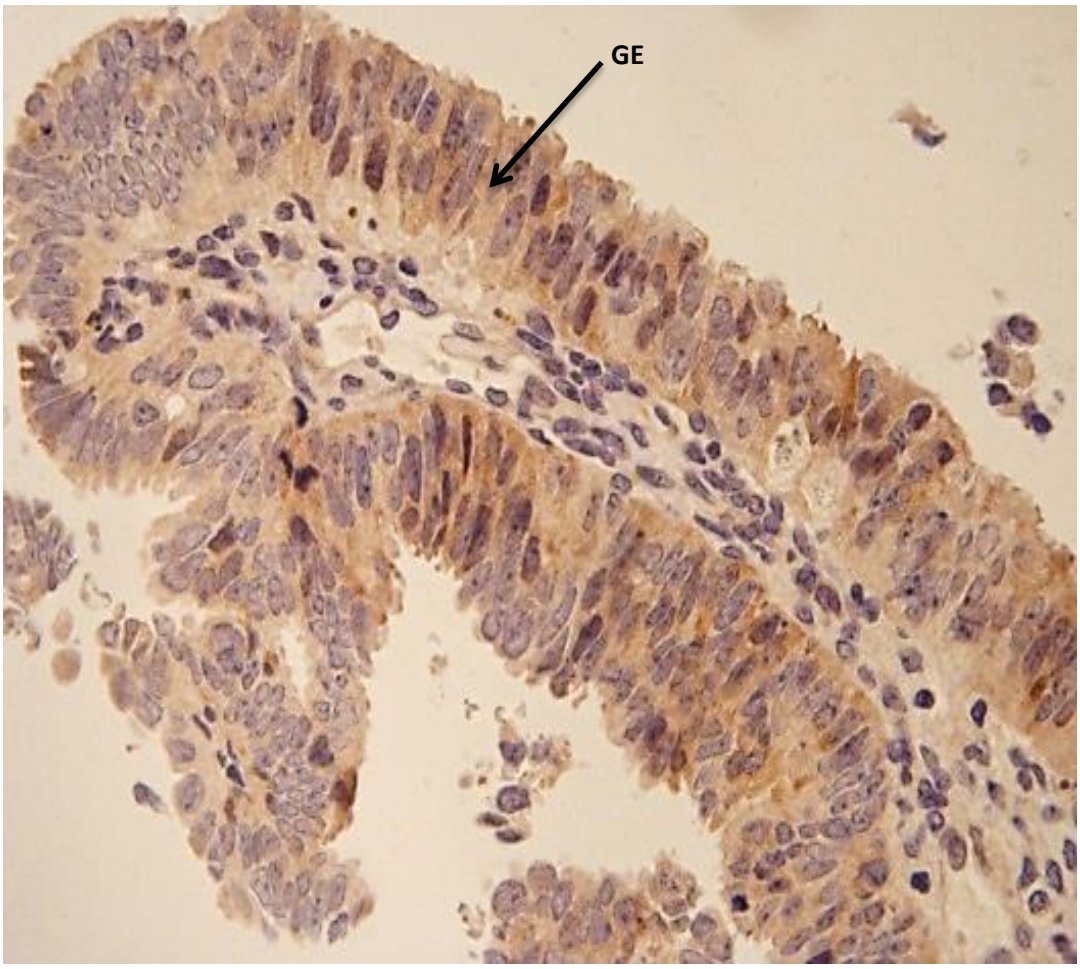


Figure 24(x400) {x40 objective and x10 eyepiece} micrograph illustrating CBS staining in the cytoplasmic compartment of glandular epithelium (GE) in Type 1 EC

CBS staining was localised to the cytoplasmic compartment of the glandular epithelium.

5.3.4 3-MPST expression in post-menopausal endometrium, hyperplasias and endometrial cancer

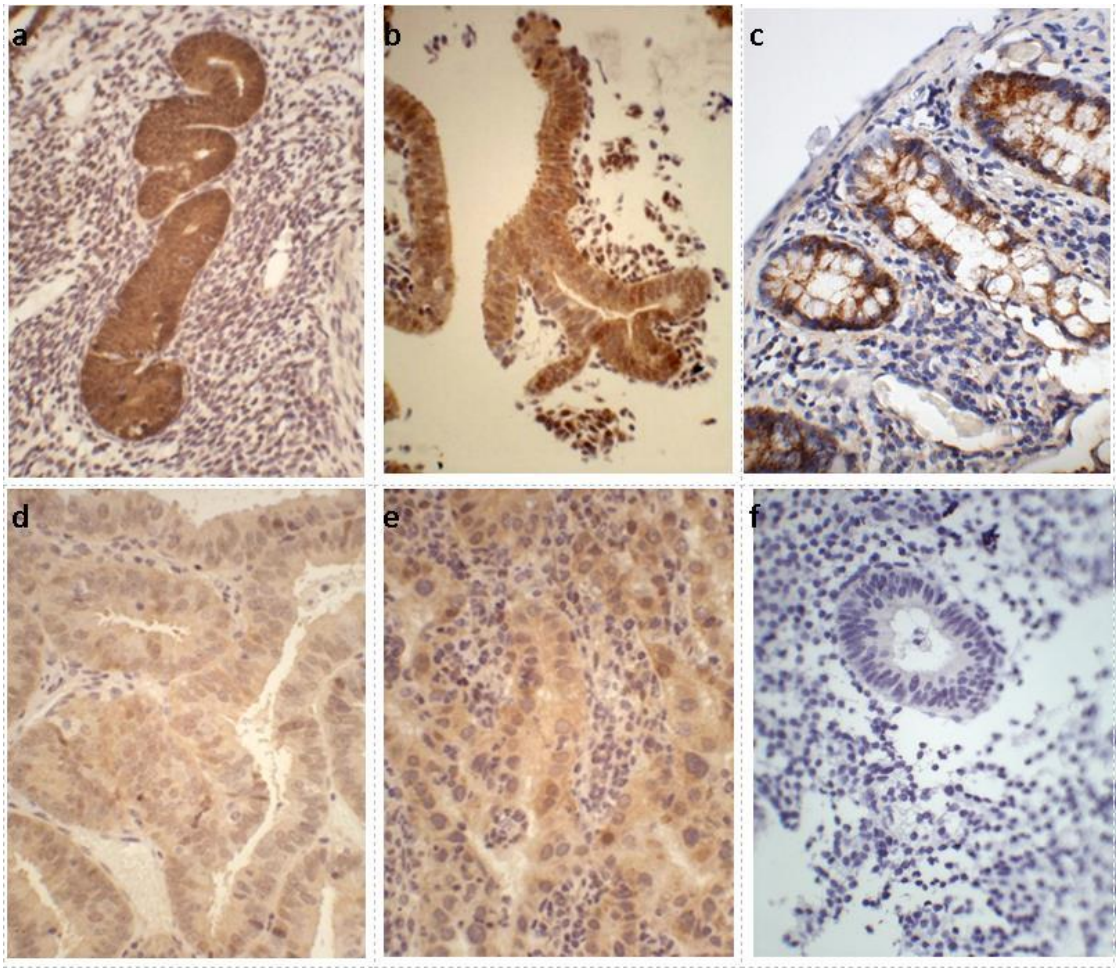


Figure 25 (x400) {x40 objective and x10 eyepiece} micrograph illustrating 3-MPST staining at 1:400 dilution. a) PM (n=16), b) CAH (n=4), c) External control (Human colon), d) Type 1 (n=43), e) Type 2 (n=18), f) IgG control.

As mentioned in the introduction (refer to page 20, 21 and table 2) due to the difficulties faced with the classification of endometrial cancers the results have been analysed by categorising EC into type 1/ type 2 and low/ high grade carcinomas.

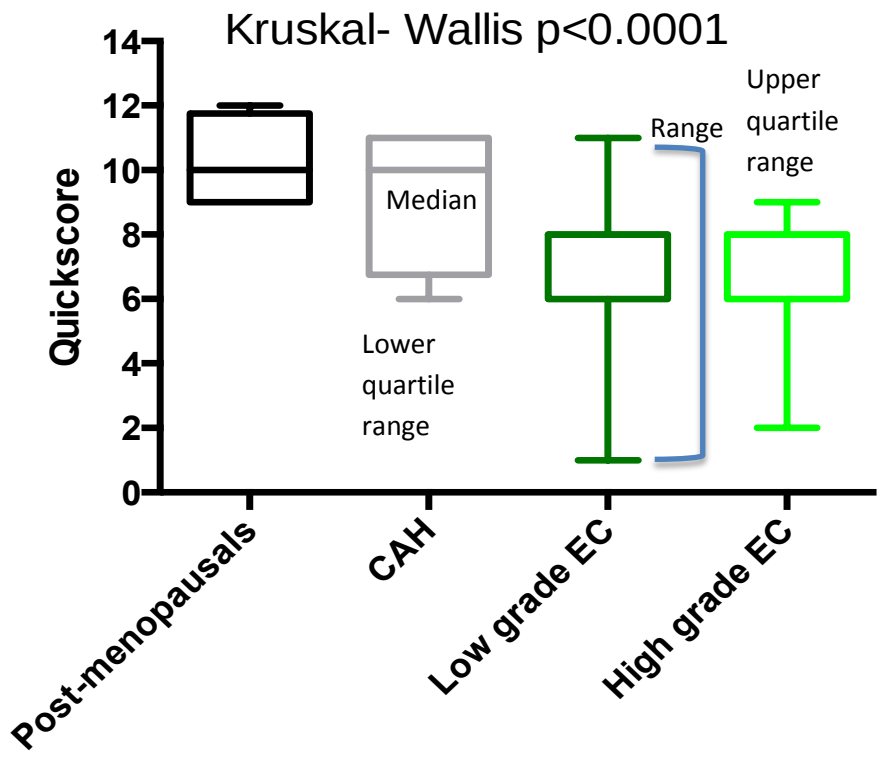


Figure 26 Graphical representation of overall loss of 3-MPST expression in cancers when compared with PM controls. PM (n=16), CAH (n=4), low grade carcinomas (n=35) and high grade carcinomas (n=26).

The modified quick score for 3-MPST staining was high in the post-menopausal endometrium. A loss of 3-MPST expression can be seen in the complex atypical hyperplasias, although not in significant amounts. Both low grade and high grade endometrial carcinomas shown an overall loss of 3-MPST staining.

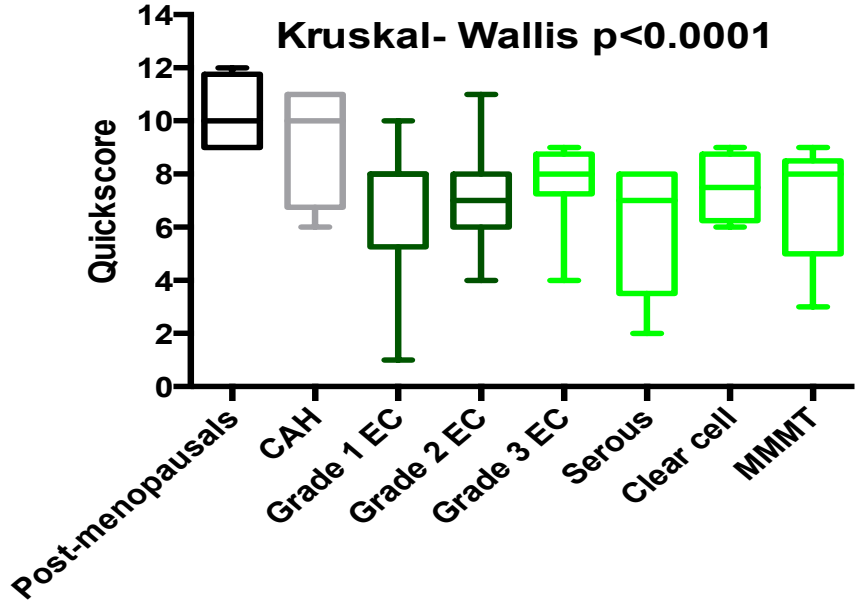


Figure 27 Graph displaying significant loss of 3-MPST staining in endometrial cancers. PM (n=16), CAH (n=4), grade 1 (n=24), grade 2 (n=11), grade 3 (n=8), MMMT (n=9), Serous (n=5), Clear cell (n=4)

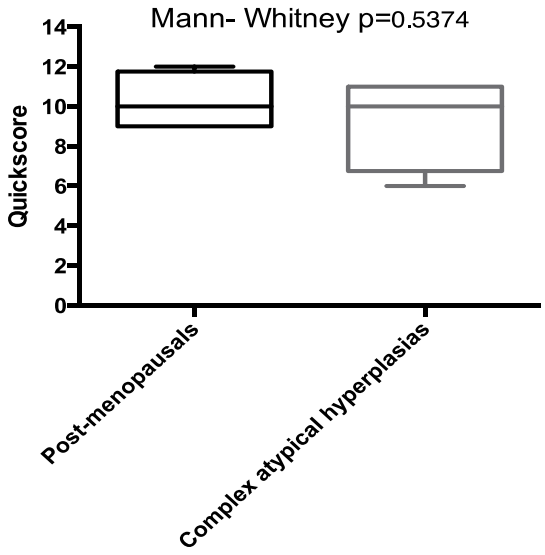


Figure 28 Change in 3-MPST expression between PM (n=16) and CAH (n=4)

No significant loss of 3-MPST staining can be seen in complex atypical hyperplasia due to the 2 groups having a similar median. Variances in values between the individual atypical

hyperplasia tissues were visible and this discrepancy will need to be investigated further by increasing the sample number in the group. If the values remain varied it could be due to the fact that the tissues that have a lower expression of 3-MPST could have a higher chance of progression to cancer.

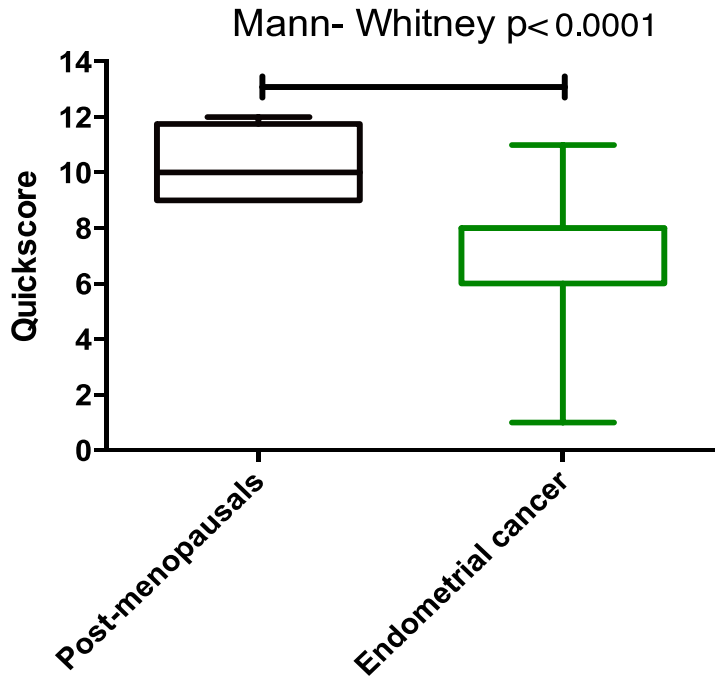


Figure 29 Graphical representation of loss of 3-MPST expression in endometrial cancers when compared with PM controls.

Significant down regulation of 3-MPST expression can be seen in proliferative cancerous endometrium when compared with atrophic PM endometrium.

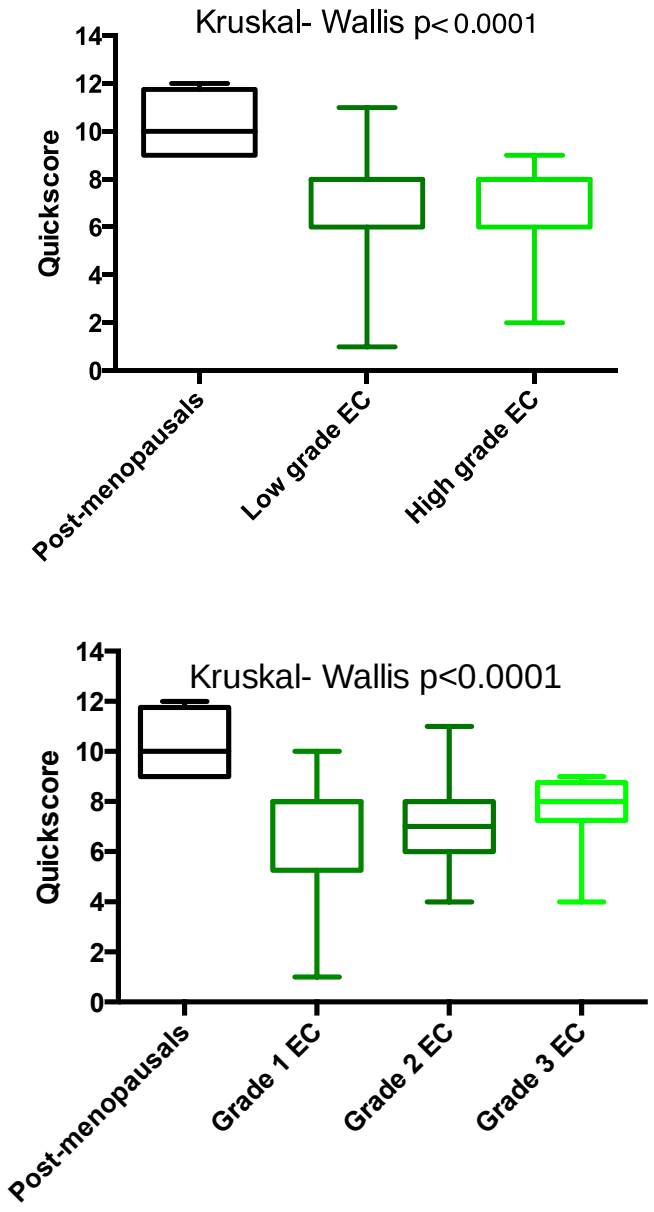


Figure 30 Early loss of 3-MPST is seen in Low grade (n=35), or grade 1 endometrioid carcinomas (n=24)

Independent evaluation of grades of type 1 endometrioid carcinomas shows a significant very early loss of 3-MPST in grade 1 endometrioid carcinomas.

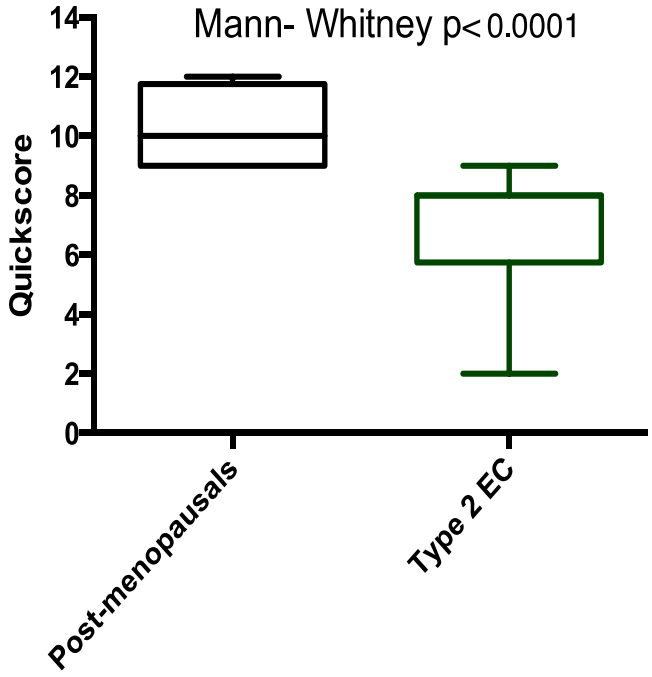


Figure 31 Graphical representation displaying loss of 3-MPST in Type 2 (n=18) endometrial cancers when compared with PM controls (n=16)

Independent comparison of type 2 endometrial carcinomas shows a similar significant loss of 3-MPST expression in these cancers as seen in type 1 endometrioid carcinomas. Therefore loss of 3-MPST could be unrelated to the oestrogen dependence and an overall feature of endometrial cancer.

5.3.4.1 **3-MPST correlation with steroid receptors**

All correlation calculations were done in conjunction with Areege Kamal.

Prior to correlation of data with steroid receptor expression the endometrial cancer cohort was divided into 2 groups. Low grade cancers (n=35) consisted of grade 1+2 endometrioid carcinomas. High grade cancers (n=26) encompassed grade 3 and type 2 carcinomas (clear cell, serous and MMMT). Spearman's rank correlation analysis of low grade carcinomas revealed no correlation between the expression of the 3 antibodies and steroid receptors.

3-MPST was found to correlate (r=0.2) weakly with progesterone receptor in high grade carcinomas after the conduction of correlational tests.

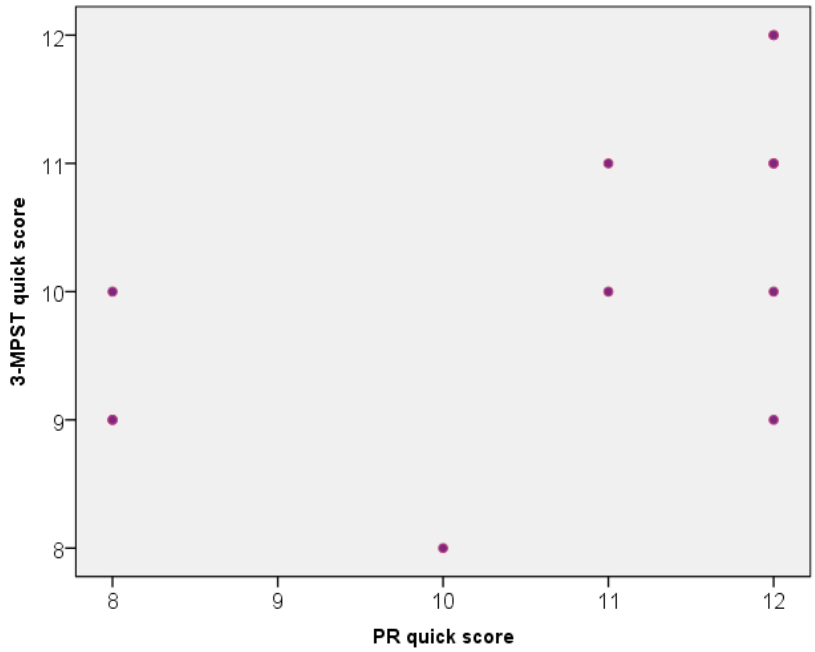


Figure 32 Graphical representation of the wear positive correlation between PR and 3-MPST in high grade endometrial carcinomas. (n=65). 1 point on graph represents more than 1 variable due to same scores achieved.

PR plays a role in cell differentiation and anatagonises the effects of ER suggesting an anti-proliferative effect on the endometrium. It is proposed to be lost in advanced stage endometrioid carcinoma and therefore a subsequent reduction in 3-MPST is to be expected. The results below show a reduction in 3-MPST in the endometrium in high grade endometrial cancers, thus providing evidence to strengthen the suggestion.

Table 21 Correlation between 3-MPST and high grade carcinomas

Correlations

			3-MPST	CBS	CSE	AR	ER-beta	PR	ER-alpha
Spearman's rho	3-MPST	CC	1.000	.009	.370	.019	.230	.263	.027
		P	.		.003	.882	.070	.034	.829
		No	65	62	62	65	63	65	65

5.3.4.2 Association of 3-MPST expression with clinic-pathological features and outcome data

The IHC modified quick scores for 3-MPST have been analysed against clinical pathological features such as myometrial invasion and lympho-vascular invasion. Myometrial invasion was categorised according to the 1991 FIGO classification into 2 groups i.e. less than 50% or more than 50% myometrial invasion. The lymph-vascular invasion was described as 'true' if present and 'false' if absent.

Comparison of 3-MPST expression with myometrial invasion has revealed no association between the two as illustrated below. Although n=62 EC samples have been stained in this project, at the time of correlation studies data for myometrial invasion was only available for 50 patients (represented as valid). This is the reasoning for discrepancy between numbers. The dots away from the box and whisker plots represent the outliers within the data set.

Case Processing Summary

	Myometrial invasion	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
3-MPST	Less than 50%	32	80.0%	8	20.0%	40	100.0%
	more than 50%	18	78.3%	5	21.7%	23	100.0%

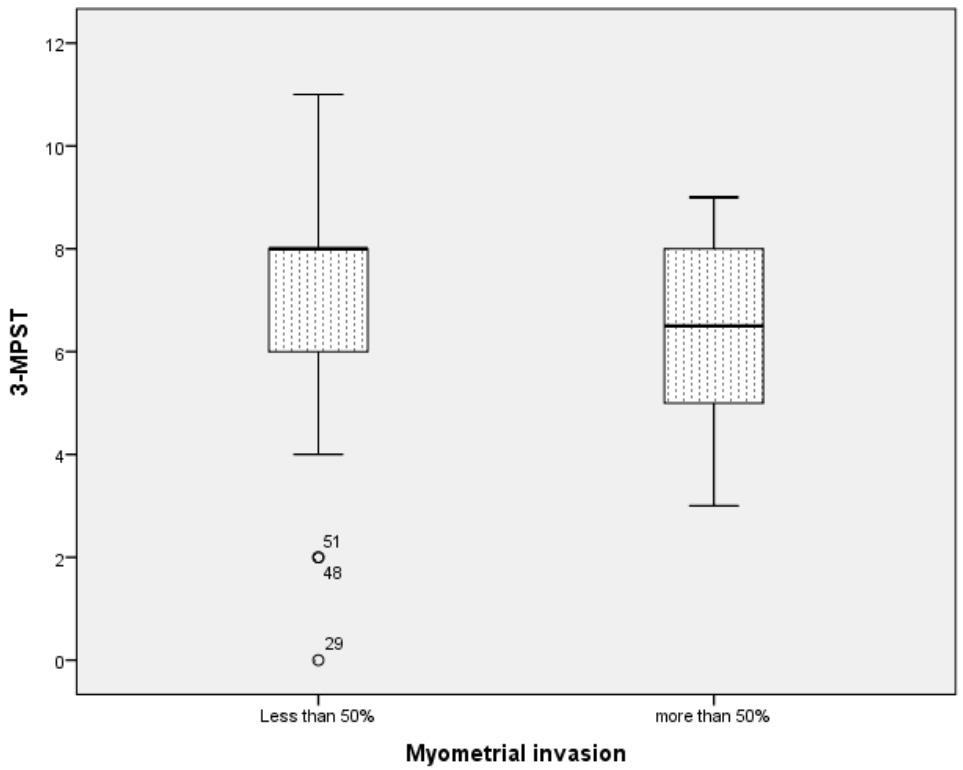


Figure 33 Graphical evidence showing no association exists between the 3-MPST expression and presence of myometrial invasion

5.3.4.3 Association between lympho-vascular invasion and 3-MPST expression in endometrial cancer

Case Processing Summary

Lympho-vascular invasion		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
3-MPST	True	17	73.9%	6	26.1%	23	100.0%
	False	34	82.9%	7	17.1%	41	100.0%

As before data for lympho-vascular invasion was only available for 51 patients in total, thereby causing an inconsistency in numbers.

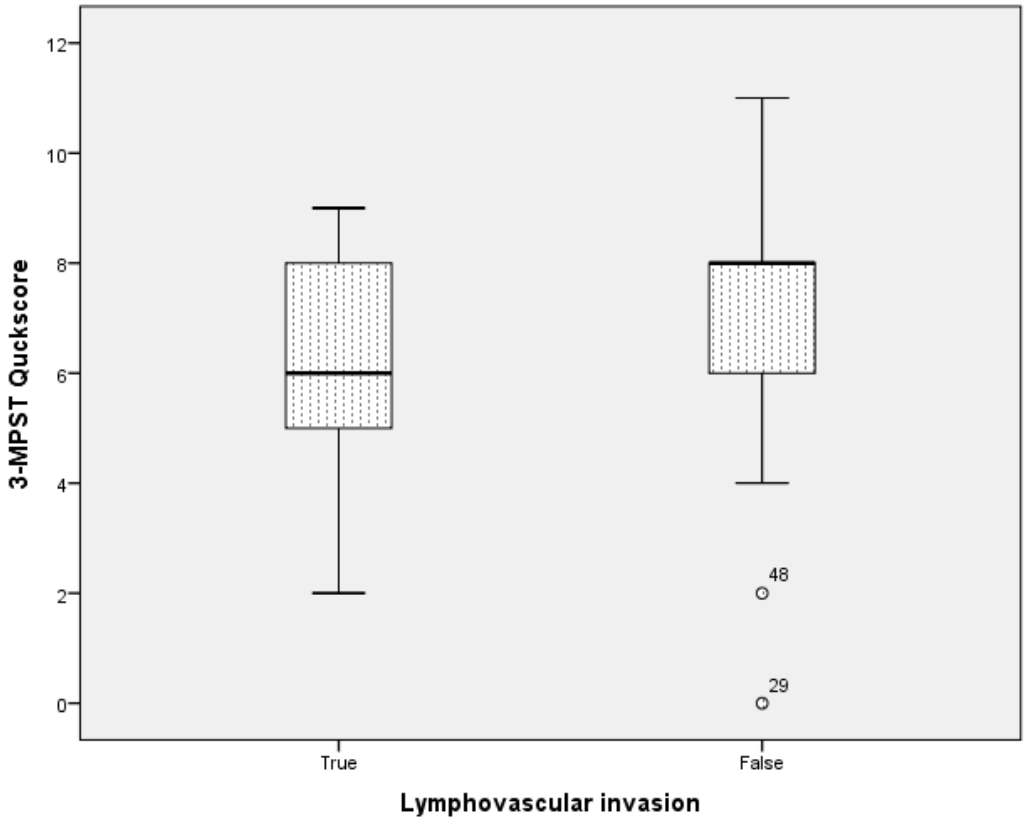


Figure 34 Graphical evidence showing no association exists between the 3-MPST expression and presence of lympho-vascular invasion

5.3.4.4 Association between outcome data and 3-MPST expression in endometrial cancer

The outcome data was categorised as '1'=event or '0'=no event. An event is either recurrence of endometrial cancer or death secondary to endometrial cancer. The IHC staining results were compared with outcome data to elicit any significant change in expression of 3-MPST in people who have had a poor outcome.

Case Processing Summary

Outcome		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
3-MPST	0	5	71.4%	2	28.6%	7	100.0%
	1	9	75.0%	3	25.0%	12	100.0%

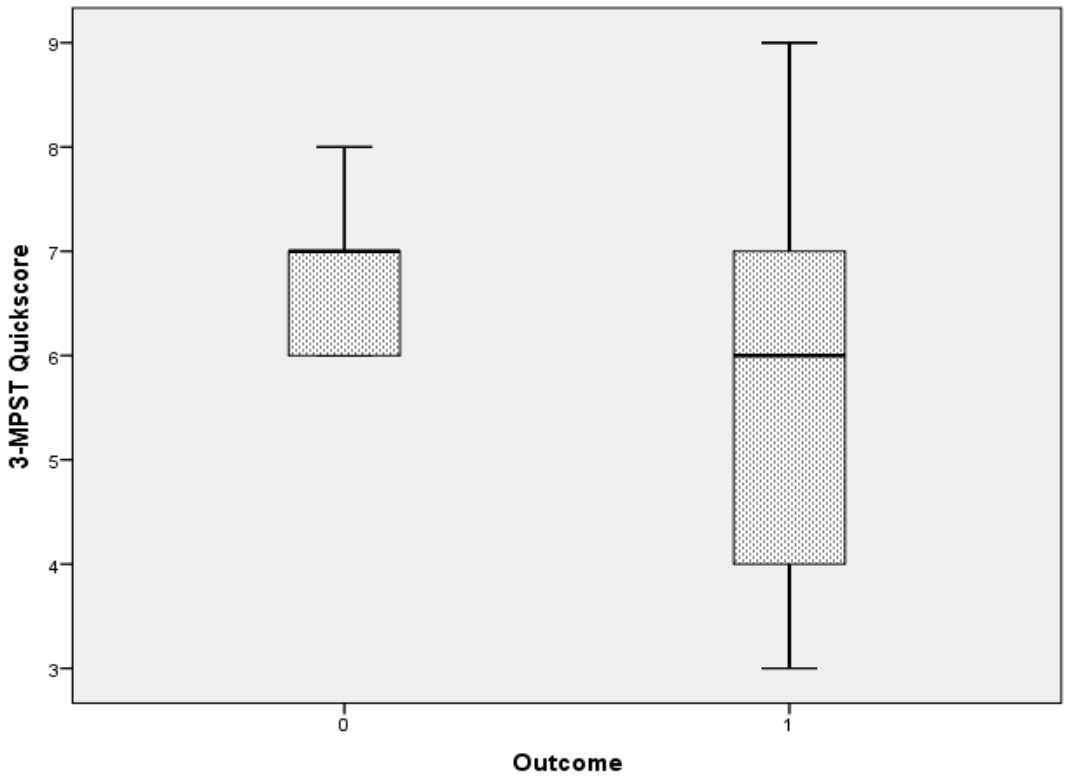


Figure 35 Graphical representation highlighting the lack of association between 3-MPST expression and outcome of patients at 36 month follow-up.

5.3.5 CSE expression in post-menopausal endometrium, hyperplasias and endometrial cancer

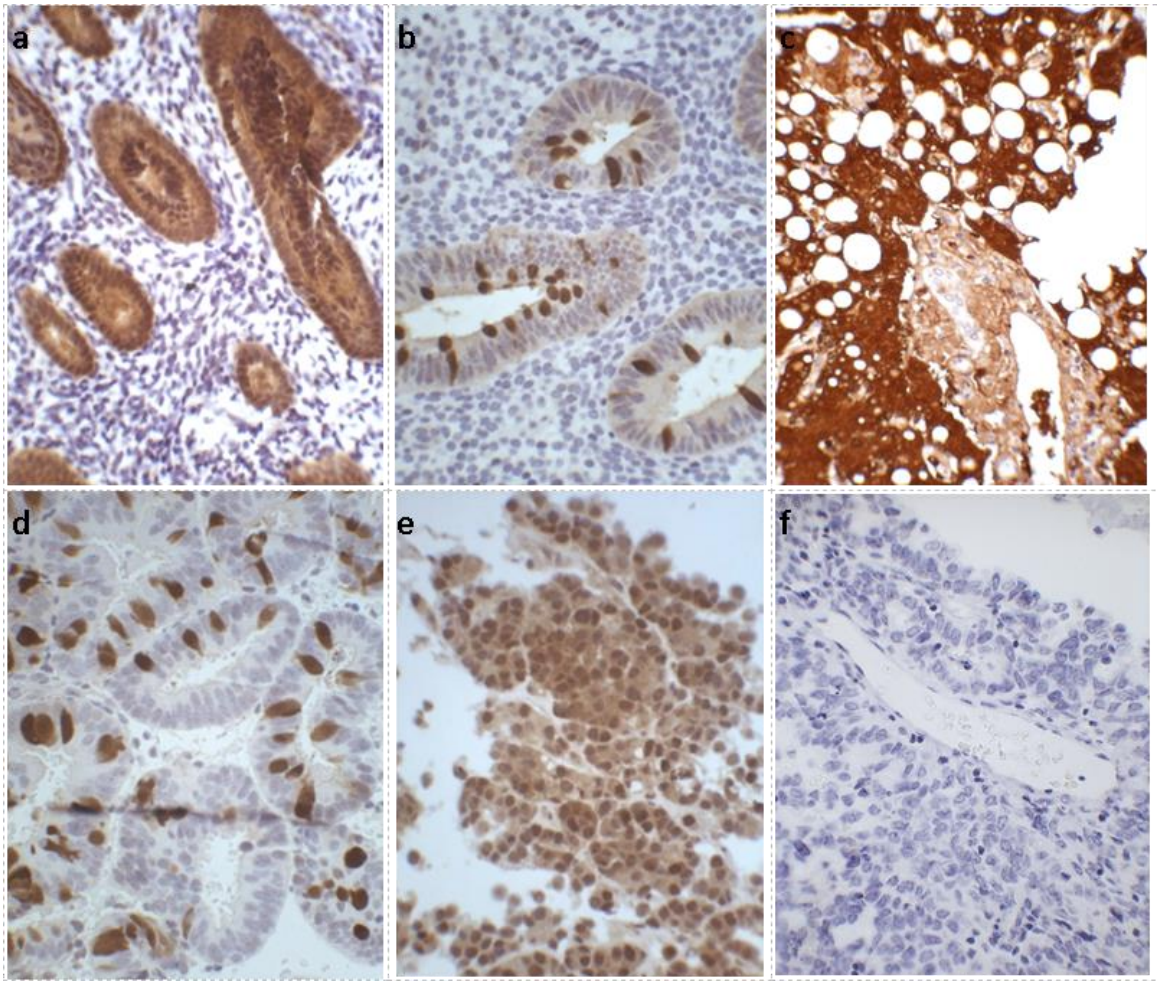


Figure 36 (x400) {x40 objective and x10 eyepiece} micrograph illustrating CSE staining at 1:400 dilution. a) PM (n=16), b) CAH (n=4), c) External control (Human colon), d) Type 1 (n=43), e) Type 2 (n=18), f) IgG control.

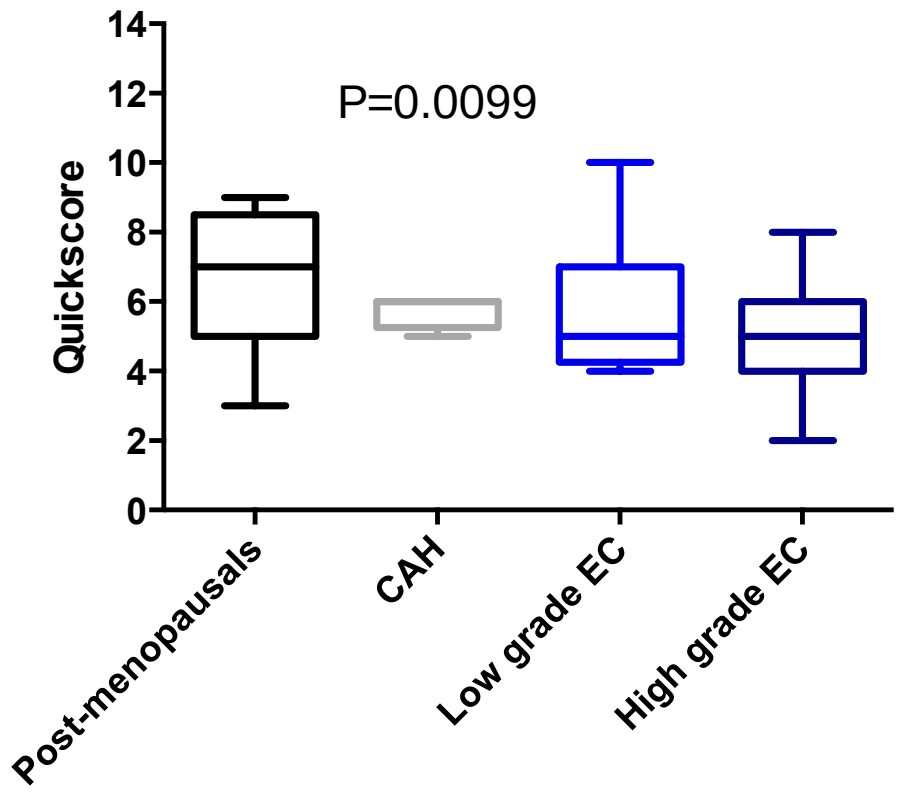


Figure 37 Graphical representation of overall loss of CSE expression in cancers when compared with PM controls. PM (n=16), CAH (n=4), low grade carcinomas (n=35) and high grade carcinomas (n=26).

The modified quick score for CSE staining was high in the post-menopausal endometrium when compared with complex atypical hyperplasias and cancers.

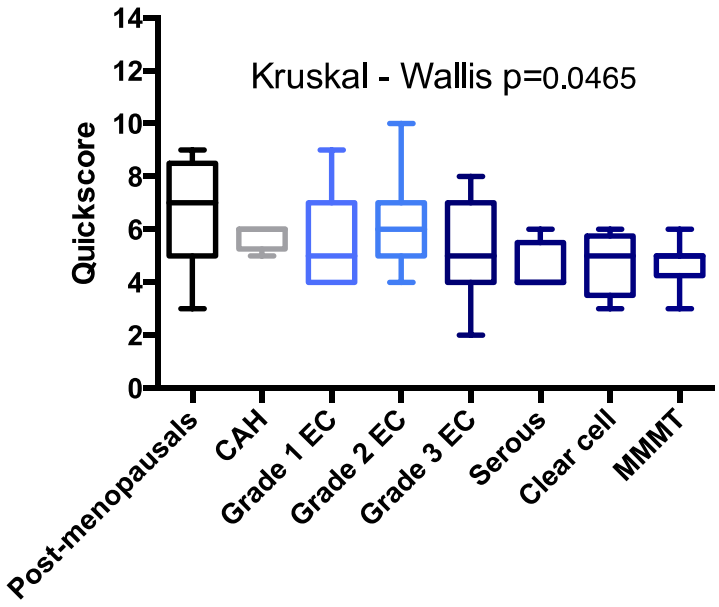


Figure 38 Graph displaying significant loss of CSE staining in endometrial cancers. PM (n=16), CAH (n=4), grade 1 (n=24), grade 2 (n=11), grade 3 (n=8), MMMT (n=9), Serous (n=5), Clear cell (n=4)

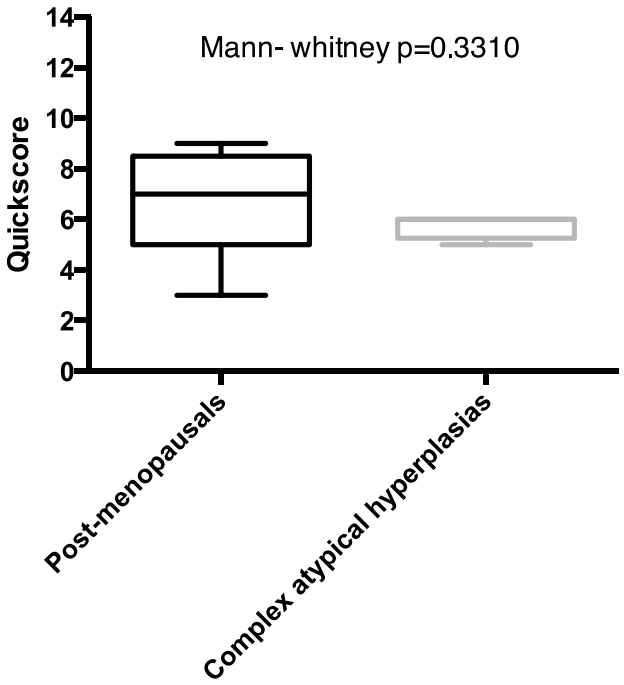


Figure 39 Change in CSE expression between PM (n=16) and CAH (n=4)

No significant reduction in CSE staining was present in CAH when compared with PM endometrium. The individual quick scores for CSE staining in CAH are similar and show consistency.

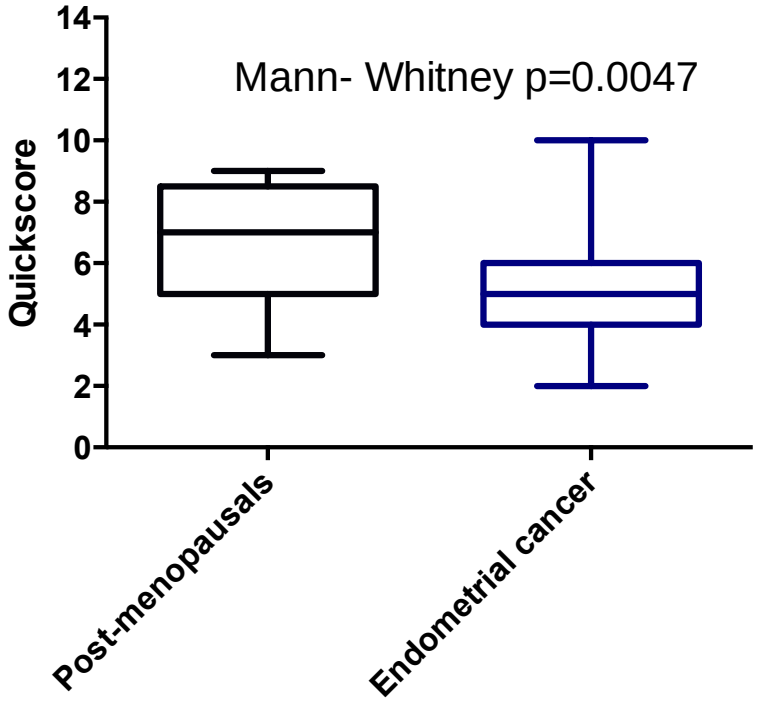


Figure 40 Graphical representation of loss of CSE expression in endometrial cancers when compared with PM controls.

An overall significant reduction in CSE staining was seen in all endometrial cancers when compared to post-menopausal controls.

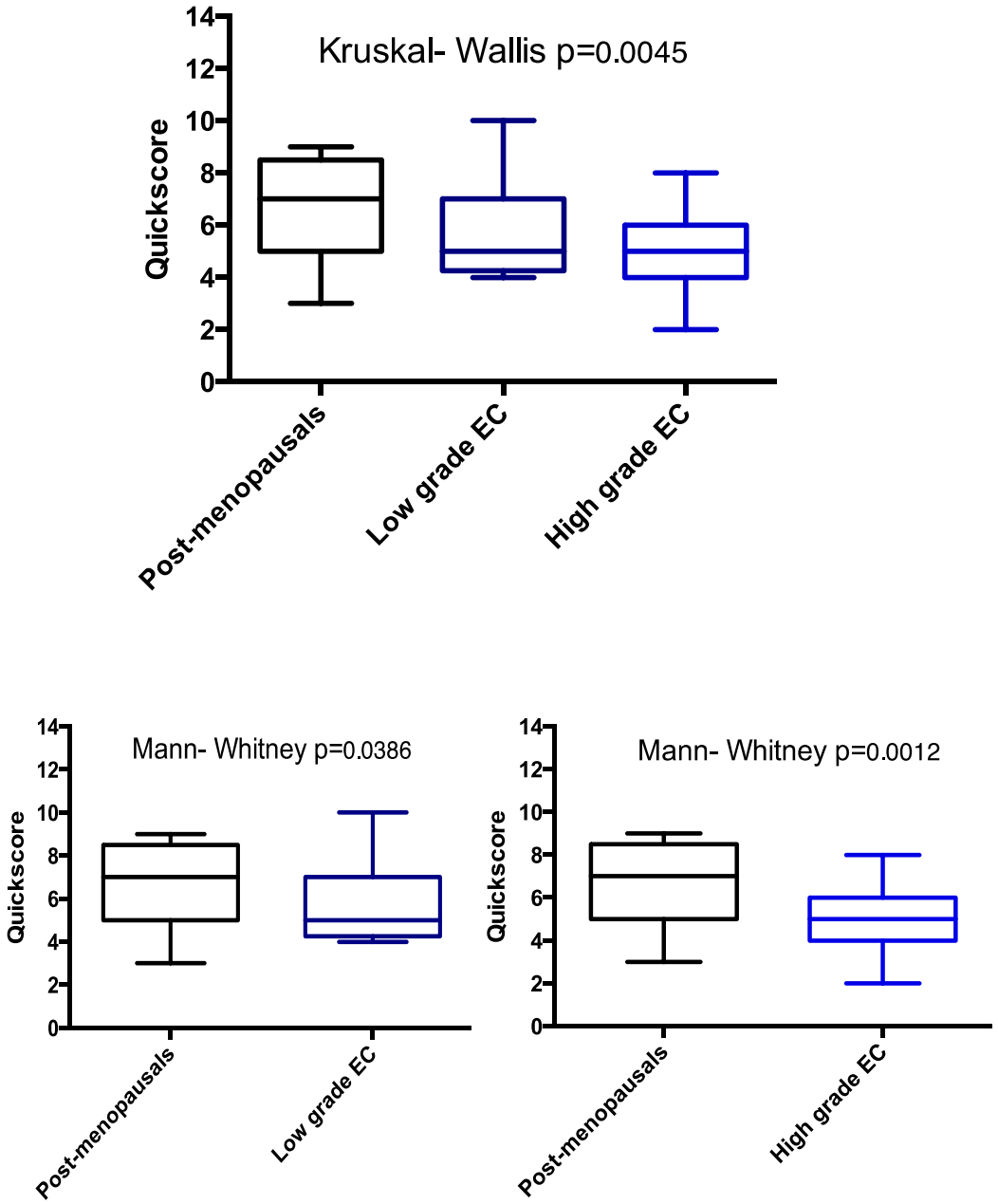
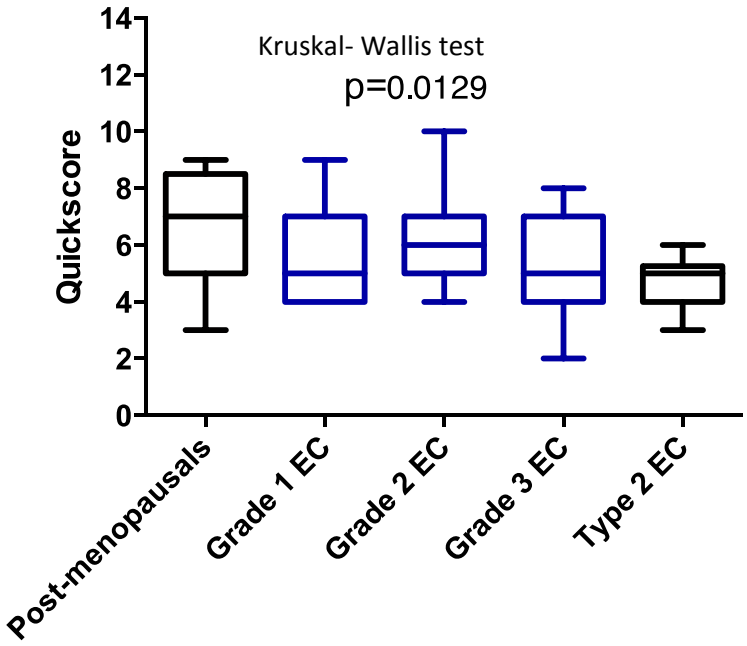


Figure 41 Graphical representation of a more significant loss of CSE expression in high grade carcinomas when compared with low grade carcinomas

Subcategorisation of the endometrial carcinomas into high and low grades has shown a greater reduction in CSE expression in high grade endometrial carcinomas when compared to low grade carcinomas.



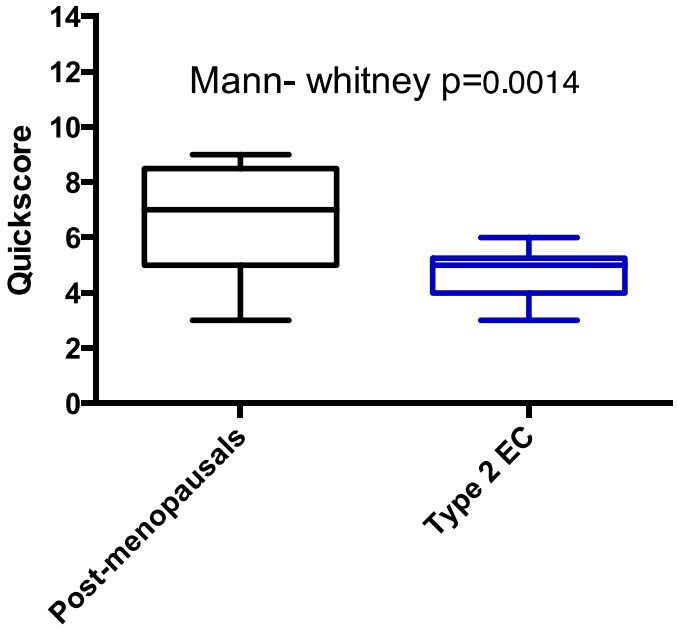


Figure 42 Graph displaying a more pronounced loss of CSE in type 2 carcinomas when compared to type 1 endometrioid carcinomas.

Categorisation of endometrial cancers into type 1 endometrioid and type 2 non-endometrioid carcinomas has revealed a greater loss of CSE expression in non-endometrioid cancers.

No correlation was found between CSE and steroid receptor expression.

5.3.5.1 CSE correlations with steroid Receptors:

High grade

Correlations

			3-MPST	CBS	CSE	AR	ER-beta	PR	ER-alpha
Spearman's rho	3-MPST	CC	1.000	.009	.370	.019	.230	.263	.027
		P	.		.003	.882	.070	.034	.829
		No	65	62	62	65	63	65	65
	CBS	CC	.009	1.000	.056	-.120	-.126	-.030	-.146
		P	.944	.	.669	.353	.335	.814	.258
		No	62	62	61	62	61	62	62
	CSE	CC	.370	.056	1.000	-.109	-.013	.177	-.070
		P	.003	.669	.	.401	.920	.168	.589
		No	62	61	62	62	61	62	62

Low grade

			3-MPST	CBS	CSE	AR	ER- beta	PR	ER- alpha
Spearman's rho	3-MPST	CC	1.000	.197	.167	.100	.139	-.031	.150
		P	.	.170	.245	.474	.326	.823	.284
		No	53	50	50	53	52	53	53
	CBS	CC	.197	1.000	.096	-.157	-.053	.084	-.235
		P	.170	.	.511	.276	.714	.562	.100
		No	50	50	49	50	50	50	50
	CSE	CC	.167	.096	1.000	-.124	-.122	-.032	.001
		P	.245	.511	.	.390	.399	.826	.995
		No	50	49	50	50	50	50	50

PM

			3-MPST	CBS	CSE	AR	ER-beta	PR	ER-alpha
Spearman's rho	3-MPST	CC	1.000	.056	.277	-.409	-.224	.606	-.320
		P	.	.864	.384	.187	.507	.037	.311
		No	12	12	12	12	11	12	12
	CBS	CC	.056	1.000	.432	-.136	-.415	-.133	.011
		P	.864	.	.161	.673	.204	.680	.973
		No	12	12	12	12	11	12	12
	CSE	CC	.277	.432	1.000	.307	-.150	.138	-.135
		P	.384	.161	.	.331	.659	.669	.675
		No	12	12	12	12	11	12	12

Figure 43 No correlation between CSE and steroid receptors was found. The total numbers of samples are inconsistent due to different numbers of samples stained for steroid receptors.

5.3.5.2 **Association with clinic-pathological features and outcome data**

Comparison of CSE expression with myometrial invasion has revealed no association between the two as illustrated below.

Case Processing Summary

Myometrial invasion		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
CSE	Less than 50%	31	77.5%	9	22.5%	40	100.0%
	more than 50%	17	73.9%	6	26.1%	23	100.0%

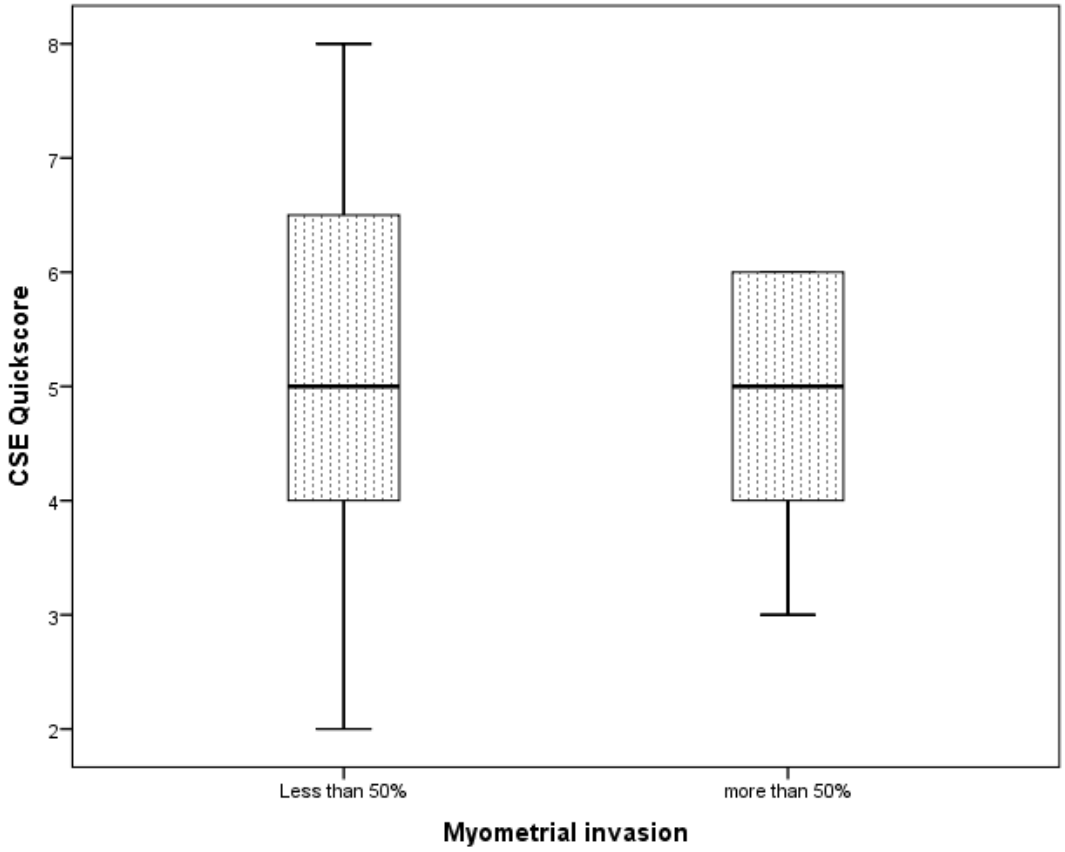


Figure 44 Graphical evidence showing no association exists between the CSE expression and presence of myometrial invasion

5.3.5.3 Association between lymph-vascular invasion and CSE expression in endometrial carcinoma

Case Processing Summary

Lymph-vascular invasion		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
CSE	True	16	69.6%	7	30.4%	23	100.0%
	False	33	80.5%	8	19.5%	41	100.0%

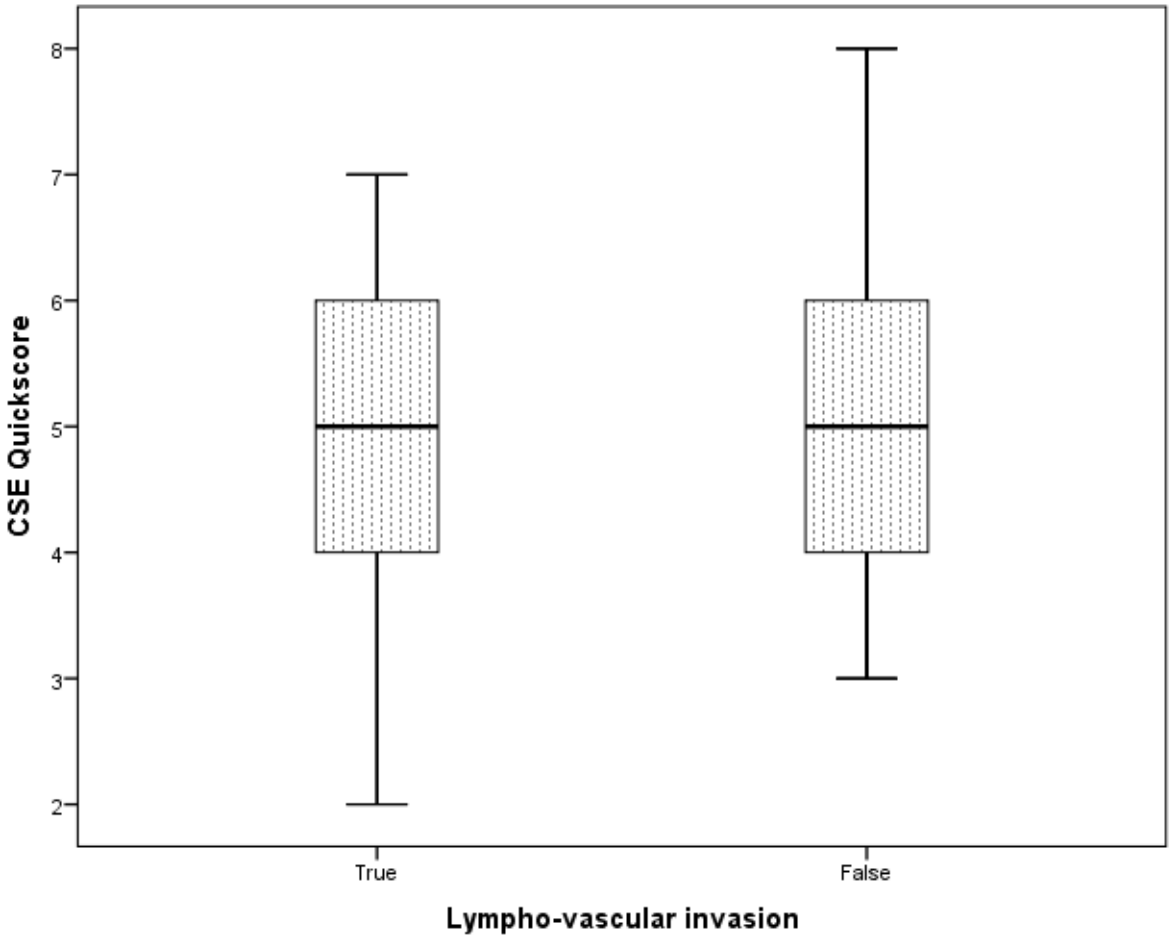


Figure 45 Graphical evidence showing no association exists between the CSE expression and presence of lympho-vascular invasion

5.3.5.4 Association between outcome data and CSE expression in endometrial carcinoma

The outcome data was categorised as '1'=event or '0'=no event. An event is either recurrence of endometrial cancer or death secondary to endometrial cancer. The IHC staining results were compared with outcome data to elicit any significant change in expression of CSE in people who have had a poor outcome. A range of CSE quick score values have been included when looking at outcome data , thereby, reducing the possibility of skewed results secondary to purely using high or low quick scores.

Case Processing Summary

Outcome		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
CSE	0	5	71.4%	2	28.6%	7	100.0%
	1	8	66.7%	4	33.3%	12	100.0%

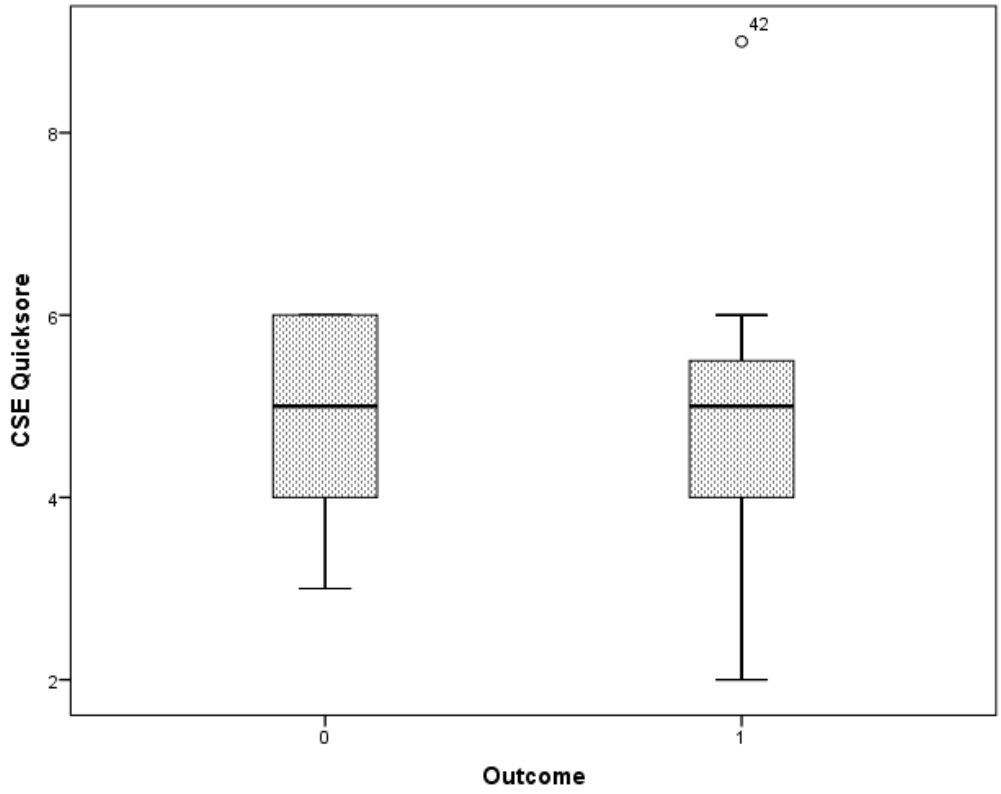


Figure 46 Graphical representation highlighting the lack of association between CSE expression and outcome of patients at 36 month follow-up

5.3.6 CBS expression in post-menopausal endometrium, hyperplasias and endometrial cancer

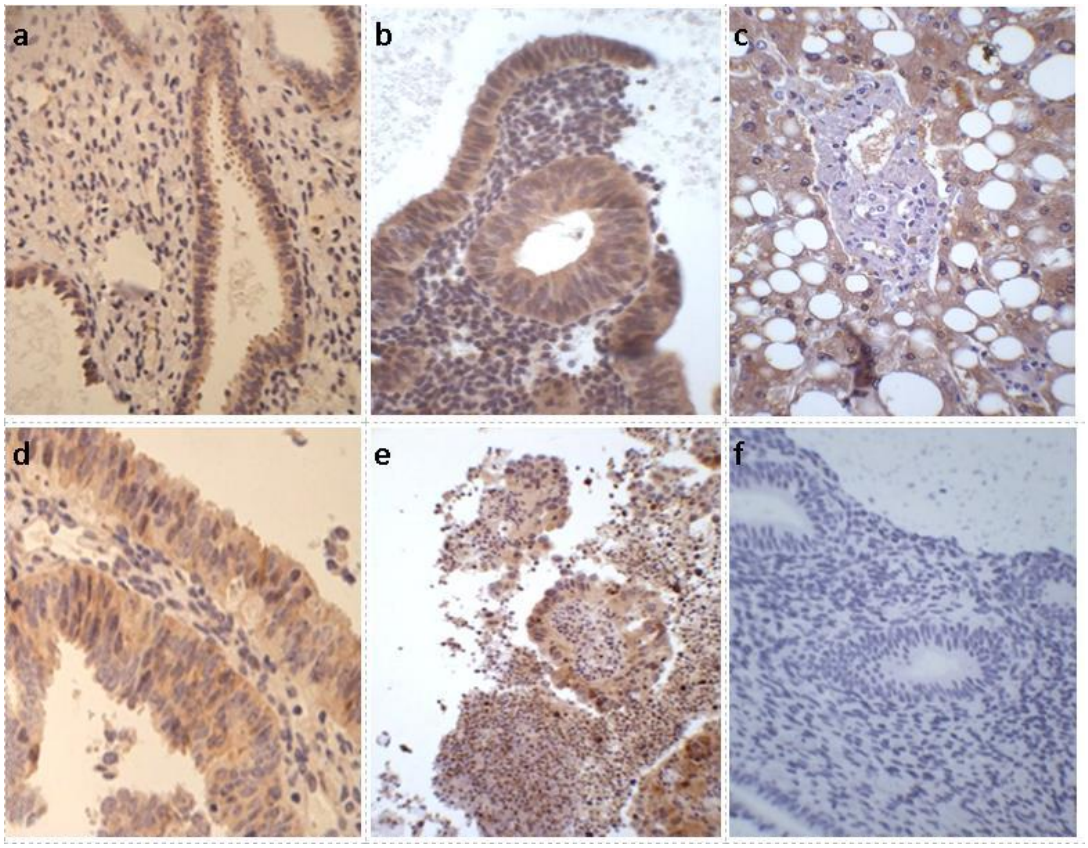


Figure 47 (x400) {x40 objective and x10 eyepiece} micrograph illustrating CBS staining at 1:400 dilution. a) PM (n=16), b) CAH (n=4), c) External control (Human colon), d) Type 1 (n=43), e) Type 2 (n=18), f) IgG control.

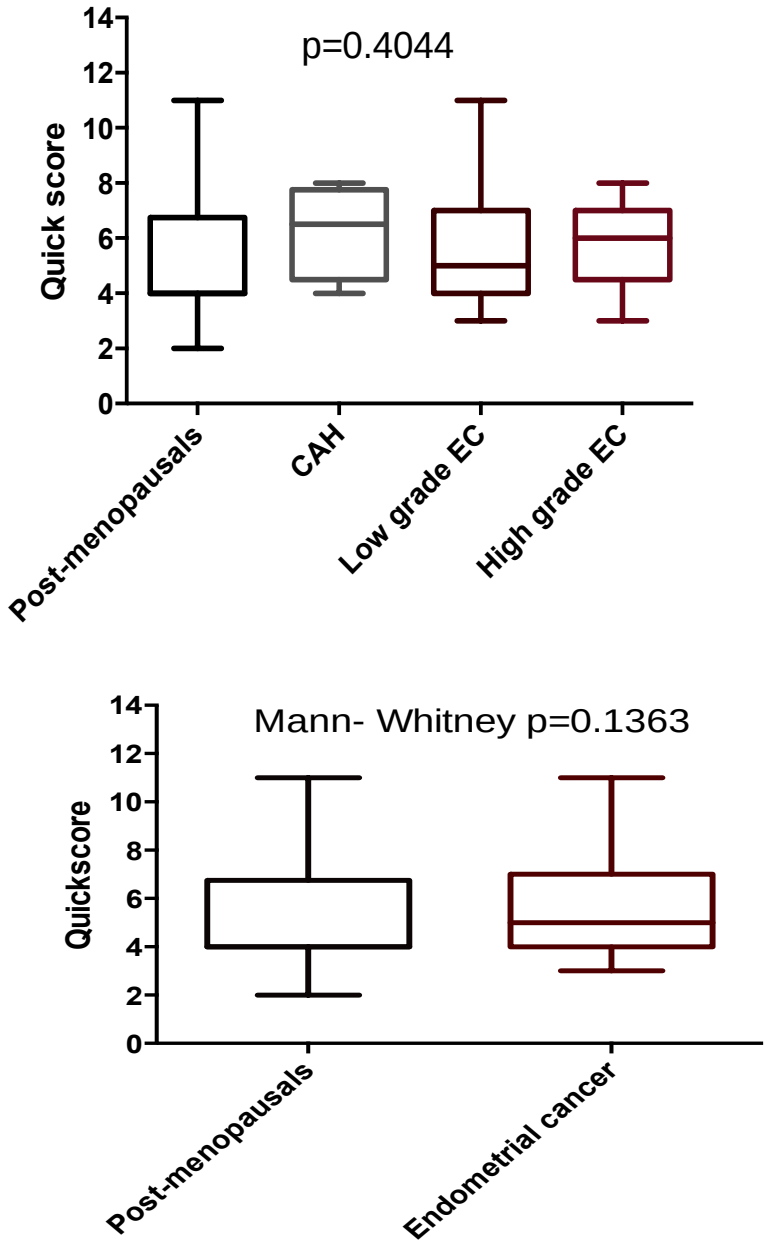


Figure 48 No significant change in CBS staining is seen between PM controls and endometrial cancers. PM (n=16), CAH (n=4), low grade carcinomas (n=35) and high grade carcinomas (n=26).

No significant change in IHC staining scores for CBS expression can be seen between all groups shown above. PM tissue stains revealed a varied expression of CBS in the glandular epithelium. The standard error margins for PM and low grade endometrial cancer groups are large showing an increased range of quick scores due to varied staining in these groups.

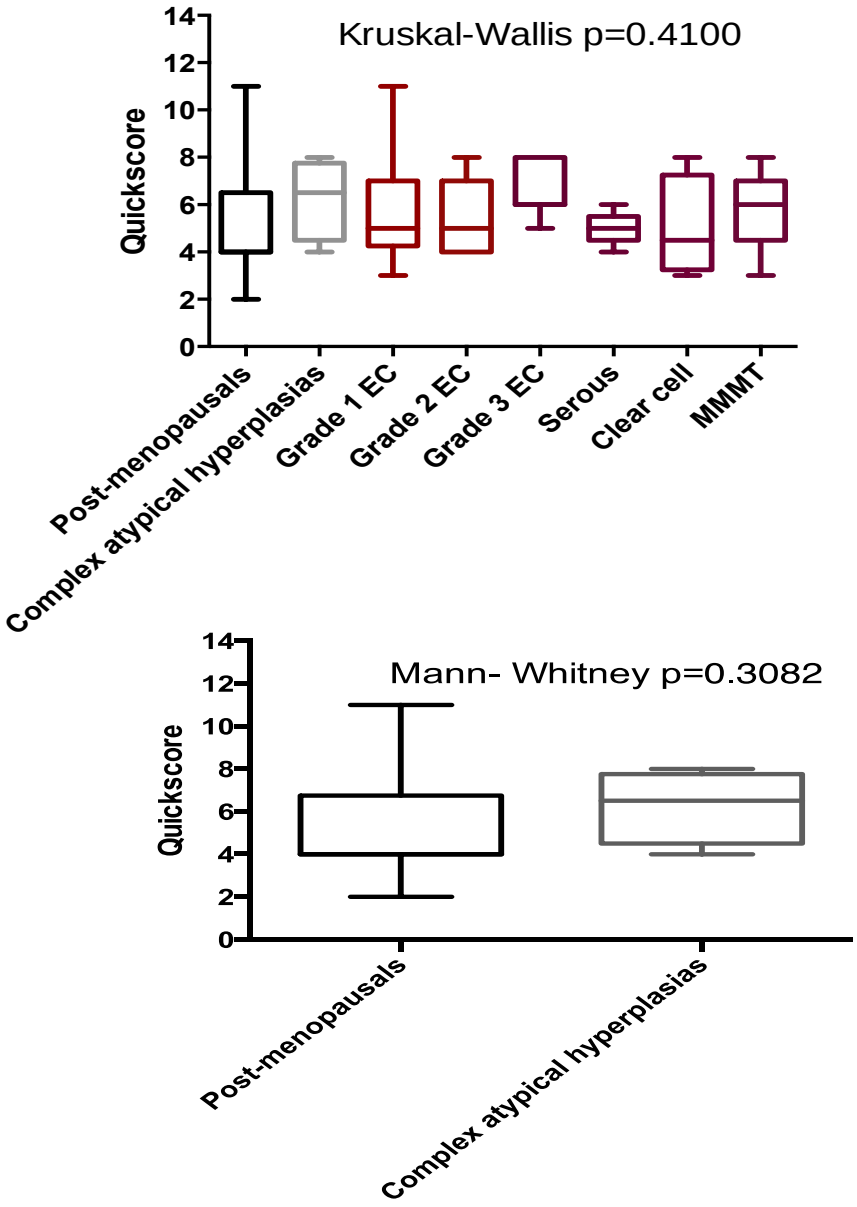


Figure 49 No change in CBS expression can be seen in CAH. PM (n=16), CAH (n=4), grade 1 (n=24), grade 2 (n=11), grade 3 (n=8), MMMT (n=9), Serous (n=5), Clear cell (n=4)

No significant change in CBS staining can be seen in the complex atypical hyperplasias.

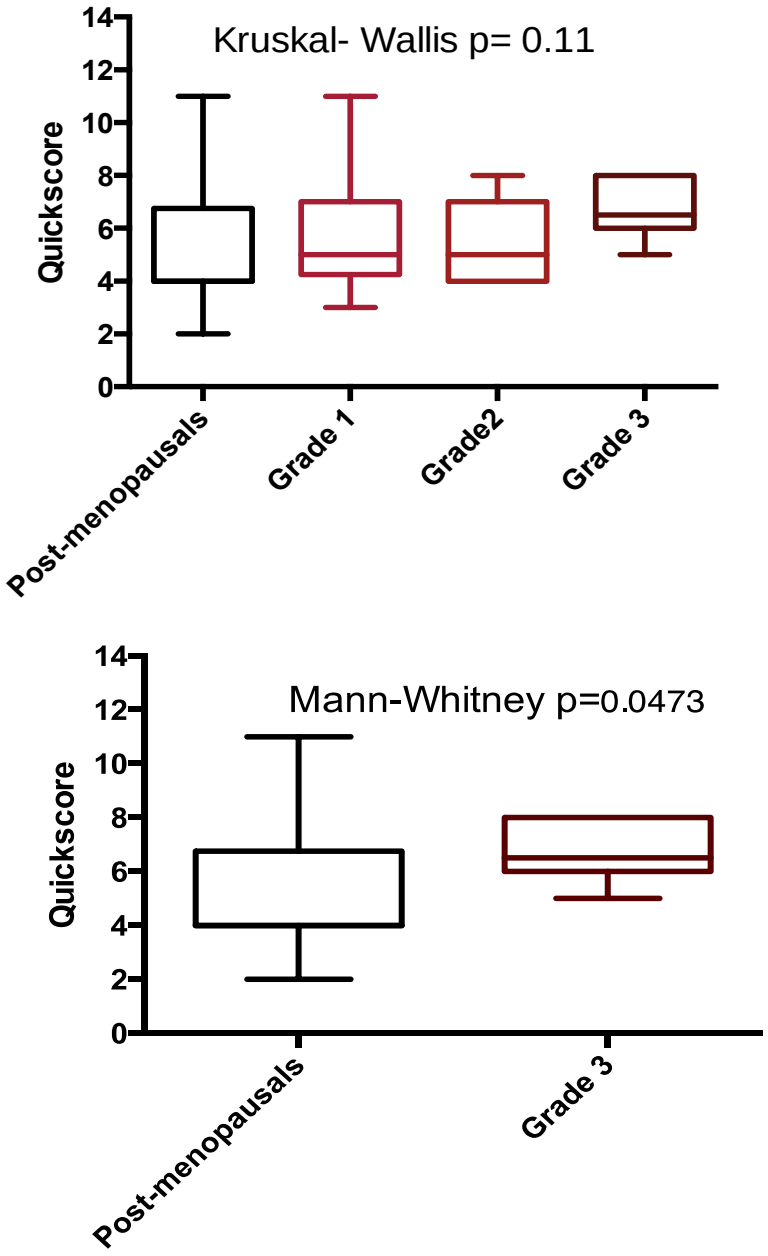


Figure 50 Significant up regulation of CBS can be seen in Grade 3 endometrioid cancers when compared to PM controls. PM (n=16), grade 1 (n=24), grade 2 (n=11), grade 3 (n=8)

A significant increase in IHC quick scores for CBS staining can be seen in grade 3 endometrioid carcinomas when compared with the PM group. Nonetheless, this slight increase in CBS expression in grade 3 carcinomas could be attributed to a compensatory mechanism within the tissue secondary to the down regulation in CSE and 3-MPST expression.

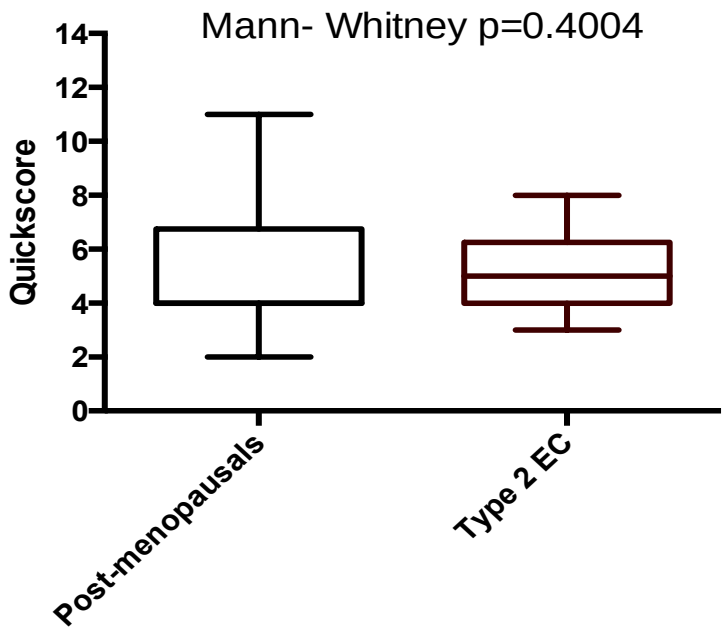


Figure 51 No change in CBS expression can be seen in type 2 non- endometrioid carcinomas PM (n=16) and Type 2 (n=18)

Comparison of CBS staining between the PM group and Type 2 non–endometrioid endometrial carcinomas showed no change.

5.3.6.1 **Correlation of CBS with steroid receptors**

Analysis of high grade (grade 3, MMMT, clear cell and serous carcinomas) cancers displayed a strong negative correlation ($p<0.007$, $r = -0.5$) between CBS and ER-alpha expression. Comparison of the IHC Staining between CBS and ER-alpha reveals weak cytoplasmic staining of ER-alpha in high grade cancer when compared to the strong cytoplasmic staining seen for CBS in high grade cancers.

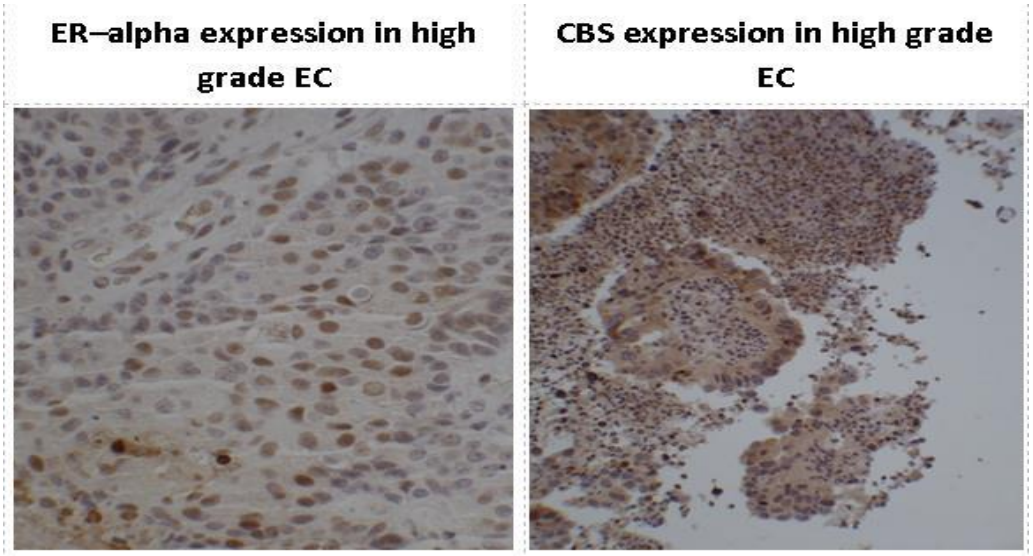


Figure 52 (x400) {x40 objective and x10 eyepiece} micrographs showing weak cytoplasmic ER-alpha staining and strong cytoplasmic CBS expression in high grade cancers

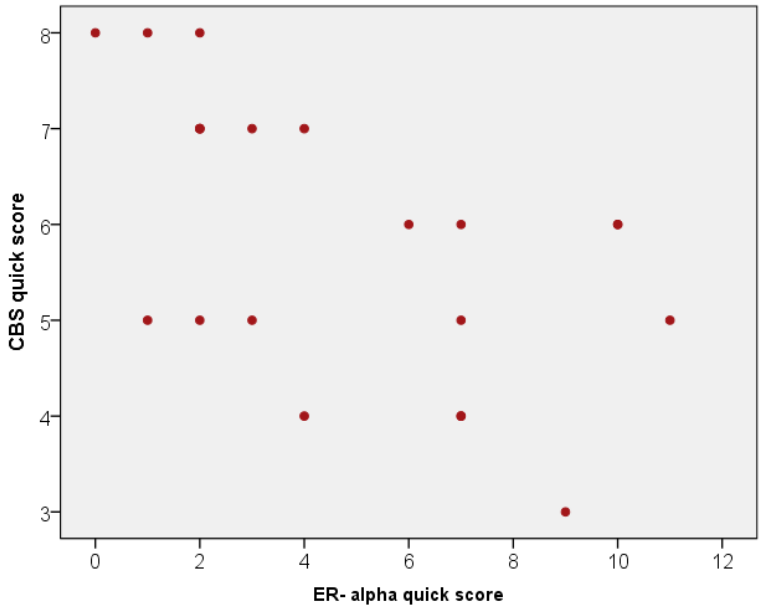


Figure 53 Graphical representation of the positive correlation between ER-alpha and CBS expression in high grade carcinomas

ER-alpha regulation in endometrioid carcinomas is proposed to be lost in grade 3 endometrioid carcinomas. Spearman's rank analysis of CBS expression when compared to ER-alpha has shown a strong negative correlation (r=-0.5). This would suggest an increase in CBS expression should be seen with a reduction in ER-alpha expression. The up regulation of CBS in grade 3 endometrioid carcinomas was shown with the IHC staining above. Although the staining could be attributed to a compensatory mechanism as previously described it could possibly be an independent increase in CBS expression. Further investigations using a larger sample number would need to be conducted in order to understand the exact mechanism behind the CBS up regulation in grade 3 endometrioid carcinomas.

Table 22 Correlation between CBS and ER-alpha in high grade carcinomas

Correlations

			3-MPST	CBS	CSE	AR	ER-beta	PR	ER-alpha
Spearman's rho	3-MPST	CC	1.000	.204	.092	.081	.117	.021	.264
		P	.	.374	.693	.708	.594	.924	.212
		No	24	21	21	24	23	24	24
	CBS	CC	.204	1.000	-.175	.007	-.060	.154	-.567
		P	.374	.	.461	.977	.797	.505	.007
		No	21	21	20	21	21	21	21
	CSE	CC	.092	-.175	1.000	.163	-.084	-.088	.198
		P	.693	.461	.	.479	.717	.705	.391
		No	21	20	21	21	21	21	21

5.3.6.2 Association between myometrial invasion and 3-MPST expression in endometrial cancer

Comparison of CBS expression with myometrial invasion has revealed no association between the two as illustrated below.

Case Processing Summary

	Myometrial invasion	Cases							
		Valid		Missing		Total			
		N	Percent	N	Percent	N	Percent		
CBS	Less than 50%	30	75.0%	10	25.0%	40	100.0%		
	more than 50%	18	78.3%	5	21.7%	23	100.0%		

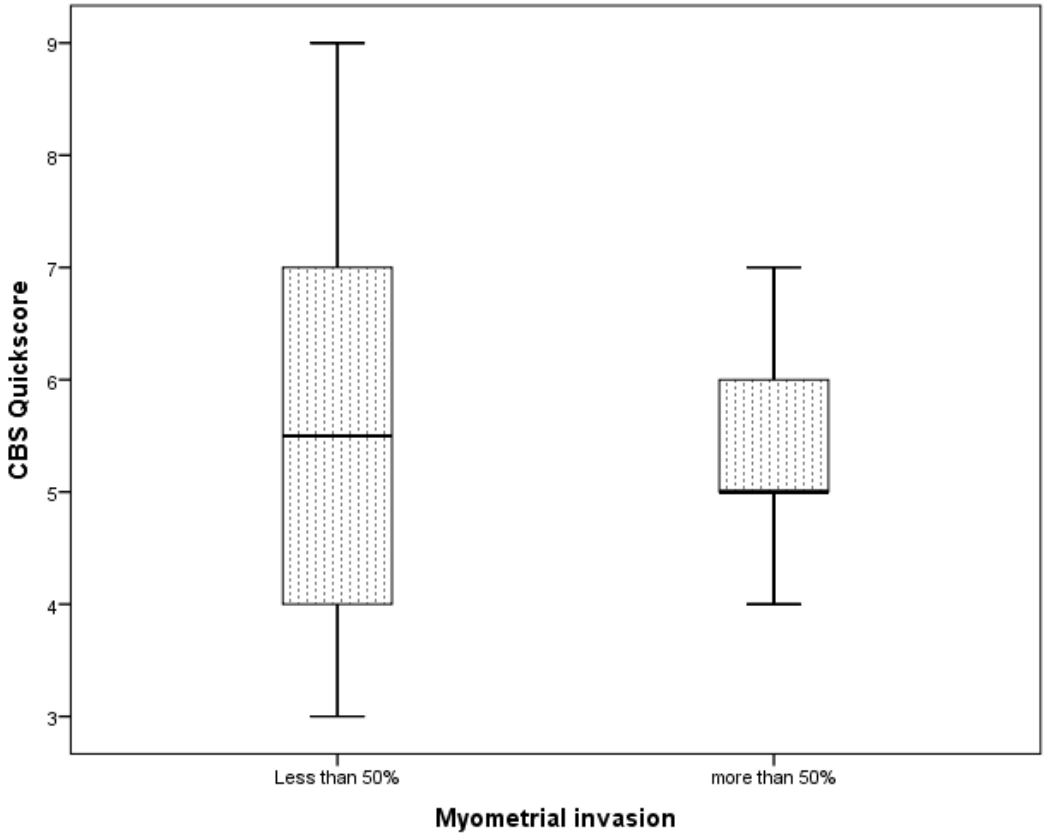


Figure 54 Graphical evidence showing no association exists between the CBS expression and presence of myometrial invasion

5.3.6.3 Association between lympho-vascular invasion and CBS expression in endometrial carcinoma

Case Processing Summary

Lympho-vascular invasion		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
CBS	True	17	73.9%	6	26.1%	23	100.0%
	False	32	78.0%	9	22.0%	41	100.0%

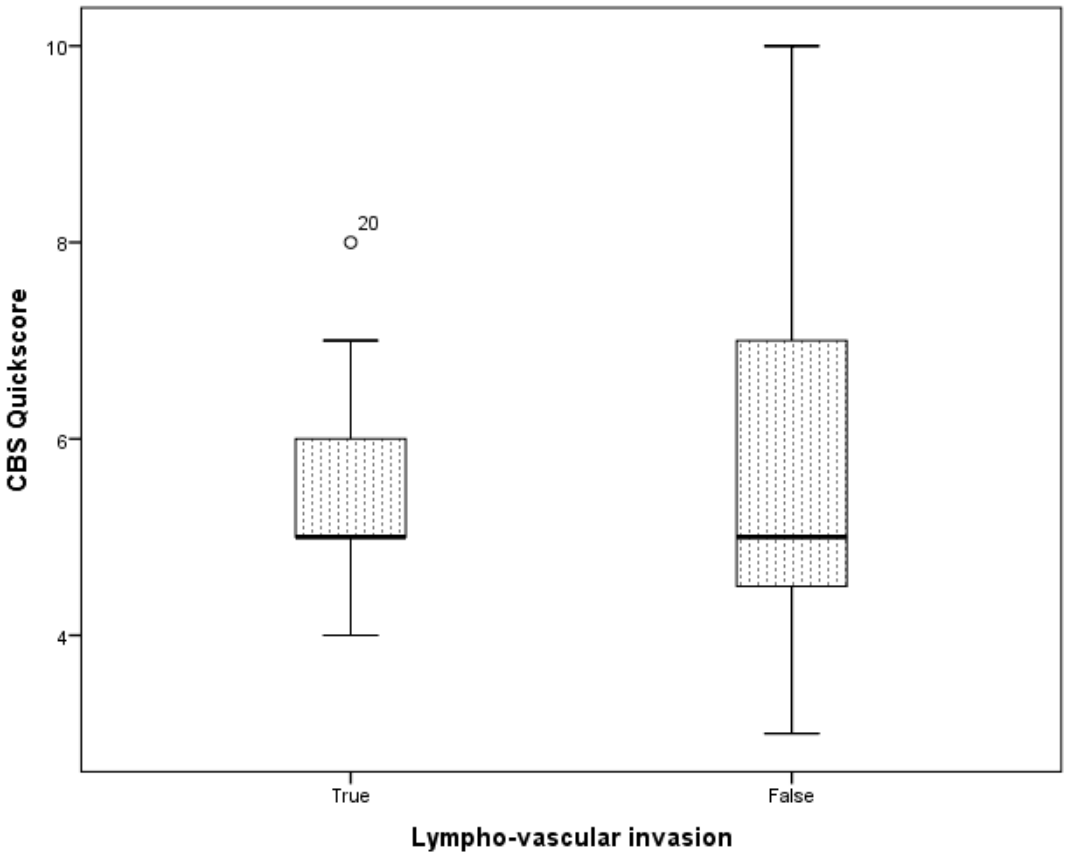


Figure 55 Graphical evidence showing no association exists between the CBS expression and presence of lympho-vascular invasion

5.3.6.4 Association between outcome data and 3-MPST expression in endometrial cancer

The outcome data was categorised as '1'=event or '0'=no event. An event is either recurrence of endometrial cancer or death secondary to endometrial cancer. The IHC staining results were compared with outcome data to elicit any significant change in expression of CBS in people who have had a poor outcome. A range of CBS quick score values have been included when looking at outcome data, thereby reducing the possibility of skewed results secondary to purely using high or low quick scores.

Case Processing Summary

Outcome		Cases									
		Valid				Missing				Total	
		N	Percent			N	Percent			N	Percent
CBS	0	5	71.4%	2	28.6%	7					100.0%
	1	9	75.0%	3	25.0%	12					100.0%

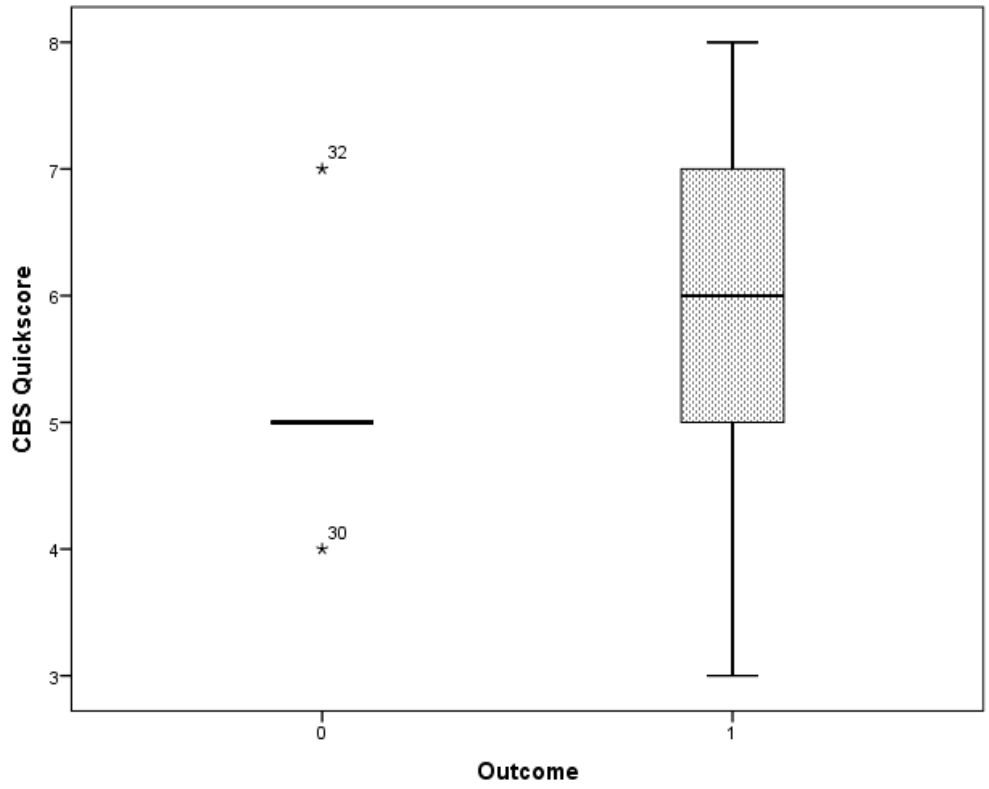


Figure 56 Graphical representation highlighting the lack of association between CBS expression and outcome of patients at 36 month follow-up

5.4 Discussion

This study reports the expression of the three H_2S synthesising enzymes; CBS, CSE and 3-MPST in atrophic post-menopausal endometrium, hyperplastic and endometrial cancer tissue. 3-MPST staining was identified in the cytoplasmic portion of endometrial glands and stromal compartments of PM, hyperplastic and endometrial cancer samples. CSE was distributed between the cytoplasmic portion of glandular epithelium and endothelial cells and CBS expression was limited to the cytoplasm of glandular epithelium. Immunostaining scores for CSE ($p=0.0047$) and 3-MPST ($p<0.001$) were significantly lower in cancerous tissues when compared with post-menopausal controls. No overall differentiation in CBS ($p=0.1363$) expression was present between all groups.

The primary aim of the study was to explore the change in expression of the 3 H_2S synthesising enzymes in endometrial cancer and hyperplastic tissue compared with healthy post-menopausal endometrium. 3-MPST expression was considerably higher in the PM endometrium when compared with endometrial cancer tissue. Although no significant loss of 3-MPST was noticed in CAH group an early drastic loss of 3-MPST in low grade endometrioid cancers suggests as possible cytoprotective role for 3-MPST in the endometrium. The cytoprotective function of 3-MPST derived H_2S was reported in a mammalian retinal degeneration by modulation of calcium ion influx[151], rodent neurology amongst others[152]. In light of this evidence it could be suggested that 3-MPST may have a similar cytoprotective role and its loss coupled with activation of other cancer pathways may result in the development of cancer. Furthermore, a reduction in expression of 3-MPST was proposed in conditions under increased oxidative stress[119]. The production of free radical and reactive oxygen species (ROS) as a by-product of normal cellular metabolism is implicated in a range of diseases. The endometrium has a high cell turnover rate and is known to use circulating lipids for the synthesis of structural lipids[120]. Additional resources are required by cancerous and hyperplastic endometrial tissue for the generation of endogenous fatty acids. This use of lipids in this pathway promotes local oxidative stress in these tissues. The antioxidant disease system within the body including superoxide dismustase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione transferase (GST) and catalase (CAT) are involved in counteracting the effects of these free

radicals[120]. Alterations in the antioxidant system and increased oxidative stress have been implicated in the pathophysiology of many disease states of the endometrium including hyperplasia and adenocarcinomas[120]. Lipid peroxidation levels and antioxidant enzymes activities in women with cancer have been explored to show an enhanced lipid peroxidation and altered uterine antioxidant activity when compared with benign diseases of the endometrium. The increased lipid hydroperoxides results in a rise in liberation of free radicals[120]. SOD was shown to regulate free radical levels by targeting tyrosine or histidine residues. SOD activity was evidenced to be suppressed in both hyperplastic and cancerous endometrium with a more pronounced reduction in adenocarcinomas[148]. Although the results concluded do not define whether changes in antioxidant activities are secondary to or a causal factor of increased oxidative stress in these disease states, a primary comparison between the lipid peroxidation and antioxidant activity was described. In addition studies looking at the effect of increased oxidative stress on cellular bioenergetics secondary to H_2S synthesised by the 3-MPST pathway have shown the inhibition of 3-MPST activity, thereby altering the positive bioenergetics role of the 3-MPST/ H_2S pathway[119].

This study reports a reduction in expression of 3-MPST in endometrial cancer; a state of increased oxidative stress. Considering previous evidence suggesting the down regulation o of 3-MPST mediated production of hydrogen sulphide is therefore justified. However, the post-menopausal samples used in this study are atrophic therefore cellular proliferation and hence strong expression of 3-MPST is not expected secondary to its role in stimulating cellular bioenergetics. Though the levels of 3-MPST are higher in post-menopausal endometrium further investigations looking at the cycling endometrium need to be considered. Once the expression of 3-MPST is defined in the proliferating endometrium it can them be compared against PM, hyperplastic and cancerous endometrium. Although the IHC staining of tissue has expressed the presence of 3-MPST the functionality of this protein has not been described. Therefore further tests would need to be conducted to understand whether the proteins have a functional role. The early and increased loss of 3-MPST seen in low grade endometrioid cancers suggest a role for the 'loss of 3-MPST' as a marker of disease. A larger sample size would need to be recruited to increase the statistical strength of these suggestions. The correlation of 3-MPST with steroid receptors revealed a weak

positive correlation between PR and 3-MPST (r=0.2). PR plays a role in cell differentiation and anatagonises the effects of ER and therefore has an anti-proliferative effect on the endometrium. It is proposed to be lost in advanced stage endometrioid carcinoma and therefore a subsequent reduction in 3-MPST is to be expected.

Angiogenesis is a pre-requisite for development and maintenance of tumour cells[4]. This facilitates the delivery of nutrients, oxygen and removal of by-products of cellular metabolism. Several previous studies have reported the role of CSE/ H_2S pathway in the regulation of vasculature within mammalian tissue including ischaemic cardiovascular disease or regulation of blood pressure[136]. In contrast, the role of CSE mediated production of H_2S and its regulation of tumour angiogenesis has not been explored. Multiple angiogenic factors are secreted by tumours, thereby promoting angiogenesis[138, 153-155]. In addition CSE mediated production of H_2S has also been shown to contribute towards the secretion of angiogenic factors such as VEGF resulting in endothelial cell migration[155]. No change in CSE expression was reported in the study conducted looking at CSE expression in colon cancer cells and they did not explore the role of CSE mediated production of H_2S in endothelial cells[4]. The suppression of CSE in colon tumour tissue using PAG on a colon cancer cell line (HCT-116) showed no changed in tumour proliferation, suggesting that CSE did not play a primary role in the supporting tumour growth and proliferation[4].

In this study the expression of CSE was only described in the glandular sections of the tumour tissue and PM samples. Micrographs (x400) {x40 objective and x10 eyepiece} revealed increased CSE expression in the endothelial cells of endometrial cancer tissue and no CSE staining in the PM tissue. The PM endometrium samples used in this study were all atrophic and therefore minimal activity is expected with respect to angiogenesis. The lack of CSE staining in the endothelial compartment of PM samples and the presence of CSE staining in endometrial cancer cells supports this theory. IHC staining and modified quick score values showed an overall reduction in expression of CSE in the glands through all histological grades in type 1 and type 2 cancers. Though the glandular expression of CSE is down regulated its function may be attributed to vascular changes and therefore further studies and scoring of the endothelial staining of CSE need to be done. Correlation of CSE

expression in endometrial cancer tissues with previously stained data for all steroid receptor expression has found no correlation between the CSE and the various receptors.

Studies looking at the role of CBS in health have proposed its importance in metabolic processes within the body[156-158]. In the absence of CBS serious clinical manifestations may develop including lens dislocation, mental retardation, atherothrombotic vascular disease and osteoporosis. The presence of CBS has also been proposed for the synthesis of a major anti-oxidant known as glutathione (GSH), thereby suggesting an important role for CBS in facilitating anti-oxidant pathways. In contrast, CBS up regulation has also been proposed in the development of Down's syndrome[156] and carcinogenesis[4, 146]. Several previous studies have demonstrated the role of H_2S donors in ATP generation and in different cell lines *in vitro*[159]. An increase in the catalytic function of the glycolytic enzyme GAPDH was proposed secondary to the presence of H_2S. A study conducted in patient matched normal mucosa and colon cancer biopsies and colorectal cancer cell lines revealed a selective up regulation of CBS in these cancers with no change seen in CSE and 3-MPST[4]. The tumorigenic potential of H_2S has also been investigated in primary serous ovarian carcinomas[146]. Patient tissue microarray was utilised to conduct *in vitro* and *in vivo* studies investigating the expression of CBS. *In vitro* effects of CBS silencing were seen to reversible with the exogenous application of H_2S donors. In cisplatin resistant orthoptic models, siRNA silencing of CBS was seen to inhibit tumour growth and sensitise ovarian response to chemotherapeutic agents e.g. Cisplatin[146].

The IHC staining of CBS showed no significant change in expression between all groups. The presence of CBS in post-menopausal endometrium may be related to the synthesis of anti-oxidant GSH. However, PM endometrium is atrophic and therefore a low glycolytic activity would suggest low rates of mitochondrial respiration and release of free radicals, thereby requiring low levels of anti-oxidants secondary to reduced oxidative stress in the PM endometrium. Although CBS was proposed to be expressed in the PM endometrium the functionality of this protein is still unknown. Further investigations would need to be conducted to highlight the function of this protein in the PM endometrium. Sub-categorisation of the cancer cohort into histological grades revealed a significant ($p=0.04$) rise in CBS expression between the post-menopausal cohort and grade 3 endometrioid

endometrial cancer group (n=8). It could be possible that this up regulation of CBS could be a compensatory mechanism due to the significant suppression of CSE and 3-MPST in endometrial cancers. Spearman's rank correlation of CBS protein expression and previous data for steroid receptor IHC staining revealed a strong negative correlation between ER-alpha and CBS (r=-0.5). Although type 1 endometrioid endometrial cancers are oestrogen regulated this regulation was proposed to be lost in grade 3 endometrioid carcinomas. Therefore, the loss of ER-alpha is expected and a subsequent rise in CBS should be seen, which is shown by IHC staining and correlation studies. Therefore, if the rise in CBS is still present once an increased sample number was stained and correlated the presence of CBS in endometrial cancers could possibly be a potential be a marker for poor disease prognosis.

The clinical pathological features of the endometrial cancers such as extent of myometrial invasion and lympho-vascular invasion have been compared against the expression of the three H_2S synthesising proteins to reveal a possible association between the two groups. Myometrial invasion was categorised according to the 1991 FIGO classification into <50% or >50% invasion into the myometrial wall. Lympho-vascular invasion was included if present and rejected if it does not exist. No association was found between the 3 proteins and the clinical pathological features. Further to this outcome data was used where '1' represents an event such as recurrence of endometrial carcinoma or death due to endometrial cancer at 36 month follow-up. Comparison of all 3 proteins with outcome data has shown no association between the expression of the 3 proteins in the endometrium and a poor outcome.

5.5 Limitations

The use of IHC staining has shown the presence of all 3 proteins in the endometrium. However, this study has not been able to show whether the proteins have a functional role in the endometrium. In addition the expression of the 3 proteins has not been described in the normal healthy cycling endometrium and would need to be investigated further to see the overall change in expression between cycling, PM and cancerous endometrium.

The large standard error margins between experimental groups can be attributed to the unequal numbers in the individual groups. Although this study was conducted using a large sample size to increase the strength of the findings it is necessary to increase the sample number of grade 2+3 endometrioid carcinomas and type 2 endometrial carcinomas.

5.6 Summary

In summary this study has identified the expression of CBS, CSE and 3-MPST using IHC in PM, CAH and endometrial carcinomas. Detailed information regarding the changes in expression of the 3 enzymes across all groups and any correlation with steroid receptors was highlighted. PM endometrium has abundantly expressed all 3 enzymes more than the CAH or endometrial cancers. The early loss of both CSE and 3-MPST in endometrial cancer suggests a role for them as possible marker of disease. No significant difference in expression of CBS was noted across all groups, although CBS was significantly up regulated in grade 3 endometrioid carcinomas. A strong negative correlation seen between CBS and ER-alpha has confirmed the possibility that H_2S may play a role in carcinogenesis in advanced endometrioid carcinomas and the increase in CBS could be a possible indicator of poor prognosis. A higher sample size will be needed to increase the strength of this suggestion. Although endometrial cancer is regulated through a vast variety of pathways, this study focused on H_2S mediated carcinogenesis as no current evidence exists with respects to this pathway in the endometrium.

Chapter 6 *In vitro* examination of effects of H_2S in endometrial cancer cell proliferation

6.1 Introduction

Hydrogen sulphide is 'third' upcoming gaseous transmitter in mammalian physiology[152]. Its potential as a regulator of vascular tone and angiogenesis was increasingly investigated in cardiovascular system[136]. *In vivo* it is naturally synthesised by colonic bacteria and by 3 enzymes including CSE, CBS and 3-MPST from the substrate L-cysteine. Though the precise mechanisms are not defined the role of H_2S was found to be varied in different tissues displaying pro- or anti-apoptotic effects on cells in culture[125, 160, 161].

To date limited research is present investigating H_2S effects on carcinogenesis both *in vivo* and *in vitro*. Previous research has reported the protective role of H_2S on colon cancer cells preventing apoptosis secondary to effects of β-phenylethyl isothiocyanate[162]. Recently evidence has shown H_2S increases colon cancer cell and primary serous ovarian carcinoma proliferation and reduced cellular apoptosis in human colon cancer cell lines i.e. HCT-116[4, 146]. Increased level of H_2S has also resulted in a reduction of survival in other cell lines such as WIDR[163].

In vitro experiments examining the effects of H_2S sulphide on cell lines with the use of H_2S donors may show discrepancies in results due to the type of H_2S donor used. Inorganic sulphide salts such as sodium hydrogen sulphide (Na HS) and sodium sulphide (Na_2S) have been extensively used in the investigations exploring the biological effects of H_2S. The salts have a very short half-life of 5 minutes when combined with water[2]. Their instant reactivity results in a large surge of H_2S production. Cell culture treatment extends over a period of days making this an unlikely source of controlled H_2S release. High levels of H_2S are also toxic to cells and so would invariably result in erroneous results and reduce the strength of the conclusions drawn looking at the effect of H_2S on cancer cell survival[2].

Studies using the synthetic H_2S donor GYY4137 (derived from Lawesson's reagent) have shown the concentration dependent death of seven different human cancer cell lines (HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS) with no effect on survival on normal human lung fibroblasts (IMR90, WI-38)[125]. This study compared the effect of NaHS and GYY4137 on survival of the above cancer cell lines. GYY4137 is known to release H_2S in a slow and controlled manner when in aqueous solution over a period of days.

H_2S measurement was under much scrutiny[130, 164]. Many methods of using spectrophotometry or fluorescence in the measurement of H_2S have been described. No one method was universally agreed as the most accurate measurement of H_2S. The choice of method used is dependent on resources and the medium in which H_2S is being detected. In this study due to the inability to directly measure H_2S, an alternate method was decided upon. The MTT assay is a proliferation assay and was used to detect the change in number of viable cells by reading the absorbance.

In this study GYY4137 was used as the H_2S donor to investigate the effect on endometrial cancer cell lines. In addition the substrate L-cysteine has also been used to treat cells to identify any changes in cell proliferation through H_2S synthesis. Normal endometrial cell lines are unable to thrive and therefore no normal endometrial cell lines are in existence. This posed a problem as we are still unaware of the effect of GYY4137 on normal endometrial cells. This study is the first to describe an effect if present of H_2S donors on endometrial cancer cell lines.

L-cysteine is a substrate for the production of H_2S via the trans-sulphuration pathway and metabolised by all 3 enzymes[165]. The addition of L-cysteine to the MFE280s and observation of its effects on proliferation of these cells will elucidate whether the enzymes CBS, CSE and 3-MPST are functional and producing H_2S. Furthermore, a range of concentrations of l-cysteine have been used to look at the change in proliferation of the MFE280s if present.

6.2 Methods

6.2.1 Demographics

The human endometrial MFE280 cell line (#98050131) was from Public Health England, Salisbury, UK. The cells were derived from a recurrent, poorly differentiated endometrial carcinoma from an elderly patient.

6.2.2 Cell culture

MFE280 cell culture

Stocks of MFE280 in 90% FBS and 10% Dimethyl sulphoxide (DMSO) were stored at -70°C. For recovery, cells were rapidly thawed in a 37°C waterbath and plated in pre-warmed media DMEM (Sigma, UK) containing 10% FBS (BioSera, UK), Primocin™ (50 mg/ml, 1:500 in media, InvivoGen) and L-glutamine (2mM, Sigma). Once established the cells were maintained in monolayer culture in an atmosphere containing 5% CO_2/air at 37°C with media changes every three days. Their confluence was monitored every 24 hours until an appropriate confluence (80-90%) had been reached. Due to the poor differentiation status of MFE280s more than 7 days were needed to allow the cell to form a confluent monolayer.

Treatment of cells

As mentioned in chapter 3 an 80mM GYY4137 stock solution in water was prepared (30mg / 1ml; Santa Cruz Biotechnology, USA) Serial dilutions in media were then produced from the stock solution at concentrations of 25 µM, 50 µM, 75 µM and 100 µM. A 200 mM L-cysteine stock solution in water was prepared (25 mg/ml; Sigma, UK) for experiments, L-cysteine was used at 1 mM and 3 mM diluted in media.

6.2.3 Cell proliferation-MTT assay

MFE-280 cells were seeded into the wells of a 96-well microtiter plate at different densities ranging from $18.5x10^4$ cells per well to $4.5x10^4$ cells per well in triplicate. The cells were allowed to attach for 24 hours after which the media was changed and cells treated with GYY4137 and l-cysteine. The treatment period was 24 and 36 hours for GYY4137 and 24 and 48 hours for L-cysteine. At the end of this period the media was changed and another 100 µl of media added to which 20 µl of 3(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) reagent (5 mg/ml in PBS) was added to each well. After 3.5 hours incubation at 37 °C the media was carefully aspirated and the insoluble formazan crystals that had formed were solubilised in acidified isopropanol (4mM HCl 0.1% Triton X-100 in isopropanol) covered with foil and gently shaken for 15 minutes on a Varishaker (DYNATECH) . The absorbance was measured at 570nm wavelength using the MultiSkan Ascent (funded by Liverpool University, Thermo Electron Corporation, UK). The software used to read absorbance was Ascent software version 2.6 (Thermo lab systems).

Absorbance readings were averaged and corrected for blank (media alone with MTT reagent) and the ratio relative to control calculated with control being 1.

6.3 Results

6.3.1 GYY4137 treatment of MFE280s in normal media

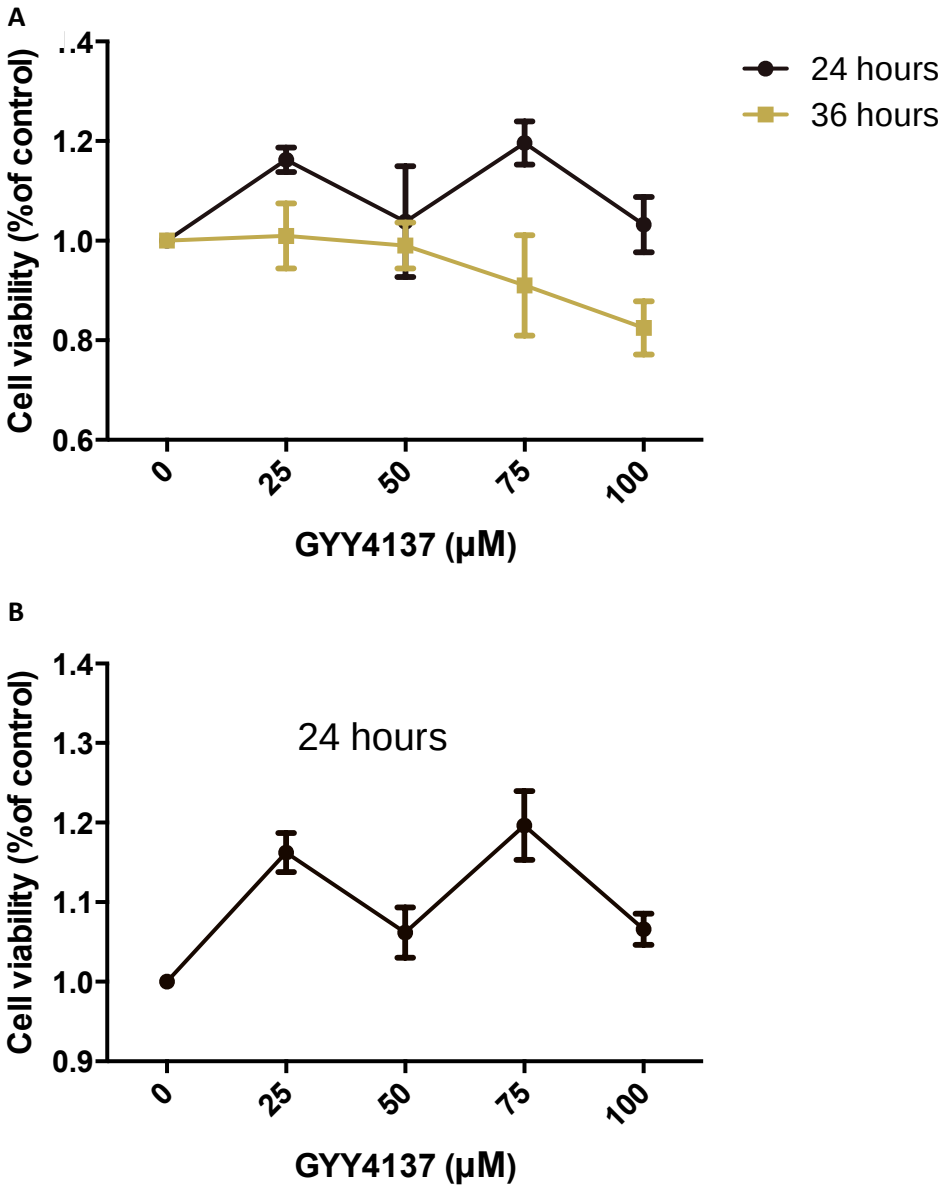
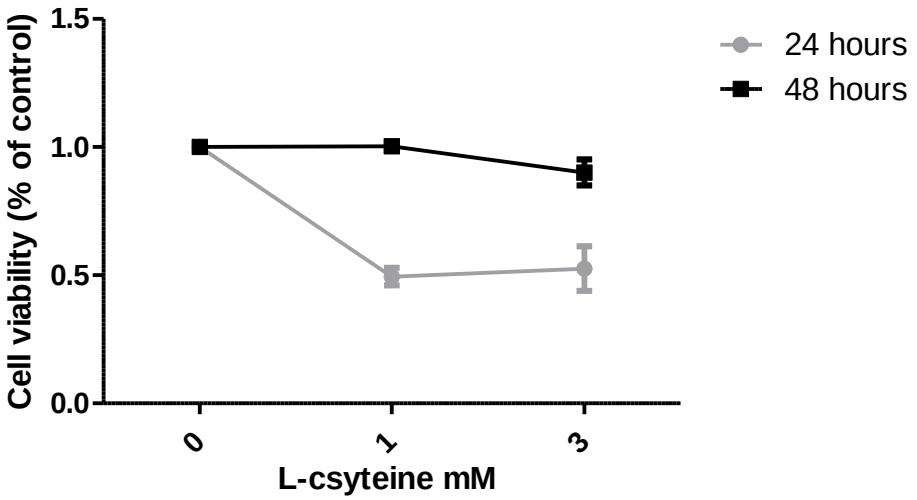


Figure 57 Graphs showing the MFE280 cell viability after treatment with GYY4137: a) 3 replicates plotted for all concentrations of GYY4137, B) 15 replicate values plotted for GYY4137 concentrations of 50 and 100 µM with 3 replicates for 25 and 75 µM.

The endometrial cancer cell line (MFE280) was treated with increasing concentrations of GYY4137 over a 24 and 36 hour period and its effect on cell proliferation assessed by the MTT assay. A positive effect on cell number was seen after 24 hour treatment which was not sustained at 36 hours. In fact treatment at the highest dose for 36 hours had a negative effect on cell number. There appears to be a biphasic response of MFE280 cells to GYY4137. The discrepancies in replicate values are addressed later in the discussion.

6.3.2 L-cysteine treatment of MFE280s



The MFE280s cell line was treated with increasing concentration of the H_2S substrate L-cysteine over a 24 and 48 hours period. The cells were maintained in reduced serum media prior to the conduction of this experiment to ensure that any change increase in proliferation would be secondary to the addition of L-cysteine and subsequent H_2S production. As shown above after 24 hours of treatment at 1mM concentration of l-cysteine inhibited cell proliferation. The 48 hour treatment period shows no effect at 1 mM on endometrial cancer cells with an inhibition of proliferation at higher concentrations.

6.4 Discussion

This study has shown the concentration dependent reduction in proliferation of the MFE280s cell line using increasing concentrations (25, 29, 75, 100 μM) of GYY4137 and L-cysteine (1 and 3 mM). However, at lower concentrations of 25 μM the GYY4137 has positive effect increasing cell viability over 24 hours. This stimulatory effect on the cancer cells could suggest a possible role for H_2S in stimulating cellular proliferation. The implications of this are discussed later in this chapter. Early investigations looking at H_2S and its alternating roles on human cells have produced evidence claiming its protective role on colon cancer cells from apoptosis due to β-phenylethyl isothiocyanate[162]. Since then recent evidence has emerged reporting the role of CBS induced production of H_2S increasing colon cancer cell proliferation and reduction of cellular apoptosis in many cell lines with no detriment to normal cell lines. In contrast, studies have shown the reduction in survival of other colonic cell lines[163]. The presence of conflicting evidence can be attributed to the different mechanisms in which the investigations have been conducted including the type of H_2S donor used, or the experimental timings and concentrations of H_2S donors or enzyme inhibitors used.

This study employs the reduction of tetrazolium to measure cell viability and proliferation. The mitochondrial dehydrogenase enzymes in viable cells cleave the tetrazolium rings to produce insoluble purple formazan crystals. These are subsequently solubilised in acidified isopropanol. The cell number is therefore directly proportional to amount of formazan, as reflected in the absorbance reading. Though NaHS was used widely in investigating H_2S its high reactivity in aqueous solutions results in a rapid release of H_2S. The experiments have been conducted over a period of days and require a sustained release of H_2S. Therefore GYY4137 was administered to cell cultures. The cells were treated over 24 and 36 hours.

A rise in proliferation of MFE280s was noticed using GYY4137 concentration of 25 μM at 24 hours. At subsequent fall in proliferation was noticed at 50 μM with a rise in proliferation seen again at 75 μM. Higher concentrations such 100 and 200 μM were shown to cause a cell death seen in preliminary experiments conducted during optimisation of GYY4137. The biphasic appearance of the graph is unconvincing for a host of reasons. Firstly, experimental errors such as pipetting errors or uneven evaporation of culture media from

the wells could possibly lead to erroneous results. Secondly, the standard error margins at 25 and 75 μM concentrations of GYY4137 are larger. This would indicate greater degree of error at these values. A total of 15 replicate values were available for 50 and 100 μM concentrations in comparison to the 25 and 75 μM concentrations, thereby tightening the standard error margins at 50 and 100 μM. Finally considering the values at 36 hours, GYY4137 is not seen to have a proliferative effect on the MFE280s. At 36 hours it can be seen that the cell viability is constant at 25 μM and reduces thereafter. Therefore more replicate values will need to be completed and added to the existing data set to ensure the increase in proliferation seen at 25 and 75 μM at 24 hours is an accurate representation of cell proliferation.

The role of H_2S donors in activation of mitochondrial ATP generation and promotion of catalytic activity enzyme GAPDH via sulphhydration was reported[166]. GAPDH is mainly involved in glycolysis but has also been implicated in non- metabolic processes, such as; transcription activation, apoptosis initiation amongst others. Proliferation, migration and invasion are all ATP dependent processes and therefore inhibition of cellular production of ATP results in an inhibition of the above functions. Studies where intracellular H_2S levels have been reduced by the inhibition of CBS in colorectal carcinomas have revealed an inhibition of tumour growth[4]. Human colon cancer biopsies and patient-matched normal margin mucosa showed an up regulation of CBS and subsequent increase in H_2S on colon cancer. Comparison of human colon cancer derived epithelial cell lines and normal colon cancer cell lines revealed a selective up regulation of CBS in colon cancer cell lines (HCT116, HT-29, and LoVo) with no change in CBS expression seen in normal colon cell lines (NCM356). Inhibition of CBS using AOAA or short hairpin RNA- mediated silencing of CBS resulted in a reduced proliferation, migration and invasion of the HCT116 cell co-cultures and reduced mitochondrial function[4]. *In vivo* studies treating nude mice with an enzyme inhibitor (AOAA) reduced the tumour blood flow, thereby reducing growth of the cancer. No change was observed in CSE or 3-MPST expression. The by-products of cysteine metabolism by CBS are H_2S and L-Cystathionine. Administration of 1 mM L-cystathionine to both NCM356 and HCT-116 showed no effect on their proliferation. It was therefore concluded that H_2S was the driving force behind the enhanced proliferation seen in these cell lines[4].

In contrast, novel evidence was published verifying the use of H_2S donors as anti- cancer drug targets. Seven different human cancer cell lines e.g. cervical carcinoma (HeLa), colorectal carcinoma (HCT-116), hepatocellular carcinoma (Hep G2), myelomonocytic leukemia (HL-60), breast adenocarcinoma (MCF-7), acute promyelocytic leukemia (MV4-11) and osteosarcoma (U2OS) have been used, excluding endometrial carcinomas[125]. All cell lines have been treated with GYY4137 or NaHS in culture medium resulting in the liberation of quantifiable levels of H_2S. Their survival was compared against cell lines treated with NaHS. The H_2S levels were measured using the methylene blue assay. The expected rapid release of H_2S from culture medium containing NaHS was confirmed peaking at 20 minutes before reaching undetectable levels at 90 minutes. The release of H_2S in the presence of GYY4137 was more controlled and sustained over 7 days and was <10% of the H_2S release seen with NaHS[125]. In addition a non- sulphur containing control of GYY4137 known as ZYJ1122 was found to be inactive in all cell lines. Further investigations involving GYY4137, NaHS and ZYJ1122 at two different concentrations (400 and 800 µM) cultured for a 5 day period showed a small increase in cell death with higher concentration of NaHS and no effect with ZYJ1122. In contrast, GYY4137 showed a significant (75-80%) increase in cellular apoptosis at a concentration of 800 µM. The concentration of GYY4137 producing an inhibitory effect is much higher than the measurement seen in the culture medium <20 µM. The intracellular accumulation of GYY4137 and concentrated release of H_2S leading to cell death was proposed.[125]

Similarly in this project concentrations above 100 µM were found to have an inhibitory effect on cell viability. Concentrations of 25 µM were found to cause a stimulatory effect over a short duration as aforementioned. Further culture studies using either monoculture or co-culture with endometrial stromal cells will allow the deduction of whether the positive effect seen in these preliminary studies is accurate. If the effect seen is found to be true then it could be possible that *in vivo* H_2S has a stimulatory role. Concentrations of 100 µM and above are high and may not be normally present in cells in the endometrium. However, as no studies exist looking at the levels of H_2S in the endometrium it is not possible to attribute any role to H_2S until this is looked at. If H_2S is found to stimulate mitochondrial respiration and increase proliferation, H_2S inhibitors may have a role in

treatment against carcinogenesis. Further investigations are needed prior to forming conclusions.

Furthermore, a xenograft model in immunocompromised mice was used to elicit the effect of GYY4137 on tumour growth[125]. Results of this study confirmed the concentration-dependent death of cancer cell lines in response to GYY4137 (400 μM -800 μM) but not NaHS. It was found that NaHS was less potent and inactive in cell lines. Furthermore, GYY4137 (400 μM, 5-8 days) was found to promote apoptosis of cancer cell line (MCF-7) by causing cell cycle arrest in the G_2/M phase and activation of apoptosis but exerted no apoptotic effects on cancer cell lines (IMR90) suggesting a cancer cell specific role of GYY4137. Though this mechanism of H_2S causing cell death has never been proposed it was shown to promote cellular proliferation through the activation of MAPK[125]. Furthermore, H_2S is a strong reducing agent and could exert its effects through altering redox reactions within cells.

The results of *in vitro* experiments conducted in this study looking at the effect of H_2S donors such as GYY4137 and H_2S substrates i.e. L-cysteine on endometrial cancer cells are novel. In most studies measuring H_2S *in vitro* was completed by using the methylene blue assay to directly measure levels of H_2S. Due to the lack of resources in the laboratories resulted in the adoption of a proliferation assay known as MTT to indirectly measure effects of H_2S.

In addition the treatment of cells with the H_2S substrate L-cysteine exhibited an overall inhibition of cellular proliferation. The cells were maintained in 5% charcoal stripped media and therefore any increase in proliferation should be secondary to the proliferative effects of H_2S. Other by-products are produced through the metabolism of L-cysteine via the trans-sulphuration pathway. In order to maximally ensure the alteration in proliferation is secondary to the H_2S produced, methods of measuring the release of by-products such as ammonia should be employed. A sharp decline in cell viability is noticed with 1 mM concentrations at 24 hours after which increasing the concentration of L-cysteine showed no change in cell proliferation. At 48 hours although no cell proliferation is evident, the cell death was not as rapid with a more controlled decline in cell viability. In addition it is necessary to consider that L-cysteine treatments were carried out with the cells growing in

5% charcoal stripped media. MFE280s were found to sensitive to changes in their nutrition. Therefore it could be possible that the inhibition of cellular proliferation could be secondary to poor cellular nutrition rather than an inhibitory effect secondary to H_2S produced from l-cysteine or released from GYY4137.

The evidence acquired so far in this study from *in vitro* experiments shows an overall reduction in endometrial cancer cell proliferation with increasing concentrations of H_2S correlating with the evidence seen in the study conducted by Zeng Wei Lee et al[125]. Furthermore, higher concentrations of GYY4137 (100 μM) were seen to cause endometrial cancer cell death. The lack of normal endometrial cell lines secondary to their inability to thrive in culture poses difficulties in eliciting the effect of H_2S on normal endometrial cells. This is a major limitation as without this knowledge it is not possible to propose the use of H_2S donors as a potential treatment for endometrial cancer cells.

6.5 Limitations

Due to the lack of normal endometrial cells it is difficult to investigate the effect of H_2S on normal endometrial cells. As the cell culture results are displaying an overall inhibitory effect in proliferation of endometrial cancer cell lines at high doses of GYY4137 it is important to know whether these concentrations are detrimental to the survival of normal endometrial cells

The MFE280s are a poorly differentiating cell line and were very sensitive to changes in media. When cultured in 5% charcoal stripped media they struggled to thrive. As the physiological state of the cells was difficult to delineate the possibility the inhibition seen could be secondary to a natural cellular apoptosis due to the cells being unable to thrive in the media.

Mitochondrial dehydrogenase enzyme activity can vary in different cells and therefore the amount of tetrazolium rings cleaved during the assay will differ. This could possibly result in divergent results. However as the replicates are compared directly to the controls this should not produce bias within the results. Furthermore, the cells have been exposed to a variety of chemical substances which may interfere with the cells ability to react with the tetrazolium compounds, producing inaccuracy in the results.

Though the addition of a slow release H_2S donor has caused a reduction in cellular proliferation, to ensure that the inhibition is secondary to H_2S a non-sulphur containing compound should also be used at similar concentration under similar experimental conditions as a control.

A dramatic reduction in cell viability is seen upon treating the cells with L-cysteine for 24 hours at 1mM concentration. It is important to measure other by products released as part of the trans-sulphuration pathway such as L-Cystathionine, Homocysteine and ammonia to ensure that the change in proliferation is due to liberated H_2S.

Other assays that could be employed to verify the results seen with the MTT assay include measuring the release of energy i.e. ATP with the use of an ATP assay kit. ATP is found exclusively in the mitochondria and a rise in ATP production would be expected in cancers.

Although the MTT assay is effective at measuring cell proliferation a direct measurement of H_2S is necessary. As aforementioned hydrogen sulphide is present in 2 forms *in vivo* and therefore it is also necessary to delineate which form of H_2S is responsible for the effects seen. The methylene blue assay was used widely and would need to be employed to increase the strength of measurement and results.

6.6 Summary

The effect of H_2S has never before been described in endometrial cancer cells. Preliminary experiments conducted in this study describe an overall reduction in cellular viability with increasing concentrations of GYY4137. Although conflicting evidence exist with respect to H_2S and its effect on proliferation, its alternative role as a tissue specific, anti- or pro-apoptotic agent was theorised. Further experiments need to be conducted in order to deduce the specific role that H_2S plays in endometrial cancer and the normal endometrium. The lack of normal endometrial cell lines in existence due to difficulties in culturing these cells means that alternate methods of exploring the presence of H_2S in this tissue is necessary, and these have been discussed later in the general discussion. The normal cellular concentration of H_2S within the human endometrium has not been measured.

Chapter 7 General Discussion

This study report describes the expression of the three H_2S synthesising enzymes; CBS, CSE and 3-MPST in atrophic post-menopausal endometrium, hyperplastic and endometrial cancer tissue. Immunostaining scores for CSE ($p=0.0047$) and 3-MPST ($p<0.001$) were significantly lower in cancerous tissues when compared with post-menopausal controls. No overall differentiation in CBS ($p=0.1363$) expression was present between all groups. *In vitro* cell cultures using an H_2S donor GYY4137 were found to cause an overall reduction in cancer cell survival. The addition of a H_2S substrate L-cysteine reduced cell proliferation at 1 mM and 3 mM concentrations over 24 and 48 hours. The primary findings of this study have elucidated the differential staining patterns of the 3 H_2S synthases. Correlation of these enzymes with steroid receptors in similar sample groups found associations between them, discussed below.

The protective role of H_2S under conditions of acute oxidative stress by scavenging superoxide anions and suppression of vascular superoxide production was suggested. *In vitro* treatment of mitochondria isolated from murine hepatoma cells with H_2O_2 has shown a decrease in H_2S production via the 3-MPST pathway[119]. An unopposed hyper oestrogenic state is a major risk factor for the development of endometrial carcinoma. Oestrogen metabolism was hypothesised as an important factor in endometrial carcinogenesis[56, 68]. In endometrial cancer and endometrial hyperplasia a 50% decrease in the antioxidant superoxide dismustase when compared with benign endometrial disease including uterine polyps and myomas was described in the presence of increased oxidative stress. Hence a reduction in clearance of free radicals occurs suggesting an accumulation of free radicals in endometrial cancer tissues[120]. The down regulation of SOD was shown to be accompanied by a 100% increase in glutathione reductase activity in endometrial carcinoma and a 60-100% increase of both glutathione and glutathione reductase in endometrial hyperplasias. A down regulation of H_2S production via the 3-MPST pathway under conditions of increased oxidative stress was proposed, suggesting a reduction in expression of 3-MPST in endometrial carcinogenesis[120].

3-MPST was expressed strongly in the PM endometrium. The findings of this study show an early loss of 3-MPST in the glandular epithelial cells of low grade endometrial carcinomas ($p<0.0001$). Although no significant loss of 3-MPST expression was noted in CAH, the score for the intensity of staining varied greatly. The variability of CAH could mean that those

with lower expression of 3-MPST could progress to EC. Only 4 CAH samples have been stained and therefore the strength of this suggestion is not strong. Further to this comparison of type 1 and 2 endometrial carcinomas against PM endometrium reveals a significant down regulation across all groups. Correlation of 3-MPST with steroid receptors revealed a weak association (r=0.2) with PR in high grade cancers and no association in low grade cancers. In health PR has an anti- proliferative effect on the endometrium with a loss of PR suggested in advanced cancers. The positive correlation between PR and 3-MPST would suggest a combined loss of both in advanced endometrial carcinomas. Evidence from this study is in agreement with this relationship, showing a loss of 3-MPST in advanced cancer.

CSE staining in the glandular epithelial cells was evidenced to be down regulated significantly (p=0.004) in endometrial cancer when compared to healthy post-menopausal endometrium. Comparison of low and high grade endometrial carcinomas with PM controls revealed a grade dependent loss of CSE expression (p=0.004), with a more significant reduction in high grade cancers (p=0.001). Comparison of type 1 and 2 cancers with PM tissue samples revealed a greater loss in type 2 non-endometrioid carcinomas (p-0.001) compared with type 1 endometrioid carcinomas. No significant difference in expression exists between the CAH and PM groups. The standard error margins for the CAH group are close together showing little variation in CSE expression within this group. This lack in variation could also be attributed to the small sample size (n=4) and a larger sample size would need to show similar lack of variance to strengthen this idea. As no significant reduction in CSE expression is seen CAH, the loss of CSE in glandular epithelium as a possible disease indicator is not possible as yet.

Evidence has claimed a role for H_2S produced by CSE in angiogenesis[133, 137, 139]. A comprehensive literature search has revealed evidence claiming the increased vascular endothelial expression of CSE in circulatory and neoplastic disease[167]. CSE mediated production of hydrogen sulphide was shown to play an important protective role in cardiovascular disease by producing vasodilation[114] and angiogenesis. Previous study in colorectal carcinoma showing an up regulation of CBS showed no change in expression of CSE but claimed that CBS mediated production of H_2S was responsible for angiogenesis. H_2S mediates angiogenesis through a variety of pathways: the mitogen activated protein kinase

pathway, PI-3K/Akt pathway and ATP- sensitive potassium channels[114]. H_2S is also known to inhibit phosphodiesterase, thereby promoting angiogenesis[104]. As most evidence at present implicates CSE in vascular endothelium, staining within the vascular endothelium in endometrial carcinomas and PM endometrium should be investigated. As aforementioned the focus of this study was placed on the CSE expression within the glandular epithelium. However, analysis of tissue micrographs stained with CSE in the vascular endothelium (refer to figure 22) has revealed minimal to no endothelial staining of CSE in PM endometrium. This is expected, as all the PM samples are atrophic. Hyperplastic samples have shown areas of endothelial staining and type 1 and 2 endometrial cancer samples have also shown positive endothelial staining for CSE. Therefore at a glance it can be deduced that CSE is being expressed by the vascular endothelium of proliferating endometrium when compared with no staining seen in PM endometrial tissue. However, quantification of intensity of staining using the modified quick score method needs to be done along with staining of normal cycling endometrium to reveal any changes in CSE expression in the vascular endothelium between the 3 phases of the menstrual cycle. Without this knowledge it is difficult to understand and deduce the role of CSE in the endometrium.

No significant differential expression of CBS was seen to exist between any of the groups involved in this study. CBS up regulation and subsequent increase in H_2S production have been shown to play a role in tumourigenesis and maintenance in colorectal adenocarcinomas and epithelial ovarian carcinomas. The physiological roles of H_2S include vasorelaxation, angiogenesis and promotion of cellular bioenergetics[7, 87]. It is these properties of H_2S that have implicated it in carcinogenesis. Loss of CBS was shown to reduce cellular viability and alter antioxidant levels, trigger apoptosis and enhance drug sensitivity[146]. Extensive research was done verifying glutathione (GSH) as an antioxidant, but selective targeting of GSH in cancer cells was a major clinical challenge[168]. CBS has therefore been suggested as a good alternate target in ovarian epithelial cells due to its minimal expression in normal cells.

This current study has shown relatively high expression of CBS in the post-menopausal endometrium. The current role and expression of CBS in the normal cycling endometrium has not been defined and would need to be clearly assessed. Although no overall difference in CBS expression was seen in this study, investigating oestrogen and non-oestrogen dependent cancers separately revealed an increase in CBS expression in grade 3 endometrioid carcinomas ($p=0.04$). Although endometrioid cancers are associated with oestrogen regulation in advanced disease a loss of oestrogen regulation was postulated via the loss of ER-alpha. Correlation studies conducted as part of this study have shown a strong negative correlation between the expression of ER-alpha and CBS ($r=-0.5$). This up regulation of CBS could be associated with advanced disease in endometrial carcinomas and may potentially occupy a role as a prognostic marker. In addition synthesis of reactive oxygen species (ROS) is evidenced to be increased in CBS silenced cells[169]. This was attributed to the reduction in GSH activity and possible reduction in superoxide dismustase activity. Many active intermediates are also released as by-products of these chemical pathways which are subsequently converted to free radicals. This enhanced production of ROS results in DNA damage resulting in mutations that may contribute to carcinogenesis[170]. H_2S was evidenced to exert tissue specific actions and its regulation via the 3 enzymes is not identical between various organs in the body. This alternating role of H_2S may explain why the results are not in agreement with published evidence in other cancers. In contrast, the up regulation of CBS in grade 3 endometrioid carcinomas could possibly be a compensatory mechanism in response to the loss of 3-MPST and CSE.

The clinical pathological features of the endometrial cancers such as extent of myometrial invasion and lympho-vascular invasion have been compared against the expression of the three H_2S synthesising proteins to reveal a possible association between the two groups. Myometrial invasion was categorised according to the 1991 FIGO classification into <50% or >50% invasion into the myometrial wall and lympho-vascular invasion was included if present and rejected if it does not exist. No association was found between the 3 proteins and the clinical pathological features. Further to this outcome data was used where 1 represents an event such as recurrence or death due to endometrial carcinoma at 36 month follow-up and 0 represents no events. Comparison of all 3 enzymes with outcome data showed no association between the expression of enzymes and outcomes of disease.

Previously literature was published investigating the use of slow release H_2S donors as anti-cancer drug targets[170]. The alternating role of H_2S in exerting pro-apoptotic or anti-apoptotic effects was reportedly previously in cultured cells, though the exact mechanisms still remain undefined[161]. H_2S donors are known activators of mitochondrial ATP generation and promoters of GAPDH catalytic activity via sulphhydration. The stimulatory effect of H_2S on cellular bioenergetics has implicated it in proliferation, migration and invasion as they are all ATP dependent mechanisms. *In vitro* and *in vivo* treatments of neoplastic and normal cell lines with the slow release GYY4137 revealed an overall anti-proliferative effect on neoplastic cells with no effect on normal cell line proliferation. The use of a rapid releasing H_2S donor showed no effect on either the neoplastic or normal cell lines. A study was conducted investigating the role of various slow release H_2S donors (GYY4137 analogues) to elicit their anticancer potential. A dose- dependent cellular apoptosis of neoplastic cells of seven differing human cancer cell lines was seen, with no effect on normal human cells[170]. As discussed conflicting evidence exists implicating the H_2S donors or enzyme inhibitors as possible therapeutic drugs targets. This conflicting evidence could be a result of the complex and ambiguous role of H_2S and its synthases in tumorigenesis.

In vitro experiments conducted in this study describe the effects of H_2S donors such as GYY4137 and H_2S substrates i.e. L-cysteine on the MFE280 endometrial cancer cell line. Results of the *in vitro* experiments have shown an overall inhibitory effect of GYY4137 and l-cysteine on endometrial cancer cells at higher concentrations. The cells were treated over 24 and 36 hours. Higher concentrations such 100 and 200 µM were shown to cause a cell death seen in preliminary experiments conducted during optimisation of GYY4137. An overall biphasic appearance of results was visible describing a rise in proliferation of MFE280s at GYY4137 concentration of 25 µM at 24 hours, after which a fall in proliferation is seen at 50 µM with a rise in proliferation seen again at 75 µM. Any concentrations higher than 75 µM were shown to inhibit cell viability. The large SEMs seen at GYY4137 concentrations of 25 and 75 can be attributed to the smaller number of replicate values plotted when

compared to the other two concentrations of 50 and 100 µM. The error in results could be due to experimental error such as pipetting errors or uneven evaporation of culture media from wells. In addition considering the values at 36 hours, GYY4137 is not seen to have a proliferative effect on the MFE280s reiterating the need for further experiments to confirm whether the rise in proliferation seen in significant or not.

Treatment of cells with the H_2S substrate l-cysteine exhibited an overall inhibition of cellular proliferation. Treatment of cells with 1mM concentration of l-cysteine at 24 hours revealed a sharp decline in cell viability with 3mM concentrations being toxic to the cells. At 48 hours no cell proliferation is evident with a gradual decline in cell viability seen. As 5% charcoal stripped serum was used to grow and maintain the MFE280s prior to treatment with l-cysteine it is possible that the decline in cell viability seen could be secondary to the cells being unable to thrive in sub-optimal conditions.

7.1 Limitations

Further studies will need to be conducted in order to quantify enzyme functionality in the endometrium. An additional technique such as quantitative real-transcriptase polymerase chain reactions (QRT-PCR) or Western blot method should be used. The use of QRT-PCR to confirm findings would have been completed, but the lack of published isotype specific-primers resulted in difficulties to adopt this method. Also published primers for CBS and 3-MPST were very expensive and could not be purchased due to lack of funding. In addition statistical analysis of IHC data involved the use of non –parametric tests. These tests are known to reduce the strength of the results and thereby produced statistically insignificant p values.

Although a large sample cohort of 81 endometrial samples have been collected and stained, variation in sample numbers within the groups reduces significance of results. Lower numbers of samples were present for CAH, grade 3 endometrioid and type 2 endometrial carcinomas. Patients who are suffering from advanced stage cancers are treated with chemotherapy or radiotherapy prior to surgery. These adjuvant therapies are known to affect the cellular architecture of the tumours and therefore would not be suitable for use in the study thus resulting in obstacles for recruitment of patients with high grade cancers. Increasing number of samples within the groups stated above should improve the significance of data.

In vitro studies using endometrial cancer cell lines required optimisation, as covered in chapter 4. The lower expression of CBS, CSE and 3-MPST in the primary cell line used; Ishikawas when compared to the MFE280s resulted in the need to use this cell line instead. MFE280s are a slow growing cell line maintained as a monolayer. They require a longer time to reach an appropriate confluence before use. Moreover, to maintain this cell line in reduced serum media (5% FBS, Charcoal stripped) resulted in increased difficulties in handling these cells. The use of a sulphur containing H_2S should be compared against another non-sulphur containing compound as a control to ensure that the change in cell viability seen is secondary to the presence of H_2S.

In addition mitochondrial dehydrogenase enzyme activity is proposed to vary in different cells and the exposure of cells to different treatments would possibly affect their ability to react with tetrazolium compounds.

During the conduction of *in vitro* studies, direct measurement of H_2S could not be done due to the lack of appropriate equipment. Therefore, for accuracy the methylene blue assay or other suggested methods of H_2S detection would need to be employed.

7.2 Future work

While this thesis has demonstrated the variable expression of CBS, CSE and 3-MPST between PM and endometrial cancer, their expression in normal cycling endometrium has not been defined. Future IHC staining needs to be conducted to allow the analysis of change in expression, if present, between cycling, atrophic and cancerous endometrium. In addition the normal endometrial samples will need to be subcategorised into the 3 different phases of the menstrual cycle to analyse any correlation between changes in enzyme expression within the 3 phases. Normal expression of the 3 H_2S synthesising enzymes can then be correlated with steroid receptors to reveal any associations. Further to this a new group consisting of hyperplasias without atypia should be included. The comparison between atypical hyperplasias and grade 1 EC should also be focused on as it is important for the prediction of progression of disease.

Expression of CBS, CSE and 3-MPST was confirmed and located with the use of IHC methodologies. To confirm the IHC results methods such as Western blotting or quantitative real-transcriptase polymerase chain reactions (QRT-PCR) should be done to quantify mRNA levels of proteins to be verified. However, as the functionality of the enzyme is unknown with the stated methods, direct measurement of enzyme function needs to be assessed. Continuous assays with the use of UV-visible spectroscopy / fluorescence + phosphorescence spectroscopies or discontinuous assays can be used depending on the experimental outcomes.

As aforementioned sample numbers between the endometrial cancer groups were not equal, thus limiting the significance of the study. Increasing the sample numbers to achieve consistency within the groups can increase statistical significance of the data. Therefore more patients with high grade cancer that have not undergone hormone therapy will need to be recruited to take part in the study.

Methods of H_2S detection can be direct or indirect. Indirect methods have already been employed in this thesis e.g. the use of *in vitro* experiments investigating the proliferative potential of endometrial cancer cells using the MTT assay. Additional assays confirming the results found with the MTT assay and others looking at effects of H_2S on invasion and

migration of cells can also be conducted. As described earlier, H_2S has autocrine and paracrine effects. Its autocrine effects include an increase in cellular bioenergetics which can be measured to identify its effect on intact cells and isolated mitochondria. Angiogenesis is a result of the paracrine effects of H_2S and could be analysed by quantification of blood vessel density. The lack of resources at the laboratories posed difficulties in direct measurement of H_2S levels in cell lines. Therefore further functional studies aimed at directly measuring H_2S production should be carried out. Since its introduction by Fischer in 1883, the methylene blue assay is a widely used, sensitive and specific method for the measurement of H_2S. Fluorescence read- out of sulphide with the use of a hydrogen sulphide probe is another method of directly measuring H_2S. The low ppb range (detection limit) of the probe allows identification of small amounts of H_2S to be identified. The probe though highly sensitive is functional in solutions and therefore the methylene blue assay would be more suited for measurement of H_2S in cell lines.

7.3 Conclusion

In conclusion this study has identified the expression of CBS, CSE and 3-MPST using IHC in PM, CAH and endometrial carcinomas. *In vitro* studies have shown an overall inhibition of cellular proliferation with the use of a H_2S donor GYY4137 and H_2S substrate l-cysteine.

3-MPST was shown to be expressed in the cytosol of glandular epithelium and within the stromal compartment of the cells. It cytoprotective role was described in many tissues and its presence in the PM endometrium could possibly be protective from carcinogenesis. Its expression was shown to be significantly lost early in low grade endometrioid carcinomas and in high grade endometrial carcinomas. This early loss of 3-MPST suggests a possible role for its use as a disease marker. Further investigations as described above would need to be completed prior to claiming any definite functions. A weak positive correlation was found between PR and 3-MPST (r=0.2) between high grade cancers. A reduction in PR was suggested in advanced cancers and the loss of 3-MPST seen in high grade cancers in this study could correlate the relationship between PR and 3-MPST.

Two types of staining have been confirmed with CSE expression including diffuse cytoplasmic staining in the cytoplasmic compartment of epithelial and endothelial cells and discrete strongly stained cells. The nature of the cells that are strongly stained is still unclear. The overall expression of CSE in the epithelial cells was demonstrated to be lost in cancers when compared to post-menopausal cells. The loss of CSE in cancers could possibly suggest a role for it as a possible carcinogenesis marker. Increasing sample numbers and employing more techniques will elucidate the role of CSE in the glandular epithelium of endometrial cells. Brief analysis of endothelial staining has shown a lack of CSE expression in the PM endometrium and an increase in endometrial cancer. As CSE was described extensively in angiogenesis future experiments will need to focus on endothelial staining of CSE.

CBS was localised to the cytoplasm of glandular epithelium. No significant overall change in CBS expression was noted between PM and cancerous endometrium. Grade 3 endometrioid carcinomas have shown a significant increase in CBS expression when compared with PM endometrium. This could be secondary to a compensatory mechanism due to the suppression of 3-MPST and CSE. In contrast, the strong negative correlation

between ER-alpha and CBS suggest a possible role of CBS up regulation to be a marker of poor prognosis.

Comparison of expression of the three enzymes with outcome data and clinic-pathologic features have shown no association of 3-MPST, CSE and CBS with poor outcomes of disease or myometrial/ lympho-vascular invasion, respectively.

In vitro treatment of the MFE280 endometrial cancer cell line with l-cysteine has shown an overall inhibition of cancer cell proliferation. L-cysteine concentrations of 1mM and 3 mM have at 24 and 48 hours have shown a reduction in cell viability. However, as the MFE280 cell line was maintained in 5% charcoal stripped media the lack of cellular viability could be secondary to the inability of the cells to thrive under suboptimal conditions. The results seen with the administration of GYY4137 produced a graph with a biphasic appearance. GYY4137 concentrations of 50 and 100 µM were seen to suppress endometrial cell viability whereas concentrations of 25 and 75 µM were seen to stimulate cellular proliferation. The SEMs for 50 and 100 µM concentrations are narrower due to a total of 15 replicates being plotted at these concentrations but 25 and 75 µM concentrations only 3 replicates have been produced. Therefore it is more likely the values at these concentrations would consist of error when compared to GYY4137 concentrations of 50 and 100 µM. More investigations will need to be done at these concentrations to ensure that they are valid.

Further investigations using established cell lines such as primary endometrial cancer cell lines in monoculture might aid in exhibition of direct effects of H_2S on their proliferative function. Using a co-culture system where the endometrial cancer cells are grown in conjunction with stromal cells and their proliferation assessed in response to H_2S (H_2S donors) is more physiologically relevant method of assessment. As H_2S was suggested to alter cellular bioenergetics the use of flow cytometry to analyse cell cycles may possibly be a more sensitive methods to assess subtle changes in response to H_2S. Other major alterations in cancer cells include their increased migratory potential and invasiveness. By using 3D cultures *in vitro* or in animal models would be suitable in assessing migration and invasion potential of cancer cells in response to H_2S.

As the expression of H_2S synthases are altered further work on examining the direct effect of known endometrial mitotic agents such as oestrogen could be examined by the

conduction of explant cultures on benign post-menopausal endometrium, to determine whether any alteration in the expression of H_2S synthases is present in response to oestrogen.

If H_2S induced alteration in cell function can be proven with the experiments mentioned above, subsequent set of experiment s may provide a novel perspective allowing the utilisation of existing H_2S synthase inducers or inhibitors to counter act the undesired effects if identified. Ultimately there is a possibility of translating further work in this area into clinically relevant novel treatment strategies.

Chapter 8 References

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Appendix

Appendix 1 Ethics approval

Liverpool Women's NHS
NHS Foundation Trust

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14th April 2011

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Dear Dharani

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

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

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

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

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

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

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

Yours sincerely

Vernon

Gillian Vernon
Research & Development Manager

Liverpool Women's

Appendix 2 Patient information leaflet and consent form

UNIVERSITY OF LIVERPOOL

Liverpool Women's NHS
NHS Foundation Trust

Ethics Submission No: 11/H1005/4

PATIENT INFORMATION SHEET

"MIPs in Endometrial Cancer Study"
Role of metastasis-inducing-proteins in endometrial cancer

Version 2.1: For patients undergoing Pipelle Sampling
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.
Thank you for reading this

Why are we doing the study?
Each year in the UK about 4500 women, commonly in their 50s and 60s, develop cancer of the lining of the womb (endometrium). The 'endometrium' builds up and is then shed each month as a 'period' before menopause. In women who unfortunately develop cancer, the cells in the endometrium multiply and behave abnormally. The survival rate of early stage endometrial cancer is good, however in extreme cases cancer cells can spread beyond the womb affecting the overall outcome of the disease. We are doing this study to better understand the changes that happen in endometrial cells. This will help us discover new targets to diagnose and design new treatment for endometrial cancer.

What is metastasis?
The spread of cancer cells beyond their origin, the womb, is called 'metastases'. This metastatic process is closely linked with the outcome of the disease, but is not fully understood. It is possible that there are special proteins that can encourage cancer cells to spread. These specific proteins are called 'metastasis-inducing-proteins' (MIPs), which we believe can cause cancer cells to invade healthy tissue. We would like to investigate the presence of these proteins in endometrial cancer cells and the role they play.

Why have I been chosen?
We are looking for a total of 160 women. We are specifically looking for 80 women who have endometrial cancer and another 80 healthy women undergoing surgery. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

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

Therefore, **NO** extra surgery will be performed for the study. A **blood sample** will also be taken from your veins if you are still having monthly periods.

How is the endometrial pipelle sample done?

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

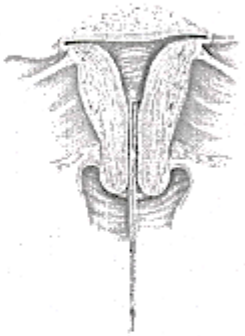

Fig 1: Pipelle Sampling

What are the possible benefits of taking part?

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

What if something goes wrong?

We do not anticipate any harm to arise while taking part in this study, as we are not carrying out any additional procedures. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action. There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

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

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Surplus tissue

You can chose to give consent for any remaining tissue, after being used for this study, to be anonymised and stored in the Liverpool Women's Hospital which can be used for future ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool Women's NHS
NHS Foundation Trust

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: The role of metastasis-inducing-proteins (MIP) in endometrial cancer

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) 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 sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree for surplus tissue to be stored in the department of obstetrics & Gynaecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3 Standard operating procedures (SOPs)

3A. Tissue Processing

Background

Formalin fixed tissue is dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine. The processing setting for most sample types is ***programme A*** that operates using the schedule provided below. The total processing time is 18 ¾ hours, therefore the processor is generally run overnight. Starting the processor at 2pm will result in completion of the cycle at 8:45 the following morning.

Programme A Processing Schedule:

4% formalin in neutral buffer 45 minutes
60% Ethanol 1 hour
70% Ethanol 1 hour
90% Ethanol 1 hour
100% Ethanol 1 hour
100% Ethanol 1 ½ hours
100% Ethanol 2 hours
Xylene 1 1 hour
Xylene 2 1 ½ hours
Xylene 3 2 hours
Wax 1 2 ½ hours
Wax 2 3 ½ hours

Equipment information:

Citadel processor (serial number CA 1390 EO 608) manufacturer: Thermo electron Corporation. Manufacturer's address: 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

Maintenance and contract:

Maintained/serviced by Thermo Fisher Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. Tel: 01928 562 541, Fax: 01928 562 512.

Solvent Information:

Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool). The wax (Histoplast PE REF8330) is purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. The 10% neutral buffered formalin is purchased from Sigma Life science, Fancy Road, Poole, Dorset, BH12 4QH.

Health and Safety Precautions

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene and formalin are harmful by contact to skin and eyes or if ingested or inhaled. Refer to the risk assessments and COSHH forms before starting procedure.

Overnight processing procedures requires the completion of permits provided in safety circular SCR14/3 (link to document provided below). A 'Yellow permit' (for long term operation) should be prepared by the DSC or laboratory manager and the copy secured in a clear plastic folder on the door outside laboratory 4. The 'Pink' permit (valid for the duration of the experiment only) should be prepared by the processor user and the document should be placed on or adjacent to the apparatus. Information that needs to be recorded on the pink copy includes:

a) experimental details

b) times and dates of experiment

c) emergency procedures

d) name, address and telephone number of the processor user

e) signature of researcher concerned and a countersignature from the laboratory manager/DSC.

PROTOCOL

A Preparation of Solvent Containers on Processing Machine:

Between processing runs, ethanol solutions are stored in labelled bottles to prevent evaporation. These are found in the metal solvent cabinet under the bench in Laboratory 4. Therefore the solvent containers must be re-filled before tissue processing commences. To

access the processor solvent container, press the ***raise*** button on the hand held controller***.*** Remove the evaporation covers from the top of the processor then press ***rotate*** or ***check/fill*** button. The rotate function will cause the operating head assembly to advance one position

and the check/fill option rotates the head three positions. Remove the container using the metal handles and add 1.5L of the required ethanol solution (the top of the bevelled edge on the solvent container is approximately 1.5L). Repeat process until all ethanol containers are filled. It is also advisable to check the level of the xylene, formalin and the wax containers on a regular basis. If fresh 100% ethanol is used to top up or prepare solutions for the processor ensure the ethanol log is updated with the volume used. The ethanol log is situated in DSC office (room 1128).

B Sample Processing Procedure

1. Label plastic cassettes with the sample ID number using pencil.

2. Carefully remove the tissue from the formalin pot using forceps and place into the small wire inserts. Secure the inserts into the plastic cassettes and re-check the sample ID. Dispose of the used formalin in the red labelled waste container which is stored below the fume hood.

2. Arrange the cassettes so they are loosely spaced in the processing baskets

3. Press ***Go To 1*** on the hand held controller**,** slot the processing baskets onto the holder over the formalin and place weight on top of the basket.

4. Press ***Lower*** button on the hand held controller (to lower the basket into the formalin).

5. Check the time is correct by pressing ***clock*** (alter time by pressing the + button or whilst holding in the clock button if necessary).

6. Select the appropriate processing time function:

Date of Processing	**Processing Time**	**Hand Held Controller Action**
Monday-Thursday	Before 2pm	Press ***delay on*** button

Monday-Thursday	After 2pm	Press ***autostart*** button
Friday	Before 2pm	Press ***days delay,*** hold this in, scroll to 2 days using the + button then press ***delay on.***
Friday	After 2pm	press ***days delay,*** hold this in, scroll to 1 day using the + button, then press ***delay on***

7. Complete the citadel 1000 user log (the folder is situated by the embedding station in laboratory 4) with sample, solvent and programme information.

8. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.

9. Press ***raise*** button on the hand held controller and remove baskets from holder.

10. Place blocks into heated reservoir in the embedding machine.

11. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue.

12. Soak biopsy inserts in xylene for several hours/overnight to remove wax residues. Leave the inserts in the fume hood to air dry

13. Remove the alcohols from the processing machine using the method described in section A and pour solutions in the labelled storage bottles using a funnel. The storage bottles are then placed in the flammable solvent metal storage cabinets.

14. Update the citadel 1000 processing user log.

C Disposal of Waste Solvents

The solvents must be changed on a regular basis (depending on time of year and processor usage) to ensure efficient processing of the tissue samples. Waste solvents must be placed in the red solvent waste containers and identified clearly with the appropriate labels provided by Lisa Heathcote or Jo Drury. Pouring the molten solution into a container and

cooling until the wax is set can discard contaminated waste wax. The wax container can then be disposed of in the LWH clinical waste. Refer to the waste disposal protocol (SOP 25) for further information.

3B: CUTTING PARAFFIN SECTIONS

Background

Preparation of 3-5 micron thick sections from wax – embedded tissue is a commonly used histological technique. This SOP will describe how to prepare these using the Microm HM335 rotary microtome and attaching sections onto aminopropyl triethoxy silane (APES) coated slides.

HEALTH AND SAFETY REQUIREMENTS

Wear a laboratory coat and nitrile gloves. Care needs to be taken when performing this procedure because there is a risk of sharps injury from the microtome blades. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. Also ensure waste wax sections are cleaned from the floor and the general working area at the end of the procedure to prevent risk of slip/trip injuries. Place all waste tissue sections in the clinical waste bags and the used microtome blades and slides into the yellow clinical waste sharps bins. DO NOT COMMENCE WORK WITH THE MICROTOME UNTIL SUFFICIENT TRAINING WAS PROVIDED AND AUTHORISATION WAS RECEIVED FROM LISA HEATHCOTE OR JO DRURY.

EQUIPMENT INFORMATION:

Microm HM335 rotary microtome is supplied by MICROM Company. Manufacturer's address: Microm UK Ltd., 8 Thame Park Business Centre, Leinman Road, Thame, OX9 3XA. Further information can also be obtained on http://www.microm-online.com.

Microscope slides (twin frost size 26 x 76mm-Printed "IVD CE" 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women's Hospital (NHS) purchasing department.

Coverslips 22 x 22mm, 22 x 40mm and 22 x 50 are purchased from Liverpool Women's Hospital (NHS) purchasing department.

Microtome blades (MB Dynasharp Catalogue Reference 3050836) are purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

Forceps, paint brush, section dryer and water bath are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

1. Carefully fill the water bath with distilled water ensuring the electrical connection points remain dry.

2. Turn on water bath from the plug and set the power switch from O to I

3. The water will need to be heated to 40°C. The best way do to this is to first turn the temperature dial to 10 (maximum setting) for 7-8 minutes and then turn the dial down to 2.5. If the temperature of the bath is above 40°C, pour in distilled water to lower the temperature.

4. Scrape away any surplus wax from the edges of the cassette to ensure a good fit in the holder then place the tissue block in the refrigerator. Cool the block for at least 30 minutes prior to cutting.

5. Ensure that the handwheel is locked in the upper range of the vertical movement by turning the lever downwards.

6. Press the reverse course feed button to move the knife carrier away from the specimen.

7. Turn the clamping lever on the blade holder to the front and swing the protective bracket forwards. Insert the feather S35 blade into the slot behind the clamping plate ensuring the blade is level on the rail (use forceps to manipulate the blade if necessary). Use half of the blade for cutting at a time. Preferably use left side first and then the right side.

8. Return the clamping lever to the original position (in line with the black frame of the blade) to lock the blade in place. The protective bracket should also be returned to the original position when the microtome is not in use to minimise the risk of sharps injury.

9. The blade angle is pre-set to 10-12C and should not require adjustment. These settings should remain the same to ensure minimal amounts of tissue are wasted when different laboratory workers prepare sections from the same block.

10. Insert the specimen against the 'fixed jaw' of the universal cassette clamp and secure by pulling the lever to the front. Ensure that each specimen cassette is always inserted in the same orientation (horizontal placement in the universal cassette clamp with the specimen number on the left) and inserted to the far left of the clamp.

11. Unlock the handwheel by turning the lever upwards. Rotate handwheel in a clockwise direction until the centre of the tissue block is level with the blade holder. Then lock the handwheel by turning the lever downwards.

12. Press the forward course feed button to move the blade carrier near to the specimen. To establish whether the blade is close to the specimen view the distance between the two at a side angle. Also, gently move the handwheel up and down to see if the specimen is not touching the blade. When you are satisfied that both blade and specimen are close to each other move the handwheel so the specimen is away from the blade, this prevents scoring the specimen block in the middle.

13. The required section and trimming thickness are set by means of a circular knob on the left of the instrument. Press the circular control knob to switch between section (FEED) and trimming (TRIM) thickness. The corresponding LED will be displayed on the operating control panel:

Green LED lights up when the FEED function is used

Yellow LED lights up when the TRIM function is selected.

14. When a new tissue block is used, select TRIM and turn the control knob until the corresponding LED is set at 20µm.

15. Unlock the handwheel and start trimming by rotating the handwheel in a clockwise direction. Once you begin to see specks on tissue in the sections change the TRIM settings from 20µm to 10µm.

16. Continue to trim on the new setting until a representative amount of tissue is exposed in the tissue block.

17. Select FEED and turn the control knob until the corresponding LED is set at the required section thickness (usually 3-5µm). Rotate the handwheel until the first section generated. Gently hold the end of the section with forceps and continue to cut until a ribbon (consisting of approximately 6 sections) is produced. Discard the first few sections when first cutting on the feed section as usually the first few are too thick.

18. Carefully float the sections onto the pre-warmed water bath. Leave or a minute or so until the wrinkles in the section disappear. Separate the sections by applying gentle pressure using forceps. Select the best sections and float onto APES-coated slides. It is considered to be good laboratory practice to attach each section in the same orientation on the surface of the slide where you can read the IVD sign.

19. Label the frosted part of the slide with the specimen ID and thickness (using a pencil). Place slides in a rack to dry at room temperature for several hours (preferably over-night).

20. When not cutting for a few minutes the specimen block moves slightly out of place which potentially can create thicker sectioning when you go back to cutting. To avoid creating thicker sections press the reverse course feed button very slightly to move the knife carrier away from the specimen by a small amount.

21. During the cutting procedure a build-up of wax on either side of the blade carrier. This can potentially cause scoring of the section. Using a soft brush, brush in an upward direction against the blade carrier to remove the wax, this is to prevent blunting of the blade and cutting down of the brush if a downward direction was used.

22. If the sections start to wrinkle during the cutting procedure, then the tissue block has become too warm and needs to be refrigerated for at least 10 minutes. To remove the tissue block, press the reverse course feed button until the specimen is safely away from the blade holder. Return the safety bracket to the upright position. Remove the tissue block by pressing the lever on the universal cassette clamp.

23. Remove any unused tissue sections from the water bath surface using kitchen roll.

24. When the microtome blade needs to be replaced, return the tissue block to the furthest position using the course feed button and lock the handwheel. Unlock the lever on the blade carrier and place the microtome blade into the yellow clinical waste sharp bin. NEVER try to remove the microtome blade when the tissue block is close to the blade edge because there is a risk of sharps injury and also the risk of damaging the tissue block.

25. When the sections have dried, place the slides into a staining rack and bake for 60 minutes in a slide drier (pre-warmed to 60°C), or 37°C overnight. Place slides in a suitable 'dust free' container until required for the immunohistochemical staining procedure.

3C APES coating procedure

Background

The Aminopropyl triethoxy silane (APES) coating procedure is used to improve tissue adhesion by producing a positive charge on the surface of the slide. The protocol described below was modified from the original method described by Maddox and Jenkins in 1991 (full reference provided in the appendices).

HEALTH AND SAFETY REQUIREMENTS

The APES concentrate is a toxic chemical that is moisture sensitive and requires storage at 0-5°C; it is an irritant and can cause burns. Normal laboratory precautions, including the wearing of gloves and the use of a fume cupboard, should be taken when handling the concentrate solution. Refer to the risk assessments and COSHH forms before starting procedure. Place all waste solutions into a labelled red solvent waste can and attach a solvent label (available from Lisa Heathcote or Jo Drury by request).

EQUIPMENT INFORMATION:

Microscope slides (twin frost size 26 x 76mm-Printed "IVD CE" 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women's Hospital (NHS) purchasing department.

APES solution (catalogue number A3648) is purchased from Sigma-Aldrich Company Ltd. UK distributor address: The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT.

Staining racks and glass jars are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

26. Place slides into plastic racks. Each rack holds 24 slides

27. Prepare a 2% APES working solution by mixing 5ml APES with 245ml 100% ethanol, or for the bulk method 40ml APES with 1960ml 100% ethanol. The APES solution is stable for several hours; therefore coat as many racks as possible to save reagents and time.

28. Prepare a glass staining trough containing 100% ethanol

Position 1: APES working solution
Positions 2 – 4: distilled water

29. Place slides racks in 100% ethanol. Dip several times to dislodge dust etc., and then drain the staining racks on absorbent paper towels.

30. Incubate the slides for 5 minutes in APES working solution.

31. Rinse slides in three changes of distilled water (2 minutes incubation for each rinse).

32. Drain slides on absorbent towels and air dry overnight, or dry in a section dryer for 1 hour at 37°C.

33. Discard the first change of distilled water. Place the staining jar containing fresh distilled water at the last rinse location (position 5). Therefore the 2nd change of water has becomes the first etc.

34. Repeat steps 5 through to 9 for each rack of slides.

35. Transfer the coated slides to a dust free container labelled ‘APES’ and the date of coating (APES slides are stable for approximately 3 months).

3D. Preparation of paraffin sections for staining

Background

Factors that prevent adhesion of sections to blank slides are usually attributed to moisture under the section, and almost always become apparent after heat antigen retrieval. The method of heating slides prior to immunohistochemical staining ensures tissue sections adhere to the glass slide. It is important the sections are not over-baked, as this could increase background staining and decrease target staining.

In order to prepare slides for staining, tissue must be deparaffinised using xylene and gradually rehydrated with descending grades of alcohol.

Health and Safety Precautions:

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene is harmful by contact to skin and eyes or if ingested or inhaled. Work in the fume hood for dewaxing procedure. Refer to the risk assessments and COSHH forms before starting procedure.

Materials

- Staining racks and glass jars are supplied by Raymond A Lamb Ltd. Manufacturer’s address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

- Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool).

- Section Dryer. Model E28.5. Supplier information: Raymond A. Lamb, Eastbourne, BN23 6QE, England.

Method

1. Switch on Section Dryer Model E28.5. Adjust temperature using the temperature dial (see below for temperature required).

2. Label slides with the stain/antibody and concentration to be used, and date in pencil.

3. Place slides for staining in metal slide racks.

4. Heat to 60°C for 60 minutes or overnight at 37°C.

5. Dewaxing solutions are in staining dishes in the fume hood in room 1125. Place the slide rack in xylene-1 for 10 minutes. Long forceps can be used to facilitate the transfer of the slide rack between dishes.

6. Drain off excess xylene, and transfer to the dish labelled “xylene-2” for 10 minutes.

7. Drain off excess xylene, and briefly blot on paper towel.

8. Place the slide rack in the dish labelled “100% ethanol-1” for 5 minutes.

9. Drain off excess ethanol, and transfer to the dish labelled “100% ethanol-2”.

10. Drain off excess ethanol, briefly blot on paper towel and transfer to 90% ethanol for 1 minute.

11. Drain off excess ethanol, briefly blot and transfer to 70% ethanol for 1 minute.

3E Immunohistochemistry

Background

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues. In order to perform the standard staining procedure, first the tissue section has to be prepared (SOP 04, 05 and 06), deparaffinised and then rehydrated (SOP 08). Antigen retrieval (SOP 09) is then performed to break any protein cross-links, therefore unmasking the antigens and epitopes in the tissue sections. The Vector ImmPRESS TM reagent kit, HRP is a two-step Immunohistochemical staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequentially non-specific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper

counts. Any endogenous peroxidase activity is quenched by incubating the specimen with Peroxidase block. The specimen is incubated with an appropriately characterised and diluted mouse primary antibody, followed by incubation with the labelled polymer. Staining is completed by incubation with 3'3-Diaminobenzindine (DAB) + substrate - chromogen which results in a brown–coloured precipitate.

Definition

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

Health and Safety Precautions

The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, such as hydrogen peroxide (H_2O_2), xylene, ethanol, and DAB (3'3-diaminobenzindine). Gloves must always be worn when working with any of these chemicals.

EQUIPMENT INFORMATION

Staining dishes

Humidified chamber

Cover slips

General points:

Ensure that solutions cover the whole specimen – if necessary use a piece of Parafilm to spread.

Antibody information:

All current antibody datasheets are held in a labelled lever arch file in room 1128.

Additional information on appropriate antibody concentration, antigen retrieval conditions and incubation time/temperature is held in an Excel spreadsheet (C:\Documents and Settings\jadrury.livad\My Documents\Dharani\Dharani abs.xlsx).

Reagent information:

TBS: 6 g/l Trizma base (T1503, Sigma-Aldrich) + 8.7 g/l NaCl (S7653, Sigma-Aldrich). Adjust pH to 7.6 with HCl.

BSA: A3803 (Sigma Aldrich)

H_2O_2: H1009 (Sigma-Aldrich)

ImmPRESS anti-mouse IgG kit (50 ml) (MP-7402, Vector Laboratories)

ImmPRESS anti-rabbit IgG kit (50 ml) (MP-7401, Vector Laboratories)

ImmPRESS anti-goat IgG kit (50 ml) (MP-7405, Vector Laboratories)

ImmPACT DAB (120 ml) (SK-4105, Vector Laboratories)

Shandon Gill 2 Haematoxylin (Thermo Scientific)

1% acid alcohol: 1 ml HCl/100 ml 70% ethanol

Shandon Consul-Mount (Thermo Scientific)

Supplier information:

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com.

Thermo Scientific, Bishop Meadow Road, Loughborough, LE11 5RG. Tel: 01509 231166 Fax: 01509 231893.

Vector Laboratories Ltd. 3, Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS. Tel: 01733 237999. Fax: 01733 237119. Web: www.vectorlabs.com.

METHOD (Paraffin sections):

1. Place slides in a staining dish containing TBS and incubate 5 minutes at room temperature.

2. Prepare 0.3% H_2O_2/TBS (2.5 ml 30% H_2O_2 + 247.5 ml TBS)

3. Incubate slides in 0.3% H_2O_2/TBS bath 10 minutes at room temperature.

4. Prepare humidified chamber by placing folded paper towels in the centre gulley's and soaking with distilled water.

5. Decant H_2O_2/TBS and tap off any remaining solution. Carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

6. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.

7. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

8. Mark area to be stained with DAKO hydrophobic marker pen ensuring that the tissue is surrounded with sufficient space to allow spreading of antibodies.

9. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.

10. Prepare antibody diluent (TBS/0.5 % BSA eg. 250 µl 10% BSA + 4750 µl TBS)

11. Prepare appropriate dilutions of each antibody, allowing 50 µl per section.

12. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

13. Place slides in humidified chamber.

14. If necessary, add one drop horse serum block to each section, spread with parafilm to ensure that the whole section is covered, and incubate 20 minutes at room temperature.

15. Tap off the serum block and proceed to step 17.

16. Steps 14 and 15 can be omitted if staining is clean without blocking.

17. Apply 50µl of the appropriate antibody to each section, spread with parafilm to ensure that the entire section is covered and incubate 30-120 minutes at room temperature or overnight at 4□C.

18. Tap off the antibody solution onto paper towels prior to placing the slides in a staining dish filled with TBS.

19. Incubate 5 minutes at room temperature.

20. Decant TBS and refill.

21. Incubate 5 minutes at room temperature.

22. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

23. Return slides to the humidified chamber and apply 1 drop labelled polymer-HRP. Ensure that the polymer matches the primary antibody.

24. Spread with parafilm to ensure that the entire section is covered and incubate 30 minutes room temperature.

25. Tap off the polymer solution onto paper towels prior to placing the slides in a staining dish filled with TBS.

26. Incubate 5 minutes at room temperature.

27. Decant TBS and refill.

28. Incubate 5 minutes at room temperature.

29. Prepare substrate/chromogen solution: 30µl/ 1ml substrate (require 50 µl per section).

30. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

31. Return slides to the humidified chamber and apply 50 µl substrate/chromagen solution.

32. Spread with parafilm to ensure that the entire section is covered and incubate 10 minutes room temperature.

33. Place slides in staining rack and immerse immediately in tap water to stop the reaction.

34. Turn on fume hood in lab 4.

35. Counterstain using filtered Gill 2 haematoxylin in lab 4. Immerse for 1 minute 30s.

36. Immerse in tap water and rinse until water is clear.

37. Dip briefly in acid alcohol, and immediately back into tap water (5 minutes).

38. Incubate 1 minute in 70% ethanol.

39. Blot off excess and incubate 1 minute in 90% ethanol.

40. Blot off excess and incubate 3 minutes in 100% ethanol.

41. Repeat step 40.

42. Blot off excess and incubate 5 minutes in xylene.

43. Blot off excess and incubate 10 minutes in xylene.

44. Remove a few slides at a time and apply sufficient mountant to cover the section.

45. Choose an appropriately sized coverslip and apply to the slide.

46. Remove air bubbles by using a cocktail stick or yellow pipette tip to gently press on the coverslip and “chase” bubbles to the edge.

47. Leave to dry in fume hood.