# OESTROGEN METABOLISM IN COLORECTAL CANCER

By

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## ABSTRACT

Colorectal cancer (CRC) is the second most prevalent cancer. Although not typically considered a hormonally responsive malignancy, oestrogens are linked to disease risk and survival. Here, it is hypothesised that oestrogen synthesis, via steroid sulphatase (STS) and 17β-hydroxysteroid dehydrogenases (HSD17β), is promoted in CRC leading to oestrogen-driven tumour proliferation. To identify potential novel treatment targets oestrogen metabolism was characterised in CRC. Oestrogen metabolism was characterised in CRC cell lines and human colon tissue using Western blotting, qRT-PCR, STS activity assay and proliferation assays. A mass spectrometry method to quantify oestrogen metabolites was developed and validated for cell culture medium.

CRC exhibited increased STS activity indicating increased intratumoural oestrogen availability. In CRC a drive towards potent oestradiol production through decreased HSD17β2 expression (oxidative enzyme) and increased HSD17β7 and 12 expression (reductive enzymes) was seen. STS over-expression increased proliferation in CRC cell line HCT116, which was at least in part mediated through G protein-coupled oestrogen receptor action. Additionally, STS activity was regulated *in vitro* and *in vivo* by the inflammatory modulator TNFα.

This thesis demonstrates that increased STS activity and oestrogen synthesis in CRC increases tumour proliferation via GPER. Thus, multiple novel targets have been identified to impede CRC proliferation.

## DEDICATION

For Phil.

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| | |
|---|---|
| **ADP** | Adenosine diphosphate |
| **AKT/PKB** | protein kinase B |
| **APC** | Adenomatous polyposis |
| **ATP** | Adenosine triphosphate |
| **AU** | Arbitrary units |
| **BCA** | Bicinchoninic acid |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CRC** | Colorectal cancer |
| **DHEA** | Dihydroepiandrosterone |
| **DM-F12** | Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 |
| **DNA** | Deoxyribonucleic acid |
| **$E_1$** | Oestrone |
| **$E_1S$** | Oestrone sulphate |
| **$E_2$** | Oestradiol |
| **$E_2S$** | Oestradiol sulphate |
| **$E_3$** | Oestriol |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **ER** | Oestrogen receptor |
| **ESI** | Electrospray ionisation |
| **FAP** | Familial Adenomatous Polyposis |
| **FBS** | Fetal bovine serum |
| **FGly** | Formylglycine |
| **FOB** | Faecal occult blood |
| **FRET** | Fluorescence resonance energy transfer |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GC** | Gas chromatography |
| **GI** | Gastrointestinal |
| **GPER** | G protein-coupled oestrogen receptor |
| **HBRC** | Human Biomaterial Resource Centre |
| **HPNCC** | Hereditary non-polyposis colorectal cancer |
| **HRP** | Horseradish peroxidase |
| **HRT** | Hormone replacement therapy |
| **HSD17β** | 17 beta hydroxysteroid dehydrogenase |
| **IA** | Immunoassay |
| **IBD** | Inflammatory bowel disease |
| **IL-6** | Interleukin-6 |
| **IS** | Internal standard |
| **kDa** | Kilodalton |
| **KO** | Knock-out |
| **LB** | Lysogeny broth |
| **LC** | Liquid-chromatography |
| **LLE** | Liquid-liquid extraction |
| **LLOQ** | Lower limit of quantification |
| **m/z** | Mass to charge ratio |
| **MAPK** | Mitogen activated protein kinase |
| **MEM** | Modified Eagle Medium |

| | |
|---|---|
| **MRM** | Multiple reaction monitoring |
| **mRNA** | Messenger ribonucleic acid |
| **MS** | Mass Spectrometry |
| **MS/MS** | tandem mass spectrometry |
| **MSI** | Microsatellite instability |
| **MTBE** | Methyl tertiary butyl ether |
| **MUSCLE** | Multi-Objective mass Spectrometry Closed-Loop Experimentation |
| **NHS** | National Health Service |
| **NICE** | National Institute for Health and Care Excellence |
| **nNOS** | Neuronal nitric oxide synthase |
| **PAP** | 3′-phosphoadenosine-5′-phosphate |
| **PAPS** | 3′-phosphoadenosine-5′-phosphosulfate |
| **PAPSS** | 3’-phosphoadenosine 5’-phosphosulfate synthase |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerise chain reaction |
| **Ppi** | Pyrophosphate |
| **PTM** | Post-translational modification |
| **PVDF** | Polyvinylidene difluoride |
| **qRT-PCR** | Real time reverse transcription polymerise chain reaction |
| **RIA** | Radioimmunoassay |
| **RPLPO** | Ribosomal protein, large, P0 |
| **RT** | Retention Time |
| **SDS-PAGE** | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| **SERDs** | Selective oestrogen receptor downregulator |
| **SERMs** | Selective oestrogen receptor modulator |
| **SPE** | Solid phase extraction |
| **STS** | Steroid sulphatase |
| **SULT1E1** | Oestrogen sulphotransferase |
| **SUMF** | Sulphatase modifying factor |
| **T** | Testosterone |
| **TBST** | Tris buffered saline with tween |
| **TEMED** | Tetramethylethylenediamine |
| **TNFα** | Tumour necrosis factor alpha |
| **TNM** | Tumour Nodes Metastasis |
| **UC** | Ulcerative colitis |
| **uPLC** | Ultra performance liquid chromatography |
| **VO** | Vector only |
| **WHI** | Women’s Health Initiative |
| **WT** | Wild-type |
| **WB** | Western Blot |

# CHAPTER 1. GENERAL INTRODUCTION

## 1.1 COLON ANATOMY AND PHYSIOLOGY

The gastrointestinal (GI) system is a long tube open at both ends with the lumen of the GI tract an extension of the external environment. It is divided into specialised structures such as the oral cavity, stomach, small intestine, large intestine and anus. Together they enable food to enter, be broken down both mechanically and chemically, facilitate nutrient absorption and allow waste to be expelled, with the whole system influenced by various hormones (Kapit et al., 2000), such as ghrelin, leptin and motilin.

The focus here is the colon, the main component of the large intestine. The colon's primary function is to absorb water and electrolytes. Prior to entering the large bowel liquid chime enters the colon from the small intestine and as it progresses through to the rectum by peristalsis water is reabsorbed, it becomes solid and is expelled as waste. Additionally, bacteria in the colon ferment soluble fibre, help produce short chain fatty acids, regulate blood cholesterol and vitamin K (Kapit et al., 2000).

The colon is commonly divided into four sections; ascending, transverse, descending and sigmoid as shown in Figure 1.1. The ascending colon begins at the caecum ascending along the right hand side of the body where it meets the transverse colon. The transverse colon sits across the top of the abdomen leading to the descending colon situated on the left hand side which connects to the sigmoid; named due to its 'S' shape and which in turn becomes the rectum (Kapit et al., 2000, Kapoor, 2013).

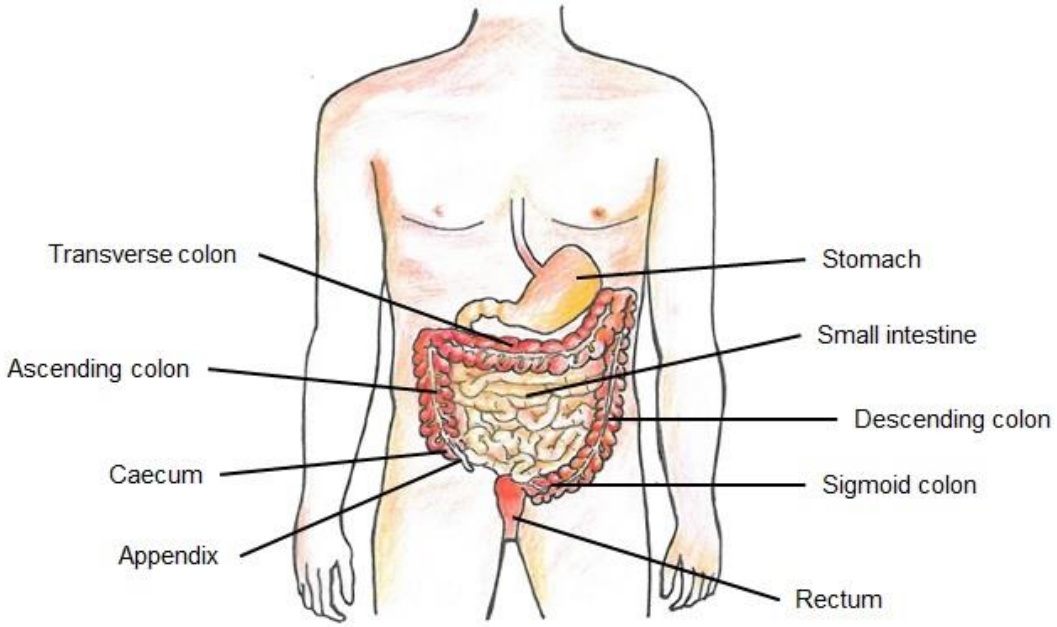



*Figure 1.1 Basic colon anatomy. The ascending colon is on the right hand side of the abdomen and begins at the caecum. It links with the transverse colon which crosses the top of the abdomen and joins to the descending colon on the left hand side. The descending colon then becomes the sigmoid colon and rectum. Drawing kindly provided by Christina Wingfield (Kapit and Elson, Taylor).*

The colon wall layers are shown in Figure 1.2 and are:

- The mucosa: the innermost layer consisting of a smooth columnar epithelial cell layer, mucus glands to lubricate the passage of faeces, (typically taking 1-3 days), connective tissue and a thin smooth muscle layer.
- The submucosa: contains blood vessels, nerves and connective tissue.
- The muscularis: a circular band of muscle and a longitudinal muscle layer important for peristalsis.
- The serosa: the outermost layer consists of connective tissue and squamous epithelial cells (Kapit and Elson, Taylor).

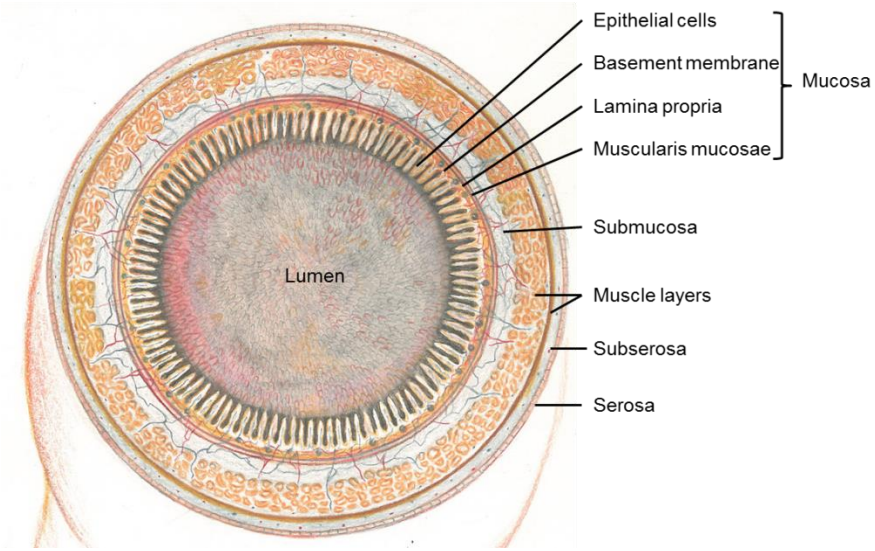



*Figure 1.2 Layers of the colon wall. The innermost layer is the mucosa followed by the submucosa which contains blood vessels, nerves and connective tissue. The muscle layers or muscularis consist of a circular band of muscle and a longitudinal muscle layer. The outermost layer is the serosa. Drawing kindly provided by Christina Wingfield (Kapit and Elson, Taylor).*

## 1.2 COLORECTAL CANCER

### 1.2.1 Incidence

Colorectal cancer (CRC) is the third most common cancer in both men and women in England, and is the second leading cause of cancer death in Europe (Office for National Statistics, 2016). Incidence has been increasing for both sexes and is more pronounced in men. However, a combination of earlier detection and new treatments has led to a steady fall in mortality (Figure 1.3). The year 2010 saw 58 and 38 new cases per 100,000 men and women, respectively. General population lifetime risk is estimated at 1 in 14 for men and 1 in 19 for women. As with many cancers, risk increases with age. In 2010 71% of new cases in men and 74% in women were in those 65 and over (Ferlay et al., 2013, Ait Ouakrim et al., 2015, Office for National Statistics, 2016).

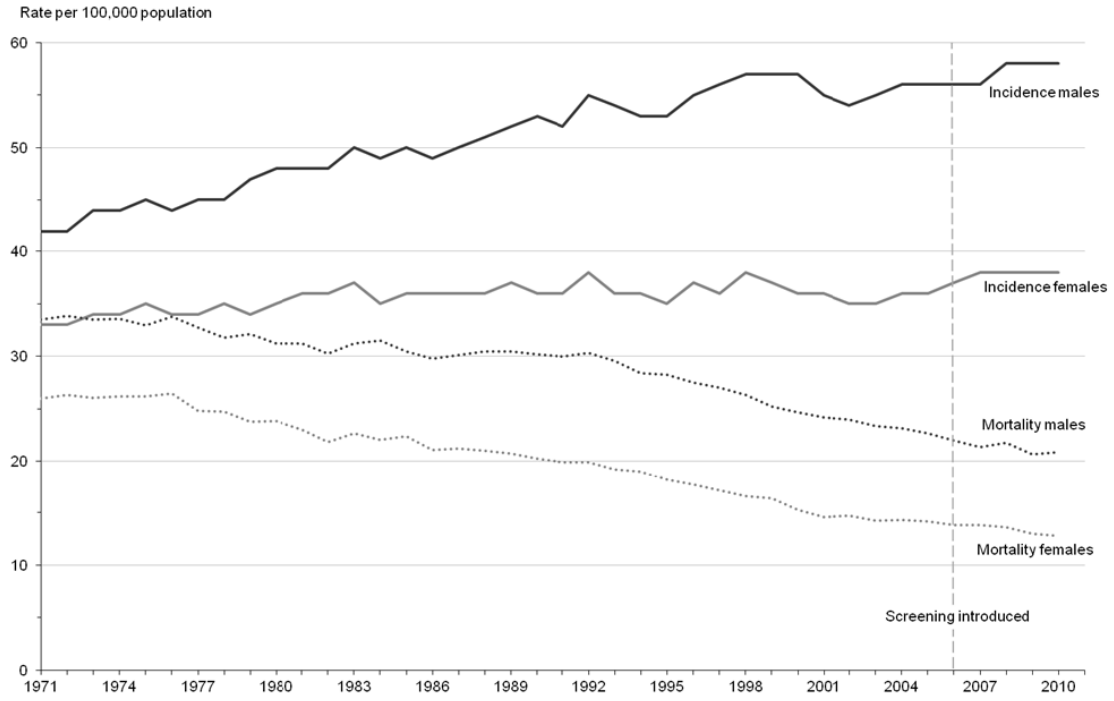



*Figure 1.3 Bowel cancer incidence and mortality rates, by sex, England, 1971−2010. Age-standardised rates per 100,000 population, standardised using the European Standard Population. Incidence has been increasing but mortality rates falling (Office for National Statistics, 2016).*

The majority (95%) of CRCs are adenocarcinomas arising from mucus producing epithelial cells. Other CRC types include, squamous cells, carcinoid and sarcomas (Cancer Research UK, 2015b). CRC is staged like most cancers using the TNM system; Tumour, Nodes and Metastases. This standardised method of staging informs of tumour size relating to layers of the bowel wall (Figure 1.2), whether it has spread to lymph nodes and metastasised. CRC can then be grouped into stages (Table 1.1) assisting with treatment decisions and predicting outcome (Cancer Research UK, 2015a). TNM is the most widely used system, particularly in clinical practice, however Dukes' classification, described by British pathologist Cuthbert Dukes in 1932 (Dukes, 1932), is sometimes used as an alternative (Table 1.2).

Table 1.1Stages of colorectal cancer using TNM method (Cancer Research UK, 2015a).

| Stage | Description |
|---|---|
| 0 | Cancer is confined to bowel inner lining |
| I | Cancer has infiltrated into muscle layer of bowel wall. T1/2, N0, M0 |
| IIa | Cancer has grown to the outer lining. T3, N0, M0 |
| IIb | Cancer has grown out of bowel wall and into surrounding structures. T4, N0, M0 |
| IIIa | Tumour is in the inner or muscle layer of the colon and has spread to local lymph nodes. T1/2, N1, M0 |
| IIIb | Tumour has grown through to the outer lining of the bowel wall, into surrounding structures and to local lymph nodes. T3/4, N1, M0 |
| IIIc | Any size of tumour with spread to more local lymph nodes. T, N2, M0 |
| IV | Spread to other parts of the body. T, N, M1 |

Table 1.2 TNM staging compared to Dukes' staging (National Institute for Health Care and Excellence, 2015).

| Stage | T | N | M | Dukes' Stage |
|---|---|---|---|---|
| 0 | Tin-situ | N0 | M0 | |
| I | T1 | N0 | M0 | A |
| | T2 | N0 | M0 | B1 |
| II | T3 | N0 | M0 | B2 |
| | T4 | N0 | M0 | B2 |
| III | T1 or T2 | N1 or N2 | M0 | C1 |
| | T3 or T4 | N1 or N2 | M0 | C2 |
| IV | Any T | Any N | MI | D |

In addition to stage, CRC is graded out of three, relating to tumour cell differentiation; one being low grade well differentiated cells and three poorly differentiated. The percentage of CRC diagnosed at each stage for men and women is shown in Table 1.3. Unfortunately, CRC is frequently diagnosed later and prognosis declines with delayed diagnosis.

Table 1.3 Percentage of colorectal cancer diagnosed at each disease stage and Five-Year Relative Survival (%) by Stage, Adults Aged 15-99, Former Anglia Cancer Network, 2002-2006. (Cancer Research, 2014)

| Stage at Diagnosis | Number of cases (%) | Five year survival (%) | |
|---|---|---|---|
| | | Men | Women |
| Stage I | 14.6 | 94.6 | 100.2 |
| Stage II | 22.2 | 83.5 | 85.9 |
| Stage III | 23.7 | 62.6 | 62.7 |
| Stage IV | 21.7 | 6.9 | 8.1 |
| Stage Not Known | 17.7 | 18.7 | 15.1 |
| All Stages | | 58.2 | 61.1 |

### 1.2.2 Signs and Symptoms

CRC does not usually cause symptoms in the early stages and so it is often diagnosed late (Table 1.3). Symptoms include a change in bowel habit, blood in faeces and abdominal pain. These are not CRC specific and often occur with benign conditions such as dietary changes and haemorrhoids. CRC is also associated with unintentional weight loss and, due to bleeding from the tumour, iron deficiency anaemia. NICE have age related guidelines for anyone presenting with these symptoms including when to undertake a faecal occult blood test (FOB) (National Institute for Health and Care Excellence, 2015b). The NHS began a screening programme in 2006 aimed at detecting early non-symptomatic CRC when survival is higher. Men and women aged 60-74 are offered FOB screening every two years and is performed by the patient in their own home making it inexpensive and convenient. If positive further more detailed investigations such as colonoscopy are undertaken. In the UK screening was only fully implemented from 2009 and so the long-term impact on diagnosis and survival is currently unknown. However, an early meta-analysis showed FOB screening reduced mortality by 16% (Towler et al., 1998). Also, a multi-centre randomised control trial showed a single flexi-test in 55-

64 year olds reduced risk of dying and developing CRC by 43% and 33%, respectively (Atkin et al., 2010). Additional earlier screening with a one-off flexible sigmoidoscopy at age 55 is expected to be fully implemented by December 2016 (Public Health England, 2015).

### 1.2.3 Aetiology

Non-modifiable CRC risk factors include advancing age, male gender, inflammatory bowel disease (IBD), family history and heritable syndromes. However, there are amendable lifestyle factors such as diet, obesity, physical activity, smoking and alcohol which can affect lifetime CRC risk. These are discussed in more detail below.

#### 1.2.3.1 Family History and Genetic Mutations

CRC is predominantly sporadic, but significant proportions, up to 35%, have a hereditary component. Approximately 5% of CRCs are due to dominantly inherited syndromes, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) (Short et al., 2015). A family history of CRC increases the risk of developing the disease, particularly if a first degree relative is diagnosed before the age of 50, reflecting complex interactions of inherited genetic susceptibility, shared behaviours and environment (Winawer et al., 1996).

FAP is a germline mutation in the adenomatous polyposis (APC) gene causing multiple benign colonic polyps, which unless removed inevitably leads to CRC by

40-50 years old (Bussey, 1987, InSiGHT, 2016). It is estimated that FAP is responsible for 1% of CRCs (Cruz-Correa and Giardiello, 2003). Currently patients are offered annual colonoscopies and due to improved survival advised to have a colectomy between ages 16-20 (The Association of Colproctology of Great Britain and Ireland, 2007, Heiskanen et al., 2000). However, patients are at continued risk of upper GI complications and so require follow-up (Vasen et al., 2008).

HNPCC arises from germline mutations in mismatch repair genes and accounts for 1-3% of CRCs. Patients are offered regular colonoscopies to screen for early colorectal disease. However, they also have increased risk of uterine, ovarian, stomach, pancreas, biliary and bladder cancer (Vasen et al., 2008, Jarvinen et al., 2000).

In 1990 Fearon and Vogelstein described CRC as an accumulation of genetic mutations. Activation of oncogenes and inactivation of tumour suppressor genes progresses normal mucosa to adenoma and then cancer in a stepwise fashion (Fearon and Vogelstein, 1990). This model, known as the chromosomal instability (CIN) or adenoma-carcinoma sequence is still used today. Acquisition of genomic instability is a key factor in CRC and three distinct pathways have been described; chromosomal instability (CIN, the most common), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) pathways (Pino and Chung, 2010). The predictable progression of sporadic CRC shares many genetic abnormalities with inherited conditions; 30- 70% of sporadic CRCs have inactivation of tumour suppressor APC. Typically there are inactivation of proto-oncogenes (e.g. Kras) and

inactivation of at least three tumour suppressor genes, e.g. APC, loss of p53 and loss of heterozygosity on the long arm of chromosome 18 (Armaghany et al., 2012) (Figure 1.4).

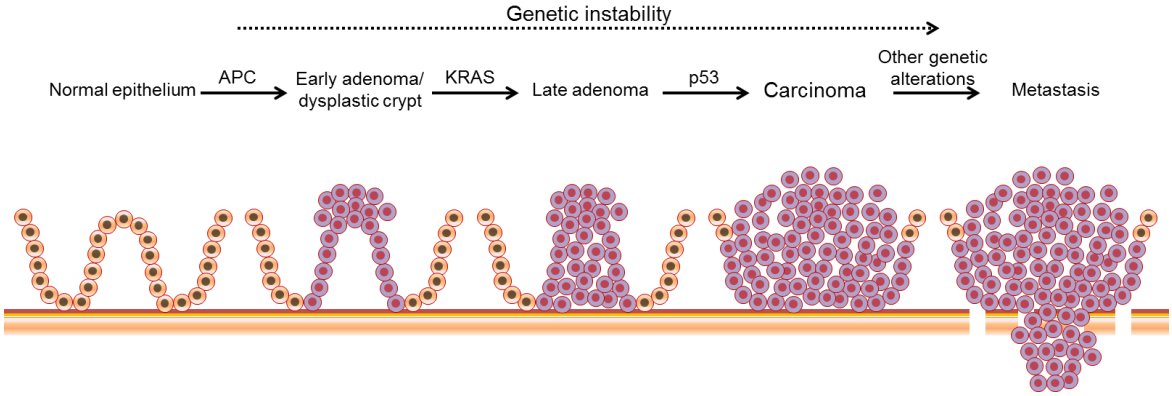



*Figure 1.4 Colon cancer chromosomal instability pathway with progression from normal epithelium to adenoma, carcinoma and metastasis by accumulation of genetic mutations. Adapted from Davies, R. J., et al. (Davies et al., 2005). This successive sequence has been estimated at approximately 15 years (Kozuka et al., 1975).*

### 1.2.3.2 Inflammatory Bowel Disease

Ulcerative colitis (UC) and Crohn's disease (CD) are inflammatory bowel diseases (IBD). They are relapsing and remitting disorders characterised by chronic inflammation within the GI tract. UC affects the intestinal mucosa of the large bowel in an uninterrupted pattern. Whereas CD is transmural and frequently discontinuous impacting on any part of the GI tract from mouth to anus (Baumgart and Sandborn, 2007).

IBD increases the risk of CRC, though the relative risk varies between studies (Rosenqvist et al., 1959, Macdougall, 1964, Farraye et al., 2010, Triantafillidis et al., 2009). Crohn and Rosenberg described IBD related CRC in 1925 (Crohn and

Rosenberg, 1925) with risk affected by disease severity, part of bowel affected and length of disease; CRC is rarely found within 7 years of colitis (Mattar et al., 2011). The current presumption is CRC risk is higher in IBD due to chronic inflammation leading to tissue damage and increasing cell turnover, multiplying the potential for DNA damage and DNA repair errors (Barral et al., 2015). NICE have guidelines for screening patients with IBD. Colonoscopy with chromoscopy is offered after 10 years since symptoms began. Based on the findings of this test, such as extensiveness of disease and amount of inflammation seen, patients are grouped into low, intermediate or high risk of developing CRC. This risk stratification guides when to offer the next colonoscopy with low risk at 5 years, intermediate 3 years and high risk after 1 year (National Institute for Health and Care Excellence, 2011b).

As discussed in section 1.2.3.1 and Figure 1.4 the majority of sporadic CRCs involve activation of oncogenes such as Kras and inactivation of tumour suppressor genes like APC and p53. Although these genes are still involved in IBD associated CRC, the timing differs. For example, in IBD associated CRC loss of APC occurs less frequently and often transpires later. Conversely, p53 mutations emerge earlier and are more common (Xie and Itzkowitz, 2008).

#### 1.2.3.3 Modifiable Factors

There are many lifestyle factors, particularly associated with Western lifestyle, which raise CRC risk. Some of which are discussed below.

Smoking and alcohol are associated with a multitude of diseases, including CRC (Zisman et al., 2006). Alcohol risk may in part be related to the frequently lower levels of folic acid found in heavy alcohol users (Giovannucci et al., 1995, Giovannucci et al., 1998). Folate is important for DNA methylation, synthesis and repair with low dietary intake associated with increased colorectal adenoma thus deficiency is thought to enhance carcinogenesis (Giovannucci et al., 1993). A recent meta-analysis study concluded folate supplementation had no effect on CRC risk (Qin et al., 2015). However, there is a difference between supplementation and deficiency. Supplementation may not reduce risk but that is not to say deficiency does not increase risk. Folate deficiency in alcohol users may not be comparable to other populations and could be increased due to other confounding variables.

Diets high in red and processed meats are also associated with CRC (Larsson and Wolk, 2006). In contrast vegetarian and pescovegetarian diets have been linked with a lower incidence of CRC (Gilsing et al., 2015, Orlich et al., 2015). Obesity, particularly visceral abdominal fat (Giorgino et al., 2005, Krotkiewski et al., 1983), physical inactivity (Koivisto et al., 1986, Regensteiner et al., 1991) and a diet high in sugar and low in fibre (Riccardi and Rivellese, 1991) increase the risk of hyperinsulinaemia and type II diabetes, which are also associated with CRC (Peeters et al., 2015, Kramer et al., 2012). Additionally, obesity is pro-inflammatory with lymphocytes and macrophages within adipose tissue secreting inflammatory cytokines such as tumour necrosis factor (TNF)α and IL-6 (Flores et al., 2015). Thus, obesity could be considered an inflammatory condition like IBD and in mice obesity induced TNFα contributes to CRC growth (Flores et al., 2012). Both obesity

and IBD are more prevalent in Western societies and associated with higher CRC risk (Burisch et al., 2014, Cordain et al., 2005, Molodecky et al., 2012). Typically IBD is associated with malnutrition and low body mass index (BMI). However, a US study found obesity was still an issue in almost a third of patients and a combination of obesity and IBD was associated with less severe disease rather than exacerbating symptoms (Flores et al., 2015). However, with active disease associated with low BMI and malnutrition, obesity likely reflects well controlled disease. The frequency of CRC between obese and non-obese IBD patients has yet to be assessed.

### 1.2.4 Treatment

Once diagnosed, CRC treatment will be guided by disease stage (Table 1.1), patient fitness and patient choice. Treatment involves many health care professionals such as surgeons, oncologists, gastroenterologists, specialist nurses, physiotherapists, pathologists, radiologists and pharmacists and so the treatment plan is a collective decision of a multidisciplinary team (MDT) (National Institute for Health and Care Excellence, 2014). In the UK treatment is guided by NICE which aims to improve outcomes of NHS users by producing evidence based guidelines and developing quality standards. The treatment described below is taken from NICE guidelines last updated in 2011: Colorectal cancer: the diagnosis and management of colorectal cancer (National Institute for Health and Care Excellence, 2011a).

#### 1.2.4.1 Surgery

Surgery is the mainstay of CRC treatment. It can be potentially curative in early disease and in advanced disease can palliate symptoms, such as stenting an obstructed bowel. Surgical intervention usually involves removing the part of the bowel affected with clear margins of normal tissue, local lymph node removal and total mesenteric excision. Occasionally isolated metastasis, such as in a liver lobe, can be surgically removed and possibly lead to 'cure' (National Institute for Health and Care Excellence, 2011a).

Surgery type depends on tumour location, but commonly the bowel is removed either through a laparotomy incision or performed laparoscopically. Left, transverse, right or sigmoid colectomies relate to the different anatomical segments of the bowel. Depending on length removed the two remaining ends may be joined together in an anastomosis or alternatively a stoma opening in the abdominal wall which can sometimes be reversed at a later date. Typically, a left sided stoma comes from the colon (colostomy) and the right side from the ileum (ileostomy). Each stoma has specific management needs, e.g. electrolyte and water loss resulting in dehydration is more common with an ileostomy.

#### 1.2.4.2 Chemotherapy, Radiotherapy and Biological Therapy

Chemotherapy and radiotherapy can be given before or after surgery to increase chance of cure, prolong life or palliate symptoms. Radiotherapy is less common for CRC but is used for rectal cancer. Common chemotherapeutic agents are Flourouracil (5-FU), Capecitabine (prodrug, metabolised to 5-FU once ingested),

Oxaliplatin, Irinotecan and Raltitrexed (usually only given if 5-FU and folic acid contraindicated) with typical combinations of:

- FOLFOX (folinic acid, fluorouracil and oxaliplatin) followed by irinotecan.
- FOLFOX followed by FOLFIRI (folinic acid, fluorouracil, irinotecan).
- XELOX (capecitabine, oxaliplatin) followed by FOLFIRI.

Biological therapies include monoclonal antibodies Cetuximab and Panitumumab (anti-endothelial growth-factor receptor), Bevacizumab (targeting vascular endothelial growth-factor (VEGF)), Ramicurimab (VEGF receptor 2) and recombinant fusion protein Aflibercept (targeting VEGF). These are sometimes used in combination with chemotherapy (National Institute for Health and Care Excellence, 2011a).

### 1.2.5 Resistance and Recurrence

There have been steady improvements in CRC outcome as shown in Figure 1.5. However, some patients are unresponsive to therapy or have disease recurrence. Why this happens is unclear and denotes that these patients receive treatment which is of little or no benefit.

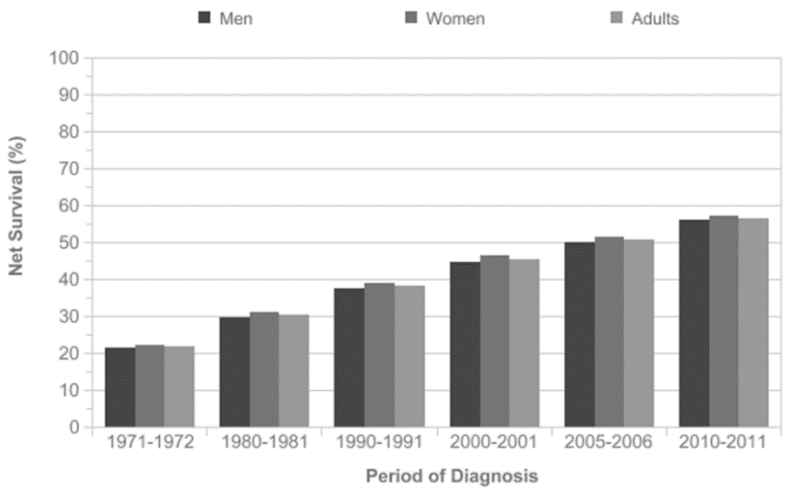



*Figure 1.5 Bowel cancer age-standardised ten-year net survival, England and Wales(Cancer Research UK, 2014)*

Therapy failure should be uncovered by clinical monitoring and radiological evidence of tumour recurrence or progression. This leads to treatment adjustments thus limiting unnecessary side effects. Along with discovering new treatments, predicting who will benefit most from current therapeutics remains an important CRC management aim to limit unnecessary therapy, side effects and cost. Current predictors include clinicopathological staging (section 1.2.1), which is a good marker for overall prognosis. Presently several protein and genetic markers help refine treatment, monitor response to therapy and inform on prognosis e.g. BRAF mutations, carcinoembryonic antigen (CEA), microsatellite instability (MSI) and KRAS (Gangadhar and Schilsky, 2010, National Cancer Institute, 2015, Bork et al., 2015, Phipps et al., 2013, My Cancer Genome, 2015). A new method has been recently described by an international consortium assessing 3443 patients from across the world. This method terms four consensus molecular subtypes (CMSs); microsatellite instability immune, canonical Wnt signaling metabolic dysregulation

and mesenchymal activation, with 87% of patients assigned to one of these groups (Guinney et al., 2015). It has been well received with hope it can more accurately predict treatment response, outcome and guide drug discovery (Wise, 2015). However, clinical trials are still needed to validate this classification method and more is still needed to help those who do not respond to current therapeutics.

## 1.3 STEROID HORMONES AND THE COLON

Steroid hormones are derived from cholesterol (Figure 1.6) with the majority synthesised in the adrenals and gonads. Although the colon is not traditionally viewed as a hormonal tissue there are over 50 hormones, genes and numerous bioactive peptides that work together to regulate secretion, absorption, digestion, motility and mucosal growth, making the gut the largest endocrine organ (Rao JN, 2010). Steroidogenic enzymes and active steroids have been detected in the intestines. Thus, the gut is a source of glucocorticoids; important for bowel homeostasis and immunoregulation (Sidler et al., 2011, Cima et al., 2004), and sex steroid oestrogen production (Sato et al., 2009); having roles both in normal GI physiology and carcinogenesis.

### 1.3.1 Oestrogens and Colorectal Cancer

Oestrogen has complex influences on the bowel with varying effects on GI physiology. For example oestradiol ($E_2$) increased contractility and GI motility in animal models (Bruce and Beshudi, 1981) and shortened the synthesis phase of the cell cycle in crypt epithelium in mice (Galand et al., 1967). Also, ovariectomy

can lead to colonic crypt atrophy in mice (Hoff et al., 1981) and healthy human females have GI motility varying with their menstrual cycle, with oestrogen and progesterone contributing to constipation (Bernstein et al., 2014, Oh et al., 2013).

In addition to normal physiology in women oestrogen has been shown to influence CRC risk. The associations between oestrogens and CRC have been suggested for a number of decades. In 1969 Fraumeni et al. noted that nuns had an increased risk of both breast and CRC compared to the general population (Fraumeni et al., 1969). Nuns are a unique population group as they are nulliparous and so have uninterrupted menstrual cycles and so a higher lifetime exposure to oestrogen. The New York University Women's Health study found that women with the highest quartile range of circulating oestrone ($E_1$) concentrations had the greatest CRC risk (Clendenen et al., 2009). A large study with a cohort of 214,162 post-menopausal women examined associations between lifetime endogenous oestrogen and CRC. It was shown that reproductive factors such as parity, age of menarche and menopause more commonly associated with breast cancer also correlated with CRC risk. Therefore, lifetime oestrogen exposure is an important risk factor for developing CRC (Zervoudakis et al., 2011).

The Women's Health Initiative (WHI) launched in 1991 was a major research program consisting of clinical trials and an observational study involving 161,808 post-menopausal women. It was designed to test the effect of Hormone Replacement Therapy (HRT, commonly conjugated oestrogens and progesterone), diet, calcium and vitamin D on conditions such as cardiovascular disease, fractures,

breast cancer and CRC. Their findings showed HRT reduced CRC risk by 40% and the combined oral contraceptive pill (OCP) reduced CRC risk by 20% (Rossouw et al., 2002, Rossouw et al., 2013, Roehm, 2015). Studies have shown differences between HRT formulations (oestrogen-progestin versus oestrogen only therapy) and protection against CRC. These findings have not been consistent as to which formulation may offer the most protection against CRC, but none appear to have found any increase in CRC risk. The reason for these inconsistencies with HRT formulations is unclear but may relate to duration of treatment within the study (Hildebrand et al., 2009, Johnson et al., 2009, Dinger et al., 2007). However, the WHI trial did note women diagnosed with CRC whilst using oestrogen plus progestin therapy had higher tumour grades, suggesting HRT had a possible role in CRC progression (Chlebowski et al., 2004). HRT may initially offer a protection against CRC, but once established promote tumour proliferation. Alternatively, HRT side effects may mask/simulate CRC symptoms delaying presentation and diagnosis. However, the finding of advanced CRC with HRT treatment in the WHI trial has not been replicated and could instead be related to study design (Prentice et al., 2009).

A higher lifetime exposure to endogenous circulating oestrogen as altered by reproductive factors appears to increase CRC risk in women. Alternatively, direct exposure to exogenous oestrogens, such as HRT, OCP or through diet may interact with and potentially alter the local colonic environment and offer, at least initially, a protective influence in women. Thus, in women there are differences in effects of endogenous and exogenous oestrogen on the gut.

Men have a lower lifetime exposure to oestrogen compared to women with the main source from adipose tissue and skin. However, men have a higher risk of CRC. Younger women with higher circulating oestrogen concentrations also have a more favourable prognosis than younger men (Hendifar et al., 2009), indicating a complex relationship between endogenous oestrogen and CRC risk. In addition, men have higher circulating androgens than women, which may also impact on the differences in CRC risk and survival between genders.

Obesity is a growing problem that affects both men and women. Adipose tissue contains aromatase, an enzyme that catalyses the synthesis of oestrogens from circulating androgens. Adipose tissue also expresses the necessary oxidoreductive enzymes (discussed in section 1.7) to alter androgen and oestrogen potency (Corbould et al., 1998). Thus, adipose is a source of oestrogen with aromatase efficiency and expression increasing with advancing age (Bulun and Simpson, 1994, Hemsell et al., 1974, Cleland et al., 1985, Siiteri, 1987). In premenopausal women the ovaries are the principal source of oestrogens (Kapit et al., 2000) however in men and post-menopausal women oestrogens are primarily produced via aromatase action in adipose and skin tissue (Hemsell et al., 1974, Cleland et al., 1985). Obesity, with increased adipose tissue mass as well as age enhances oestrogen biosynthesis. Therefore, it may be oestrogenic effects combined with associated diet and physical activity factors (section 1.2.3.3) that contribute to the risk of CRC in obesity.

## 1.4 OESTROGEN SYNTHESIS AND METABOLISM

The aromatase and sulphatase pathways produce active oestrogens from circulating precursor steroids (Figure 1.6). Within the colon aromatase activity is low (English et al., 1999, Foster, 2013, Sato et al., 2009) therefore it is thought that oestrogen is primarily produced through steroid sulphatase (STS) action.

Oestrogen sulphates are biologically inactive as they have no significant affinity to oestrogen receptors (ER) (Zhu et al., 2006). STS converts circulating sulphated oestrogens ($E_1S$ and $E_2S$) to active oestrogens such as $E_1$ and $E_2$. The reverse reaction is catalysed by oestrogen sulphotransferase (SULT1E1).

$E_1S$ is the most abundant oestrogen precursor in the circulation of men, non-pregnant women and postmenopausal women (2-4 nmol/l in males, 2-5 nmol/l in premenopausal females and 0.5-2 pmol/l in postmenopausal females (Mueller et al., 2015)). Bound to albumin and with a half-life of up to nine hours, $E_1S$ is considered a reservoir for biologically active oestrogens $E_1$ and $E_2$ (Reed et al., 2005, Ruder et al., 1972). Once desulphated, $E_1$ can be further reduced by 17β-hydroxysteroid dehydrogenase enzymes (HSD17β) to $E_2$, which has the highest binding affinity for ERs (Zhu et al., 2006).

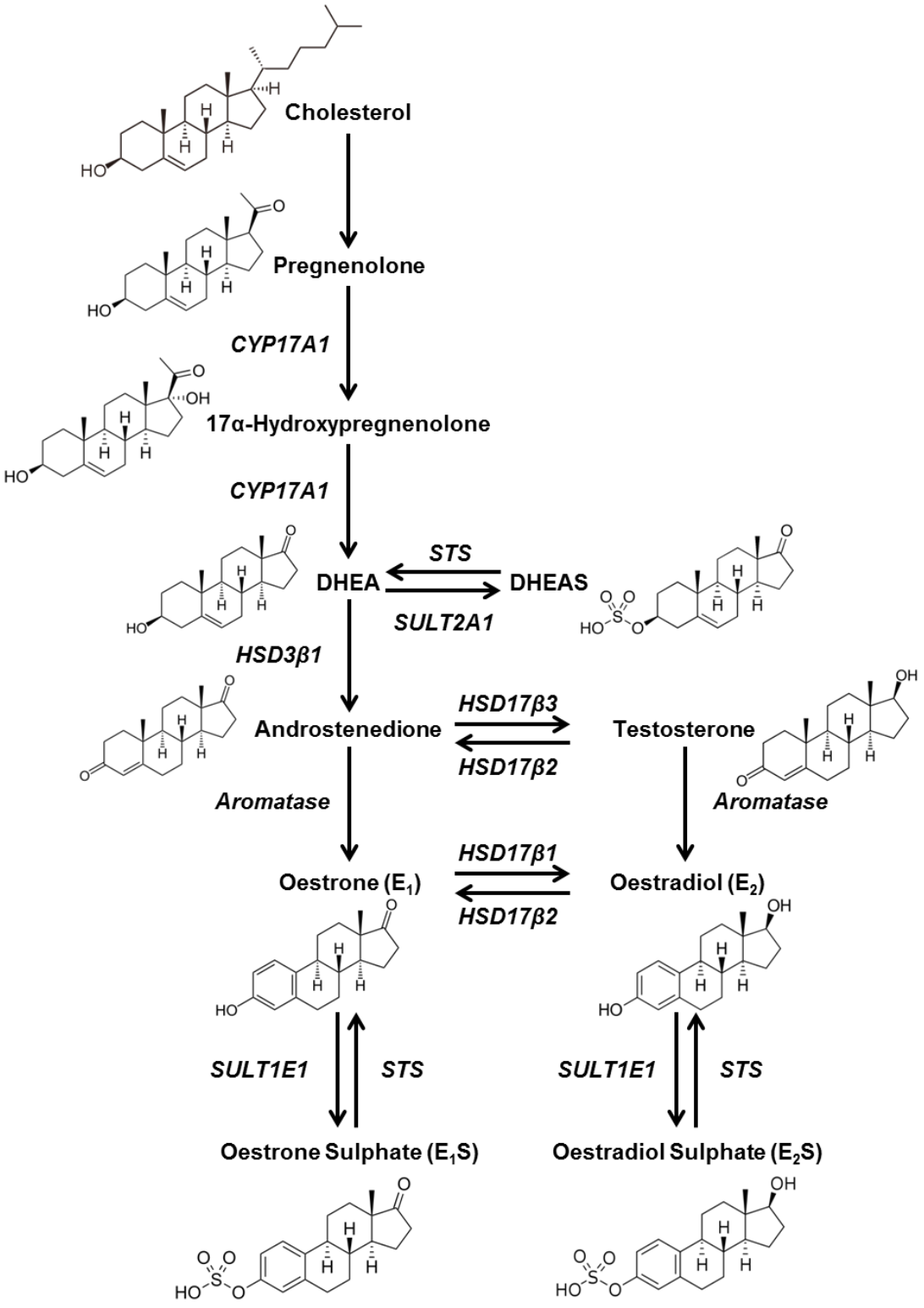



*Figure 1.6 Simplified diagram of oestrogen metabolism showing active oestrogens $E_1$ and $E_2$ can be generated through either the aromatase or steroid sulphatase (STS) enzyme pathways. Oestrogen sulphotransferase (SULT1E1); 17β Hydroxysteroid dehydrogenase type 1, 2 and 3 (HSD17β1, 2, 3 respectively).*

In relation to CRC, oestrogen enzymes STS, SULT1E1 and HSD17βs and oestrogen receptors will now be discussed in more detail.

## 1.5 STS

STS, also known as arylsulphatase C, is a member of the sulphatase family, catalysing the hydrolysis of sulphate ester bonds from a wide range of substrates. STS is the principal enzyme for desulphating steroids from their inactive to active forms and so activity can impact on local concentrations of $E_2$, dehydroepiandrosterone (DHEA) and testosterone (T) (Secky et al., 2013, Purohit et al., 2011). STS is a membrane-bound microsomal enzyme, mainly localised in the rough endoplasmic reticulum (Sato et al., 2009, Ghosh, 2007) and is virtually ubiquitously expressed in small quantities in human tissues, with placenta and liver having the greatest activity (Nardi et al., 2009, Dalla Valle et al., 2007, Reed et al., 2005, Warren and French, 1965).

The STS gene is located on the short arm of chromosome X and mapped in Xp22.3-Xpter (Yen et al., 1988, Reed et al., 2005, Suzuki et al., 2011, Nardi et al., 2009). STS is synthesised as a membrane-associated precursor with 63 kDa mass and asparagine-linked oligosaccharide chains. Endoglucosaminidase H cleaves these chains resulting in a final size of 61 kDa with a four day half-life (Conary et al., 1986). X-ray crystallography has shown STS to be a monomer with a “mushroom-like” shape; two hydrophobic anti-parallel alpha helices protruding from a spherical molecule. The hydrophobic tail is implanted in the luminal membrane of the endoplasmic reticulum and beside it is a long narrow pocket with the enzyme

reaction site at the base. To be desulphated substrates and products have to travel through the endoplasmic reticulum membrane (Hernandez-Guzman et al., 2003, Nussbaumer and Billich, 2004, Thomas and Potter, 2013).

### 1.5.1 STS Regulation

There is limited data on the molecular regulation of STS transcription. The STS gene spans over 200 kbp with the regulatory region in the first 100 kbp and coding region downstream (Nardi et al., 2009). Measuring STS activity is an important part of assessing peripheral oestrogen metabolism as STS can undergo various post-translational modifications. It holds four potential N-glycosylation sites however digestion by endoglycosidase H showed only two (Asn47 and Asn259) are used (von Figura et al., 1998, Stein et al., 1989). All four of N-linked sites are glycosylated to some extent however only mutations in these two glycosylation sites decreased activity (Stengel et al., 2008). Since glycosylation alters STS function there does not necessarily need to be a change in mRNA or protein expression to alter activity.

Another STS post translational modification is cysteine (C)75 to form formylglycine (FGly) at the active site. This is facilitated by coenzyme FGly-generating enzyme (FGE) which is encoded by the gene sulphatase-modifying factor 1 (SUMF1). Further hydration forms gem-diol hydroxylformylglycine (Thomas and Potter, 2013, Schmidt et al., 1995). Like STS, FGE is also glycosylated and found in the endoplasmic reticulum. There is some evidence that FGE is secreted by cells, acting in a paracrine manner to influence STS activity in neighbouring cells (Preusser-Kunze et al., 2005, Zito et al., 2007). Additionally, it is suggested that a paralog of

SUMF1, SUMF2, lacks the catalytic domain of SUMF1 but can dimerise with SUMF1 inhibiting its action and reducing STS activity (Zito et al., 2005). This adds another factor in STS regulation but further research is needed to clarify SUMF2's role.

### 1.5.2 STS Regulation in Disease

STS mRNA and activity have been shown to be regulated by products of inflammation such as NFκB and TNFα. These components are released with metabolic inflammation for example high fat diets, overfeeding and obesity (Hotamisligil et al., 1995, Hotamisligil et al., 1993, Shoelson et al., 2003). Liver STS expression was induced in mouse models of metabolic disease such as ob/ob mice, high fat diet and type 2 diabetes models. In these instances STS overexpression appeared beneficial with improved body weight and insulin sensitivity, decreased hepatic steatosis and inflammation. In female mice this was thought to be through active oestrogen generation as these effects were lost with ovariectomy and in males by reduced inflammation in white adipose tissue and skeletal muscle (Jiang et al., 2014).

In chronic liver disease STS was induced by inflammatory activated NFκB, increasing circulating oestrogens which may contribute to this disease associated male feminisation. Also, there appears to be a negative feedback loop with STS activated oestrogens attenuating NFκB mediated inflammation (Jiang et al., 2016). Human and animal data is also supported by cell line studies. Comparable effects have been demonstrated in human MG-63 preosteoblasts with NFκB inducing STS

expression (Dias and Selcer, 2016). In addition to metabolic disease, STS was induced by IGF-II via a PI3-kinase/Akt-NFκB pathway in prostate cancer PC-3 cells (Sung et al., 2013). Whereas, the prostate cancer cell line LNCaP had increased STS activity in response to IL-6 and TNFα without alteration in STS mRNA levels (Suh et al., 2011). Similar effects have also been shown in the breast cancer cell line MCF7 (Newman et al., 2000). Together these data suggests STS is regulated as part of the inflammatory response in multiple tissue types.

STS activity augmentation by cytokines IL-6 and TNFα without mRNA changes suggests post-translational modifications are involved (Honma et al., 2002, Reed et al., 2005, Newman et al., 2000, Suzuki et al., 2011, Purohit et al., 1996, Purohit et al., 1997). Another possibility is these cytokines alter membrane permeability, increasing substrate accessibility and thus there is more product available to desulphate rather than a true increase in STS activity (Purohit et al., 2002). However, neither posttranslational modification nor membrane permeability alterations in these conditions have been confirmed.

In addition to inflammation STS expression appears be affected by oestrogens. For example in MCF7s STS transcription was up regulated by $E_2$ via direct binding to oestrogen receptors (ER) and activation of oestrogen response elements (ERE) in the promoter regions (Zaichuk et al., 2007, Secky et al., 2013). Additionally, treatment with anti-oestrogen ICI182780 reduced both basal and $E_2$ stimulated expression of STS mRNA. Oestrogens also induced ERα degradation in an auto regulatory feedback loop which was prevented by pretreatment with proteosomal

inhibitor MG132. STS mRNA increased after exposure to $E_2$ and MG132, whereas MG132 alone reduced STS mRNA (Zaichuk et al., 2007, Fan et al., 2004). As STS provides the mechanism, through desulphation, for active oestrogens it is logical that it would be involved in feedback mechanisms to regulate oestrogen concentrations within tissues.

Sex steroids have a role in immune function and inflammatory processes (Cutolo et al., 2002, Bouman et al., 2005). Epidemiological and immunological evidence implies that female sex hormones influence chronic inflammatory disease (Ngo et al., 2014). Inflammatory cells, such as macrophages and T cells have been shown to express ERα and ERβ (Straub, 2007). Furthermore, STS is frequently dysregulated in cancer and a hallmark of cancer is inflammation (Hanahan and Weinberg, 2000). Therefore, it is unsurprising that STS is also regulated by inflammatory mediators. However, this has yet to be examined in the colon and is explored in more detail in Chapter 7.

### 1.5.3 STS in Colorectal Cancer

#### 1.5.3.1 Oestrogens

Sato and colleagues showed CRC tumours had higher oestrogen concentrations compared to normal colonic mucosa with higher total oestrogen and $E_1$ concentrations associated with reduced survival. Importantly, oestrogen concentrations were concordant with STS and SULT1E1 expression rather than aromatase (which was expressed at low levels). Additionally, STS and SULT1E1 ratios correlated with prognosis; tumours negative for STS and positive for

SULT1E1 had an improved outlook, whereas those positive for STS and negative for SULT1E1 were associated with adverse clinical outcome (Sato et al., 2009). As discussed in section 1.5.1 STS can undergo post-translational modification and these correlations were using mRNA and protein and did not include any direct measurement of enzyme activity. Also demonstrating oestrogen pathway changes in CRC, English and colleagues noted HSD17β2 was frequently reduced with no alteration in aromatase (English et al., 1999). Increased $E_1$ from the sulphatase pathway together with a fall in HSD17β2 would procure $E_2$, the most active oestrogen. Therefore, oestrogens generated through the sulphatase pathway may contribute to CRC progression and poor survival. The findings from these two groups show the consequences of local oestrogen metabolism and the sulphatase pathway in CRC. As these enzymes can be targeted by hormonal therapy they offer potentially new CRC treatments.

It is unknown how HRT (usually sulphated oestrogens and progesterone) influences the progression from adenoma to carcinoma and metastasis. As discussed in section 1.3.1 combination HRT reduces CRC risk by 40%, but women diagnosed with CRC while using HRT had higher tumor grades (Rossouw et al., 2002). Evidence suggests higher STS expression is a frequent finding in CRC although further study would be needed to confirm this. For example STS, SULT1E1 expression and activity in tumours of HRT users versus non-users has not yet been examined.

#### 1.5.3.2 Androgens

Androgens may act similarly to oestrogens in CRC with joint protective and cancer promoting effects. STS can desulphate DHEAS, a source of androgens as well as oestrogens. However, with minimal aromatase activity in the colon (English et al., 2001) any DHEAS desulphation would be almost exclusively for androgen production. The colon does express cytosolic and nuclear androgen receptors (AR) (Marugo et al., 1992, Stebbings et al., 1988) and functional membrane AR have also been identified in colon tumors (Gu et al., 2009). The effect of androgens in CRC is unclear with some studies showing apoptotic and others anti-apoptotic effects. *In vitro* testosterone induced apoptosis, whereas DHEA enhanced survival in serum-deprived Caco-2 CRC cells (Anagnostopoulou et al., 2013). However, *in vivo* testosterone has been found to accelerate cell proliferation in the small intestine and induced colon cancer in rats, with CRC growth reduced following castration (Tutton and Barkla, 1982). A more recent study supports these findings with castration protecting against colonic adenomagenesis (development of adenomas), reversed by testosterone supplementation in rats and mice (Amos-Landgraf et al., 2014). However, there are no definitive studies on whether androgens influence CRC progression.

Human studies exploring the effect of androgen treatment on the colon have been inconsistent. Higher serum DHEAS concentrations in men are associated with slightly lower CRC risk (Alberg et al., 2000). However, a large study of 107,859 prostate cancer patients assessed CRC incidence and androgen deprivation therapy (Gillessen et al., 2010). CRC risk rose with increasing androgen deprivation

therapy and duration; highest incidence of CRC with orchidectomy, followed by GnRH agonist therapy and no androgen deprivation therapy (Lin and Giovannucci, 2010).

## 1.6 SULT1E1

SULT1E1 is an enzyme that sulphates oestrogens and androgens rendering them biologically inactive. Conjugation to sulphate or glucuronide groups at carbon position three of the four-carbon ring steroid backbone increases hydrophilic properties and facilitates circulatory distribution and excretion. Importantly, these sex steroids can then become active again through enzymatic deconjugation, such as desulphation by STS. Sulphated oestrogens have a longer half-life than the unconjugated forms; the half-life of $E_1S$ is 10-12 hours and unconjugated active oestrogens 20-30 minutes (Ruder et al., 1972). Therefore, circulating inactive steroids act as reservoirs for active oestrogen production, enabling local steroid metabolism by peripheral tissues (Reed et al., 2005, McNamara et al., 2013).

Figure 1.7 shows how oestrogens can be inactivated by the addition of sulphate from universal sulphuryl group donor 3'-phosphoadenosine-5'-phosphosulphate (PAPS). In brief, PAPS synthase (PAPSS) is bifunctional with an NH2-terminal APS kinase domain and a COOH-terminal ATP sulphuryl domain. These act in two steps with the sequential action of ATP sulphurylase and APS kinase to form PAPS (Franzon et al., 1999, Venkatachalam, 2003, Harjes et al., 2005). There are two human protein isoforms of the PAPSS enzymes; PAPSS1 and PAPSS2 (77% homology) and they do not complement each other (van den Boom et al., 2012,

Franzon et al., 1999). In brain and skin PAPSS1 dominates and in liver, cartilage and the adrenal glands PAPSS2 is more highly expressed.

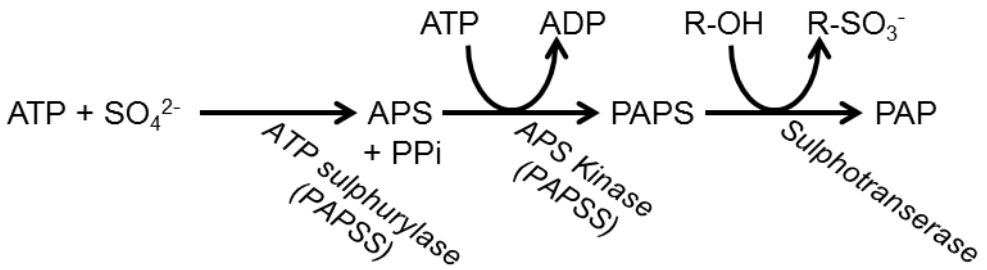



*Figure 1.7 Diagram depicting the two step process of 3'-phospoadenosine 5'-phosphosulphate (PAPS) synthesis and the subsequent usage of PAPS as a co-substrate for sulphotransferase reactions. PAPS synthase (PAPSS) catalyses two sequential reactions to form the universal sulphate donor, 3'-phospoadenosine 5'-phosphate (PAP). First the adenosine triphosphate (ATP) sulphyurylase domain combines sulphate ($SO_4^{2-}$) with ATP to form adenosine 5-phophosulphate (APS) and pyrophosphate (Ppi). Secondly, APS combines with ATP, catalysed by the APS kinase domain creating PAPS. Sulphotranserases, such as SULT1E1 acting on oestrone, can then catalyse the transfer of the sulphate to create oestrone-sulphate and PAP.*

### 1.6.1 SULT1E1 Structure and Location

Sulphotransferases fall into two main groups; cytosolic or membrane bound in the Golgi apparatus. SULT1E1 is a cytosolic family member with gene located on chromosome 4. It is the principal enzyme for oestrogens, catalysing the sulphoconjugation of $E_1$ and $E_2$ at the 3-hydroxyl terminus with great efficiency (Coughtrie, 2002, Cole et al., 2010, Meinl and Glatt, 2001, Her et al., 1995). SULT1A1 and SULT1A3 are also capable of sulphating oestrogens, but have much lower affinities. These are 92.5% identical to each other and approximately 50% identical to SULT1E1 (Thomas and Potter, 2013).

Human SULT1E1 contains 294 amino acids and forms a dimer of 35 kDa with two $E_1$ or $E_2$ bound per subunit (Zhang et al., 1998, Pasqualini, 2009). The crystal structure with cofactor (PAPS) complex has been reported (Pedersen et al., 2002) and together the substrate and PAPS binding site form an open tube through the core of the SULT1E1 protein. Residues of the substrate binding site are largely hydrophobic and surround the substrate closely positioning the hydroxyl group so it can act as acceptor in the sulphate transfer (Thomas and Potter, 2013).

SULT1E1 is expressed in the liver and small intestine and is the dominant sulphotransferase in lung tissue. However, expression is absent in the kidney (Riches et al., 2009). SULT1E1's role may be particularly important during fetal development (Duanmu et al., 2006) as ablation in mice caused placental thrombosis and fetal loss (Tong et al., 2005).

### 1.6.2 SULT1E1 Regulation

Sulphotransferase genes are part of phase two metabolism and so transcriptional regulation is complex. Glucocorticoids have been shown to effect oestrogen function by glucocorticoid receptor-mediated transcriptional up-regulation of SULT1E1 and inactivating oestrogens (Gong et al., 2008). SULTs are also subjected to substrate negative feedback. Hence endocrine disruptors instead of activating oestrogen receptors (ER) can instead bind to SULT1E1.In the presence of STS, inactivating SULT1E1 could lead to increased oestrogen activation (Wang and James, 2006, Kester et al., 2002).

For sulphotransferase reactions, the sulphate molecule is donated from the universal sulphate donor, PAPS (Figure 1.7). As PAPS is exclusively produced by PAPSS1 and PAPSS2, supply can be considered a rate limiting step. Thus, sulphation is at least in part regulated by PAPSS availability (van den Boom et al., 2012). Additionally, PAP, the by-product of sulphation inhibits SULT1E1 and therefore assists through a negative feedback loop in the regulation of sulphation (Gulcan and Duffel, 2011, Zhang et al., 1998).

### 1.6.3 SULT1E1 in Colorectal Cancer

SULT1E1 and STS expression have been linked to prognosis in CRC with high SULT1E1 expression associated with a more favourable prognosis (Sato et al., 2009). However, there is little further examination of SULT1E1 in the colon. In more traditional hormonal cancers, such as breast and endometrial cancer, common single nucleotide polymorphisms (SNPs) in SULT1E1 have been shown to affect disease free survival and frequently increase risk. This demonstrates the importance of SULT1E1 in tumourigenesis (Choi et al., 2005, Hirata et al., 2008).

PAPSS is important for SULT1E1 action however there have been no studies on PAPSS activity and expression in colon cancer. However, overexpression of SULT1E1 and PAPSS1 in MCF7 cells, leading to increased oestrogen inactivation through sulphation, resulted in growth inhibition by arresting cell cycle and inducing apoptosis. Paradoxically, PAPSS1 and 2 expression in breast cancer tissue have been found to be increased. This may be an initial adaptive response to protect cells from oestrogenic mitogenicity by sulphation inactivation (Xu et al., 2012).

## 1.7 17βHYDROXYSTEROID DEHYDROGENASE

17βHydroxysteroid dehydrogenases (HSD17β) are responsible for the final step in formation of active androgens and oestrogens and have a key role in sex steroid biology. There are multiple isoenzymes with variable tissue expression aiding oestrogen and androgen intracrine biosysnthesis (Labrie et al., 1997). Nicotinamide adenine dinucleotide (NAD(H)) or, its phosphate form, (NADP(H)) are required for activity. Reductive actions are important for active sex steroid production, whereas oxidation decreases hormone activity (Torn et al., 2003). To date 15 HSD17β enzymes have been recorded with type 6 and 9 not identified in humans. All bar type 5, (an aldo-keto reductase), are short-chain dehydrogenases/reductases. HSD17βs are all similar in size and have highly conserved motifs e.g. Rossmann fold. Despite this, homology is actually low with variable cellular localisation (Duax et al., 2000, Duax et al., 2005, Lukacik et al., 2006, Day et al., 2008b). Kinetic studies have shown these enzymes to be multifunctional, but *in vivo* the majority appear to have substrate and directional preferences. HSD17βs implicated in oxidising $E_2$ to $E_1$ or reducing $E_1$ to $E_2$ in the colon are discussed below.

### 1.7.1 HSD17β Oxidation of Oestradiol to Oestrone

Oestrogen oxidative activity decreases ER stimulation through reduced affinity and is the primary reaction in the normal colon (English et al., 1999). This reaction is predominantly via HSD17β2 however additionally HSD17β4 and 8 have been considered as *in vitro* they have demonstrated oestrogen oxidative capability (Day et al., 2008a, Luu-The et al., 2006, Aka et al., 2009). Currently in the normal colon only expression of HSD17β2 and 4 have been identified (English et al., 2000).

#### 1.7.1.1 HSD17β type 2

The HSD17β2 gene is located on chromosome 16q24 and is 387 amino acids. The protein sequence shares approximately 20% sequence identity with HSD17β1, the primary reductive enzyme (Labrie et al., 1997). HSD17β2 is microsomal, located on colonic epithelium luminal surface and is able to metabolise orally administered steroids such as HRT and contraceptives (Puranen et al., 1999).

In 1999 English and colleagues quantified the expression of HSD17β2 and 4 in human CRC tissues, with expression reduced compared to normal tissue. They predicted this would result in higher local $E_2$ concentrations (English et al., 1999), although they did not confirm this (such as through LC-MS/MS analysis) and sample size was small including just 10 males and 14 females. It was postulated that an increase in the $E_1/E_2$ ratio in CRC may be important for CRC progression; $E_1$ being protective and $E_2$ detrimental. However, another study with a slightly larger sample size of 35 females and 39 males found reduced HSD17β2 expression in CRC, with presumed increase in $E_2$ synthesis, was associated with improved survival in females (Oduwole et al., 2003). As both $E_1$ and $E_2$ possess binding affinity to oestrogen receptors these data are conflicting as to which oestrogen is the more protective. However, neither study took into account enzyme activity or other oestrogen metabolising enzymes such as STS and SULT1E1. In CRC it is likely more complex than one type of oestrogen being “good” and another being “bad”. The response may instead reflect the oestrogen receptor status of the tumour (section 1.8). Additionally, other potential factors to consider are gender, age, pre and post-menopausal, genetics and length of oestrogen exposure.

#### 1.7.1.2 HSD17β type 4

HSD17β4 is peroxisomal and expressed in virtually all tissues including intestine (Labrie et al., 1997). It is a 736 amino acid protein with an N-terminal dehydrogenase domain and a larger C-terminal domain containing hydratase and lipid carrier moieties (Breitling et al., 2001). *In vitro* it has $E_2$ oxidoreductase activity (Adamski et al., 1995), conversely *in vivo* it is instead involved in peroxisomal fatty acid β-oxidation (Breitling et al., 2001, Qin et al., 1997) with mutations causing an autosomal-recessive disorder of peroxisomal fatty acid β-oxidation called D-bifunctional protein (DBP) deficiency (Pierce et al., 2010, McMillan et al., 2012). Expression is highest in the liver, heart, prostate and testis with up-regulation demonstrated in prostate cancer (Zha et al., 2005). In CRC oxidative oestrogen activity has been shown to be reduced with down-regulated HSD17β2 and 4 mRNA and protein expression (English et al., 1999). However, the activity of each of these enzymes on oestrogen oxidation was not separately determined. In breast tissue HSD17β2 and 4 were both expressed, but only HSD17β2 had an effect on oestrogen oxidation. Thus, *in vivo* HSD17β4 may only have a minor role in steroid metabolism (Miettinen et al., 1999).

#### 1.7.1.3 HSD17β type 14

HSD17β14 is a cytosolic enzyme and has so far been identified by Northern blot in the brain, liver and placenta. It has been shown to oxidise $E_2$, testosterone and 5-androstenediol (Lukacik et al., 2007). Breast cancer patients receiving tamoxifen treatment and whose tumours expressed HSD17β14 had a more favourable outcome (Sivik et al., 2012), possibly by increased oxidation of $E_2$ to $E_1$. Additionally,

*in vitro* transfection of HSD17β14 in breast cancer cell line MCF7 lead to reduced $E_2$ concentrations (Jansson et al., 2006). Therefore, HSD17β14 may also be important in oestrogen metabolism. However, it is unlikely to play a role in CRC as no or low expression was seen by Northern blot in the small intestine, colon, pancreas, spleen and gonads (Lukacik et al., 2007).

### 1.7.2 HSD17β Reduction of Oestrone to Oestradiol

The uptake and desulphation of circulating $E_1S$, followed by reduction of $E_1$ would result in the most potent oestrogen, $E_2$. If oestrogens were implicated in CRC proliferation and disease progression then limiting $E_1$ to $E_2$ conversion could potentially benefit patients. In humans reduction of $E_1$ to $E_2$ is primarily via HSD17β1, the best characterised out of the HSD17βs. However, in the colon despite HSD17β1 and three transcripts being detected by RT-PCR they were not confirmed by Northern analysis (English et al., 1999, English et al., 2000, English et al., 2001). Thus, any reductive reactions must be through an alternative enzyme.

#### 1.7.2.1 HSD17β type 7

HSD17β7 is a 334 amino acid long membrane associated reductive enzyme and approximately 37 kDa. In rodents it was reported to catalyse $E_1$ to $E_2$ and is also a prolactin receptor-associated protein (Nokelainen et al., 1998, Krazeisen et al., 1999). However, sequence and promoter analysis suggested its main role in cholesterol biosynthesis, reducing zymosterone to zymosterol (Marijanovic et al., 2003, Ohnesorg and Adamski, 2006). This was supported by HSD17β7 KO mice; they are fertile but do not produce viable foetuses due to severe brain

malformations, likely due to the reliability of the brain on *in situ* cholesterol biosynthesis (Shehu et al., 2008). However, HSD17β7 is overexpressed in breast carcinoma (Oncomine datasets (Rhodes et al., 2004)) and inhibition of this enzyme in MCF7 cells resulted in lower $E_2$ and higher DHT concentrations (measured by immunoassay) attenuating proliferation. HSD17β7 inhibition in MCF7 xenograft mouse studies resulted in tumour shrinkage, reduced HSD17β7 expression, decreased plasma $E_2$ and elevated DHT (Wang et al., 2015). Thus, this suggests instead of, or in addition to cholesterol metabolism, HSD17β7 has a role balancing $E_2$ and DHT sex steroids. There are currently no published studies on HSD17β7 in CRC.

#### 1.7.2.2 HSD17β type 12

HSD17β12 is an endoplasmic reticulum protein with high homology to HSD17β3. *In vitro* studies have shown it efficiently reduces $E_1$ to $E_2$ (Luu-The et al., 2006, Blanchard and Luu-The, 2007), however others have suggested that HSD17β12 is not very efficient at this reaction in breast cancer (Day et al., 2008b). A study of 41 matched breast cancer tissues found HSD17β12 was expressed in 83% of breast cancers and expression was higher in cancerous tissue (Song et al., 2006). However, *in vitro* knockdown of HSD17β12 in T47D breast cancer cells by siRNA did not impact on $E_1$ to $E_2$ metabolism and *in vivo* the same group found HSD17β1 was the better endocrine target in breast cancer (Day et al., 2008a). Thus, the role of HSD17β12, at least in breast cancer, is unclear (Aka et al., 2009). Expression has been identified in many lipid metabolising tissues such as liver, kidney, heart and skeletal muscle, but also in endocrine-related organs such as pancreas,

pituitary, adrenal, testis and placenta and GI tract. Therefore, HSD17β12 may be involved in both lipid biosynthesis regulation and steroid metabolism (Sakurai et al., 2006).

### 1.7.3 HSD17β Inhibitors

Oestrogen metabolism can be inhibited through its receptors and enzymes STS and aromatase, with much success in breast cancer. The advantage of inhibiting HSD17βs is that expression patterns vary between tissue types and between normal and cancerous cells. This potentially allows for more tumour direct and/or tissue specific inhibitors to modify steroid pathways and thus minimise unwanted side effects. The current focus is on HSD17β1 inhibitors for breast cancer and endometriosis and HSD17β3 inhibitors for prostate cancer (Day et al., 2008b). However, HSD17β inhibitors are still under development with none yet reaching clinical trials.

## 1.8 OESTROGEN RECEPTORS

Currently three main oestrogen receptors have been identified; oestrogen receptor alpha (ERα/ ESR1), oestrogen receptor beta (ERβ/ ESR2) and G protein-coupled oestrogen receptor (GPER/GPER-30).

### 1.8.1 Oestrogen Receptor Alpha and Beta

ERα, located on chromosome 6q25 was identified in 1966. ERβ was then later discovered in 1996 and is found on chromosome 14q23-24.1. The most potent

oestrogen, $E_2$, has a similar binding affinity for both of these receptors (Kennelly et al., 2008). ERs are part of the nuclear hormone receptor family and conventionally function as ligand-regulated transcription factors (Edwards, 2005). Whereas nuclear ERs mediate the typical genomic action of oestrogen, membrane ERs have rapid, non-genomic actions (Li et al., 2015).

Mouse ERα KO are infertile due to behavioural and physiological changes (Lubahn et al., 1993, Ogawa et al., 1998, Prossnitz and Hathaway, 2015). Additionally multiple organ systems are affected such as impaired bone growth (Vidal et al., 1999, Vidal et al., 2000), loss of cardiovascular protection (Hodgin et al., 2001) and increased adipose tissue associated with glucose intolerance and insulin resistance (Heine et al., 2000). ERβ KO mice are also infertile however in females this is due to ovulation defects and in males for unknown reasons (Antal et al., 2008).

#### 1.8.1.1 ERα and ERβ in Colorectal Cancer

In the colon these two ERs appear to have opposing functions; ERα is pro-mitogenic (Xu and Thomas, 1994, Di Domenico et al., 1996) and ERβ is pro-apoptotic (Qiu et al., 2002, Arai et al., 2000). In normal colon ERβ predominates and is fundamental to colonic cell homeostasis (Fang et al., 2012, Fang et al., 2010). ERα colonic expression is controversial with studies finding either low (Cavallini et al., 2002) or no (Witte et al., 2001) expression in the colon and CRC. However, meta-analysis has found that ERβ is decreased in CRC suggesting it is a tumour-suppressor gene protecting against CRC when activated by oestrogens (Niv, 2015).

ERβ KO mice develop colonic hyperproliferation, loss of differentiation and disordered apoptosis (Kennelly et al., 2008) with a fall in ERβ attenuating apoptosis. Recent research also proposes a role for micro RNA (miRNA) in CRC progression. $E_2$ acting on ERβ has been shown to down-regulate miR-135b with loss of ERβ increasing miR-135b expression, which may increase DNA mismatch repair activity promoting carcinogenesis (He et al., 2012).

Selective oestrogen receptor downregulators (SERDs), such as Fulvestrant and selective oestrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, are used with great success in treatment for breast cancer. SERM action varies in different tissues e.g. acting as ER antagonists in breast tissue, but agonists in bone, protecting against osteoporosis (Howell et al., 2004, National Institute for Health and Care Excellence, 2015a, National Institute for Health and Care Excellence, 2011c). Effects in the colon are however not well documented. HCT116 CRC cells treated with raloxifene and an ERβ gene constructed into an adenoviral vector had reduced proliferation both *in vitro* and *in vivo* as xenografts in mice (Tu et al., 2012). However, a treatment recreating the protective effects of ERβ in the CRC by upregulating expression would be challenging. A large population study of 85,411 women showed taking tamoxifen for five years for breast cancer led to a moderate increased risk of CRC (Newcomb et al., 1999). CRC develops slowly over years (Kozuka et al., 1975, Fearon and Vogelstein, 1990) and so the effects of SERMs on the healthy colon, adenoma and carcinoma with loss of ERβ may differ and change over time. However, as discussed below, the ultimate effects of SERMs

and SERDs in the colon are further complicated by the fact they also function as GPER agonists (Prossnitz and Barton, 2014).

### 1.8.2 G Protein-Coupled Oestrogen Receptor

GPER shares structural homology with angiotensin II receptors and other chemotactic peptide receptors. Originally it was presumed the ligand for GPER was a peptide; however now it has been shown that GPER is a Gs-coupled receptor that promotes oestrogen dependent activation of adenylyl cyclase (Filardo, 2002, Feng and Gregor, 1997). Ligands include $E_2$, tamoxifen (Vivacqua et al., 2012, Vivacqua et al., 2006b), genistein (Vivacqua et al., 2006a) and aldosterone (Rigiracciolo et al., 2016).

The functional location of GPER is unclear. Immunohistochemical analysis on breast cancer cells has detected GPER predominately intracellularly (Filardo et al., 2006). Contrastingly, GPER has characteristics of a plasma membrane ER and has been shown to have a short half-life of less than 30 minutes on the plasma membrane (Cheng et al., 2011). Tissues such as uterine epithelia (Gao et al., 2011) and myometrium (Maiti et al., 2011) have shown GPER as a plasma membrane receptor (Filardo and Thomas, 2012).

GPER maps close to the genetic locus defining familial hyperaldosteronism II on short arm chromosome 7, 7p22.3 (Carmeci et al., 1997, Lafferty et al., 2000). It has a molecular mass of approximately 44 kDa and lesser amounts near 60 and 80 kDa. The identity of these larger masses are unclear, however they are eliminated after

removal of N-linked glycan chains with glycosidases and the 44 kDa band shifts to 38 kDa, which is the estimated molecular weight of the unmodified core protein (Filardo and Thomas, 2012, Filardo et al., 2007). This receptor can promote modest mitogenic responses as well as homeostatic responses. 17β-oestradiol and the GPER selective agonist G1 are linked with proliferative responses in cancer cells such as breast (Santolla et al., 2015), endometrium (Vivacqua et al., 2006b), ovary (Albanito et al., 2007), testis (Chevalier et al., 2011, Franco et al., 2011) and thyroid (Vivacqua et al., 2006a). Contrastingly some studies have found GPER activation can inhibit proliferation such as in prostate cancer cell lines PC-3, DU145 and LNCaP *in vitro* and PC-3 xenografts *in vivo* (Chan et al., 2010). Despite GPER showing greatest effects in reproductive tissue, KO mice do not have gross reproductive anomalies and instead females have several metabolic deficiencies including impaired glucose tolerance and reduced bone growth (Martensson et al., 2009, Prossnitz and Hathaway, 2015).

#### 1.8.2.1 GPER in the Colon

GPER expression has been demonstrated in colon myenteric neurons. In functional studies intact mice treated with G15 (GPER antagonist) had shortened colon transit in proestrus and oestrus. Oestrogen treated ovariectomised mice had higher colon transit time than vehicle, but this was inhibited by G15. Within the myenteric plexus GPER stimulation with G1 led to nitric oxide production and GPER co-localised with neuronal nitric oxide synthase (nNOS) (Li et al., 2015). A supporting study found rats treated with oestrogen had increased relaxation responses to electric stimulation in isolated colon tissues along with increased nNOS expression in the

myenteric plexus (Shah et al., 2001). GPER, through nNOS in colonic myenteric neurons may contribute to reduced colon transit time and constipation.

There is little documented about GPER in CRC, however, in more traditional hormonal cancers, like breast, endometrial and ovarian cancer GPER has been linked to disease progression (Smith et al., 2009, Girgert et al., 2014, Skrzypczak et al., 2013, Liu et al., 2014). In breast cancer GPER correlates with tumour size and metastasis (Filardo et al., 2006) with an inverse relationship between expression and disease free survival in patients treated with tamoxifen (Ignatov et al., 2011). Tamoxifen is a GPER agonist and so GPER may contribute to drug resistance. *In vitro* studies on GPER-expressing breast cancer cell lines have shown downstream GPER activation includes MAPK, PI3K/Akt and related survival/ proliferation pathways stimulating cell growth (Filardo et al., 2002, Filardo et al., 2000, Pandey et al., 2009).

GPER has been identified in mouse colon myenteric neurons (Li et al., 2015), but lacks the characterisation of ERα and ERβ in CRC (section 1.8.1.1) such as expression and effect on proliferation.

## 1.9 HYPOTHESIS AND AIMS

There is compelling evidence that CRC is hormonally responsive with tumour oestrogen metabolism frequently altered and impacting on survival. Reproductive factors such as parity, age of menarche and menopause affect CRC risk with higher endogenous lifetime oestrogen exposure increasing likelihood (Zervoudakis et al.,

2011, Fraumeni et al., 1969, Clendenen et al., 2009). Tumours with higher oestrogen content, increased STS and reduced SULT1E1 enzyme expression are associated with a poorer outcome (Sato et al., 2009). However, the oestrogen metabolism pathway has yet to be fully elucidated in CRC or targeted therapeutically in CRC. Oestrogen metabolism involves several enzymes e.g. STS, SULT1E1, HSD17β1, 2, 7 and 12, PAPSS1 and 2. Previously their role in CRC has been examined individually with no information currently on the complexity of their interactions. This may explain some of the conflicting results on oestrogen and CRC risk reported in the literature. Additionally, which oestrogen receptors are important in the colon remains controversial.

Utilising available research tools such as real time PCR, Western Blotting, cell culture, activity assays and LC-MS/MS this thesis examines oestrogen metabolism in human colon tissue, human colorectal cancer tissue, CRC cell lines and xenograft mouse models. This novel and multi-directional approach aims to identify therapeutically targetable oestrogenic changes in CRC. The hypotheses are:

- Active $E_2$ synthesis is promoted in CRC through increased STS activity and changes to HSD17β enzyme ratios.
- $E_2$ enhances tumour proliferation through GPER action.
- $E_2$ synthesis and GPER can be targeted therapeutically to reduce tumour proliferation and extend survival.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 CELL CULTURE

Cell lines (Table 2.1) were grown in 75 $cm^2$ tissue culture flasks with vented cap (T75, Corning Ltd, UK) in media supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Poole, UK) and 1% penicillin-streptomycin (10,000 units/ 100 ml, Invitrogen, Paisley, UK) at 37$^oC$ with 5% $CO_2$. To reflect that the majority of CRCs are adenocarcinomas, only CRC cell lines originating from adenocarcinomas were chosen and included HCT116 and HT29 cells grown in McCoys 5A (modified) Medium with L-Glutamine (Life Technologies, Grand Island, NY, USA), Caco-2 cells cultured in Minimum Essential Medium (MEM) plus Earle's Salts and 5 mM L-Glutamine (Life Technologies, Grand Island, NY, USA) and Colo205 in RPMI-1640 medium with 5 mM L-glutamine and $NaHCO_3$ (Sigma-Aldrich, Poole, UK). Cells used for positive controls included breast cancer cell lines MCF7 and MDAMB231 grown in RPMI-1640 medium and placental choriocarcinoma cell line JEG3 cultured in DMF-12 media (Life Technologies, Grand Island, NY, USA). All cells were adherent except Colo205 cells which grew in part suspension.

MCF7 and HCT116 cells were kindly gifted from Professor C. McCabe and Caco-2 cells from Dr C. Tselepis (The University of Birmingham). All other cell lines were obtained from ATCC. To ensure high quality data all gifted cells were verified through DNA Diagnostics Centre with cell markers checked against recommended online cell line databases (DNA Diagnostics Centre, 2015).

Table 2.1 Summary of ATCC cell line basic characteristics (ATCC, 2016).

| Cell Line | Type | Gender | Description |
|---|---|---|---|
| HCT116 (CCL-247) | Colorectal carcinoma | Male | Adult<br>No previous treatment |
| Colo205 (CCL-222) | Colorectal adenocarcinoma | Male | 70 years old<br>Dukes' stage D<br>From metastatic site: ascites<br>Caucasian<br>Previously treated with 5-FU |
| Caco-2 (HTB-37) | Colorectal adenocarcinoma | Male | 72 years old<br>Caucasian |
| HT29 (HTB-38) | Colorectal adenocarcinoma | Female | 44 years old<br>Caucasian<br>p53 mutation |
| MCF7 (HTB-22) | Breast adenocarcinoma | Female | 69 years old<br>Caucasian<br>From metastatic site: pleural effusion<br>ER+ |
| MDA-MB-231 (HTB-26) | Breast adenocarcinoma | Female | 51 years old<br>From metastatic site: pleural effusion<br>Caucasian |
| Jeg 3 (HTB-36) | Placenta choriocarcinoma | Female | One of six from the Woods strain of the Erwin-Turner tumor model by Kohler and associates |

Cells were reviewed three times per week for media change or if 80% confluent split into new T75 flasks with new passage number noted. Passage numbers used were 50-70 for HCT116 cells, 5-20 for Colo205 cells, 20-40 for Caco-2 cells, 60-90 for HT29 cells, 5-20 for MCF7 cells, 5-20 for MDA-MB-231 cells and 8-20 for Jeg3 cells. To split cells medium was removed and cells washed twice with phosphate buffered saline (PBS, Oxoid, Hampshire, England). To trypsinise 1-3ml of Tryple express (Invitrogen, Paisley, UK) was applied and cells incubated at 37$^{o}$C for 2-5 minutes.

Trypsin was deactivated by FBS containing media and cells gently mixed into suspension. For seeding 10 µl of cell suspension was pipetted into a haemocytometer (Immune Systems Ltd) and cells counted at 100 x magnification. The average of three counts was used to calculate number of cells per µl.

## 2.2 PROTEIN EXTRACTION

### 2.2.1 Cells in Culture

Cells in suspension were pelleted by centrifugation at 450 rcf for 5 minutes. Medium was removed and cells washed twice with PBS. Radioimmunoprecipitation assay buffer (RIPA Buffer, Sigma-Aldrich, Poole, UK) with protease inhibitor cocktail (1:100, Sigma-Aldrich, Poole, UK, 1ml RIPA Buffer per 0.5-5 x $10^7$ cells) was used to lyse the cells and protein lysate stored at -80$^o$C in a microcentrifuge tube.

### 2.2.2 Plated Cells

For plated cells media was removed and cells gently washed twice with PBS. As Colo205 cells grow part in suspension the PBS wash was collected and centrifuged to pellet cells as per section 2.2.1. For 6 well plates 250 µl of RIPA Buffer with protease inhibitor cocktail was applied to lyse cells and 500 µl for T25 flasks. Plates or flasks were frozen at -20$^o$C, thawed at room temperature and scraped to collect protein lysate. Lysate was stored in a microcentrifuge tube at -80$^o$C.

Protein lysate was clarified by centrifugation at 13, 000 rpm at 4$^{o}$C for 10 minutes. Supernatant was then transferred avoiding cell debris pellet to a clean microcentrifuge tube.

### 2.2.3 Tissue

Human colon tissue (≤ 30 mg frozen in liquid nitrogen) was weighed and placed on dry ice. RIPA buffer (≤ 1 ml) with protease inhibitor cocktail and phosphatase inhibitors (Thermo Scientific, UK (both 1:100 dilution)) were used to homogenise tissue.

Different methods of homogenisation were tested to balance time, cost and yield. However, due to limited tissue availability it was not possible to do a direct comparison between the methods:

1. Homogenised in RIPA using a TissueRuptor device for approximately 40 seconds.
2. Crushing tissue with a micro pestle (Sigma-Aldrich, Poole, UK) in RIPA until homogenised, freezing at -80$^{o}$C for 30 minutes and repeating the crushing process
3. Utilising a Qiagen Tissuelyser II on loan (Qiagen, Manchester, UK). This involved a steel bead inserted into the microcentrifuge tube with the tissue and RIPA which was then shaken until homogenised.

Micopestle method was mainly employed due to low costs and similar protein yields to other trialled methods. Once homogenised the soluble fraction was separated by centrifuging at 13,000 rpm for 10 minutes at 4$^{o}$C. The supernatant was then

transferred, avoiding the pelleted cell debris, to a new microcentrifuge tube and stored at -80ºC. To reduce freeze-thaw mediated degradation supernatant was aliquoted and stored at -80ºC.

### 2.2.4 Protein Quantification

A bicinchoninic acid (BCA) assay (Bio Rad, Hertfordshire, UK) was used as per manufacturer's instructions to quantify concentration of protein harvested from tissue or cells. In brief, 4 µl of sample (in duplicate) or pre-prepared standard (0, 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 mg/ml) was pipetted into a 96 well plate. Solution B was added to Solution A in a 1:50 dilution and 80µl of combined reagents added to each sample. The plate was incubated at 37ºC for 30 minutes. BCA assay utilises the reduction of $Cu^{2+}$ to $Cu^{+}$ by proteins in alkaline solution. Chelation of copper with protein in alkaline solution containing sodium potassium tartrate forms a light-blue complex. BCA reacts with the reduced cuprous cation forming a water soluble BCA/copper complex and turning the solution purple. This colourimetric change can be measured at 560 nm on a Wallac Victor$^3$ 1420 multilabel counter and protein concentration calculated from the standard curve.

## 2.3 RNA EXTRACTION

RNA was extracted from cells using RNeasy® kit (Qiagen, Manchester, UK) as per manufacturer's instructions (see 2.3.3 for details).

### 2.3.1 Cells in Culture

Cells in suspension from T75 culture flasks (section 2.1) were pelleted by centrifugation at 300 rcf for 5 minutes and media removed. RLT Buffer (10 µl β-ME (Sigma-Aldrich, Poole, UK)/ 1 ml RLT buffer) was added to the cell pellet and mixed by pipetting. The lysate was then homogenised by passing it through a blunt 21 gauge needle with RNase free-syringe several times and RNA extraction continued as described in section 2.3.3.

### 2.3.2 Tissue

Human colon tissue (≤ 30 mg frozen in liquid nitrogen) was weighed and placed on dry ice. Homogenisation techniques were as described in section 2.2.3 using RLT Buffer. Once homogenised lysate was stored at -80$^{o}$C, or RNA extraction was completed as described below.

### 2.3.3 RNA Extraction

70% ethanol was added to homogenised lysate and mixed by pipetting. This mix was transferred to an RNeasy spin column with collection tube and centrifuged > 8000 rpm for 15 seconds with flow through discarded. Note on-column DNase treatment was not performed. RW1 Buffer was added with further 15 seconds centrifugation at > 8000 rpm with flow-through discarded. Twice RPE buffer was added followed by centrifugation at > 8000 rpm for 15 seconds and 2 minutes with flow-through discarded. Ethanol carry over was reduced by a further 1 minute centrifugation at full speed. To elute RNA the column was placed in a new collection tube with 40 µl RNase free water and centrifuged at > 8000 rpm for 1 minute. RNA

yield and purity was calculated in ng/µl using a NanoDrop Spectrometer (NanoDrop Products, Wilmington, DE, USA). Purity was quantified using absorbance at 260 (nucleic acids) and 280 nm (proteins). A 260/280 nm ratio of around 2.0 is accepted for RNA. Additionally 260/230 nm gives information of other contaminants, with a good result being 2.0-2.2. RNA was stored at -80$^{o}$C until reverse transcribed which was typically one week.

## 2.4 REVERSE TRANSCRIPTION

To synthesise cDNA from cell lines a Tetro cDNA Synthesis Kit (Bioline Reagents Ltd, London, UK) was used as per manufacturer's instructions. Up to 5 µg of RNA (extracted as described in section 2.3) was added to a premix containing 4 µl 5x RT Buffer, 1 µl Ribosafe Rnase Inhibitor, 1 µl Tetro Reverse Transcriptase (200u/µl), 1 µl 10 nM dNTP mix, 1 µl Primer Oligo $(dT)_{18}$ and totalled to 20 µl with DEPC-treated water. This was mixed by pipetting and incubated at 45$^{o}$C for 30 minutes with reaction terminated by 5 minute incubation at 85$^{o}$C before being stored at -20$^{o}$C.

Maximal cDNA yield was generated from human RNA samples using a more sensitive kit, SensiFAST™ (Bioline Reagents Ltd, London, UK). Up to 1 µg of RNA was added to 4 µl of 5 x TransAmp Buffer, 1 µl Reverse Transcriptase and totalled to 20 µl of Dnase/Rnase free water. This mix was run on an Eppendorf Mastercycler Gradient PCR Thermal Cycler using the program 25°C for 10 min (primer annealing), 42°C for 15 min (reverse transcription), 85°C for 5 min (inactivation) and 4°C hold (or chill on ice).

## 2.5 PICOGREEN ASSAY

Concentration of cDNA was determined using PicoGreen assay (Invitrogen, Paisley, UK) as per manufacturer's instructions. In brief 1 µl of cDNA was added (in duplicate) to 99 µl 1x TE Buffer and 100 µl 1x PicoGreen and compared to a standard curve as analysed on Wallac Victor$^3$ 1420 multilabel counter, (excitation ~480 nm, emission ~520 nm). PicoGreen is a fluorescent asymmetric cyanine dye that on binding to double stranded DNA (dsDNA) forms a luminescent complex exhibiting a > 1000 fold increase in fluorescence compared to free dye in solution. The amount of fluorescence is proportional to the concentration of dsDNA and so cDNA.

## 2.6 QUANTITATIVE POLYMERASE CHAIN REACTION

Relative concentrations of specific mRNA were determined using quantitative real-time PCR. Pre-optimised specific TaqMan™ (Applied Biosystems, Poole, UK) gene expression assays were used and are listed in Table 2.2. Genes were validated in duplex as shown in Appendix II.

Bound to the 5' end of the oligonucleotide probe is a fluorescent reporter dye (FAM or VIC) and at the 3' end a quencher (TAMRA). Intact, the proximity of the quencher reduces fluorescence emitted from the reporter by fluorescence resonance energy transfer (FRET). If the target sequence is present, the probe anneals and is cleaved during extension by 5' nuclease activity of Applied Biosystems hot-start DNA polymerase. Subsequently the quencher and reporter dye are separated and the emitted fluorescent signal by the reporter dye increases (Figure 2.1). As the PCR

reaction cycles, the reporter dye signal intensifies and is detected in real time with fluorescence proportional to the amount of amplicon produced; the higher the copy number, the sooner fluorescence will appear.

Table 2.2 Table of TaqMan® gene expression assay used.

| Gene | Code |
|---|---|
| **RPLPO** | 4310879E |
| **PP1A** | Hs04194521_s1 |
| **HPRT1** | 4310890E |
| **HSD17β1** | Hs00166219_g1 |
| **HSD17β2** | HS00157993_m1 |
| **HSD17β7** | Hs00996127_m1 |
| **HSD17β12** | Hs00275054_m1 |
| **STS** | Hs00165853_m1 |
| **SULT1E1** | HS00193690_m1 |
| **PAPSS1** | Hs00193745_m1 |
| **PAPSS2** | Hs00190682_m1 |
| **GPER** | Hs00173506_m1 |
| **ERβ** | HS00230957_m1 |
| **ERα** | HS00174860_m1 |
| **TNFα** | Hs01113624_g1 |
| **gapdh** | 4352932E |
| **il-6** | Mm00446190_m1 |
| **sts** | Mm04214605_u1 |
| **hsd17β2** | Mm00500430_m1 |

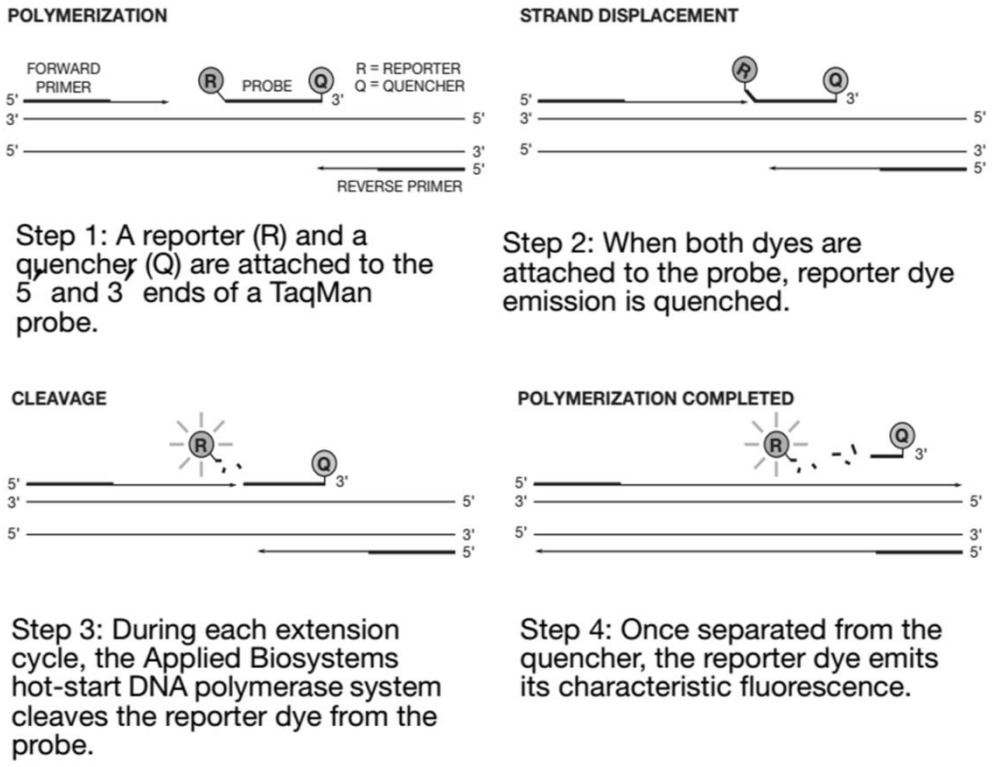



*Figure 2.1 Principles of real-time PCR. From Real-Time PCR Systems Chemistry Guide, PN 4348358 used with permission from Thermo Fisher Scientific (Thermo Fisher Scientific, 2005). Representation of how the 5' nuclease chemistry uses a fluorogenic probe to enable detection of a specific PCR product.*

Loaded into a 96 well plate was cDNA (minimum 20 ng calculated using PicoGreen), mastermix and probes totalling 12 µl. The reaction program was 50ºC 2 minutes, 95ºC 10 minutes, followed by 40 cycles of 95ºC for 15 seconds then 60ºC for 1 minute. The cycle threshold (Ct) was used for statistical analysis; ΔCt (gene of interest Ct minus internal standard gene Ct), with graphical display given in arbitrary units (i.e. $1000*(2^{-\Delta Ct})$). If levels of expression were undetermined an arbitrary Ct of 42 was used to allow for numerical analysis.

It is recommended to use more than one internal control (Sorby et al., 2010) in real time PCR to improve accuracy. However, human tissue sample availability limited

the number of real-time PCR reactions performed. GeNorm and Normfinder software were used to select a single internal standard control gene for human colon tissue samples and cell lines (Appendix II). RPLPO was the most stably expressed gene in colon cancer cell lines and on research performed by Dr Rowan Hardy (University of Birmingham) GAPDH was most consistently expressed in wild-type and mice overexpressing human TNFα (Tg-TNF).

## 2.7 PROLIFERATION ASSAY

Two types of proliferation assay were performed in this project and which method used is stated for each experiment.

### 2.7.1 CyQUANT

Cell proliferation was analysed using CyQUANT® (Life Technologies, Grand Island, NY, USA) as per manufacturer's instructions. CyQUANT® uses a green fluorescent dye that when binding to cellular nucleic acids emits fluorescence (480/520 nm). The fluorescence emission correlates with cell number.

Cells were seeded at 5000 cells/well in a 96 well plate in replicates of six covering 4, 24, 48, 72 and 96 hour time points. Four hours gave initial cell count once cells had adhered after seeding. After each time point media was completely removed and plate stored at -80$^{o}$C until ready for analysis. To analyse plate buffer and green fluorescent dye were added to each well and read on a plate reader (Wallac Victor3TM 1420 Multilabel Counter, PerkinElmer) using fluorescence 480/520 nm.

The counts at 4 hours were averaged and the percentage change at each subsequent time point calculated.

### 2.7.2 BrdU

To monitor changes in proliferation in response to oestrogens 5-bromo-2'deoxyuridine (BrdU) proliferation assay (Roche products Ltd, UK) was performed as per manufacturer's instructions.

The optimal cell seeding density and BrdU incubation was calculated for each cell line. Cells were seeded at 1000-10,000 cells per well with BrdU incubation times of 2, 4 or 6 hours. The optimal results for Caco-2 cells were 4000 cells, HCT116 and HT29 500 cells all with 4 hour BrdU incubations.

After treatment (such as $E_1$ 1, 10, 100 and 500 nM, $E_2$ 1, 10, 100 and 500 nM, with SFBS and complete media controls for 48 hours each in 6-8 wells of a 96 well plate), 100 µM BrdU was added. BrdU is an analog of thymidine and is incorporated into the DNA of proliferating cells. After 4 hours media containing BrdU was removed and cells fixed and DNA denatured in one-step using FixDenat. After 30 minutes this was removed and monoclonal antibody to BrdU containing horseradish peroxidase (HRP) added for 90 minutes. This antibody binds to the BrdU in the newly synthesised DNA. Anti-BrdU was then removed and cells washed three times in PBS. This was a colourimetric assay and when the substrate containing tetra-methylbenzidine (TMB) was added it catalysed the conversion of HRP on the anti-BrdU from colourless to blue, measurable on a Wallac plate reader at 405 nm

absorbance. Colour would develop over 5-30 minutes, with intensity proportional to BrdU incorporation. Even at the higher oestrogen concentrations all cells proliferated and on visual observance looked healthy, suggesting no toxic effects at these doses.

## 2.8 STS ASSAY

This assay has been previously described (Purohit et al., 1995, Duncan et al., 1993) and has been adapted for CRC cell lines and tissue. Differences between the STS activity assay performed in protein lysate and intact cells are described in sections 2.8.1 and 2.8.2 respectively.

Conversion of $E_1S$ to $E_1$ by STS was measured using [6,7-$^{3}$H] $E_1S$ ($4 \times 10^5$ dpm, Perkin-ElmerLS, Boston, MA, USA) adjusted to a final concentration of 20 μM with unlabeled $E_1S$ in PBS (protein lysate) or serum free cell media (intact cells). [4-$^{14}$C] $E_1$ ($1 \times 10^4$ dpm, Perkin-Elmer) was also included in the reaction mixture to monitor procedural losses. The dose of $E_1S$ was chosen to ensure the reaction was not saturated (did not reach total count) and did not have any toxic cellular effects (as assessed visually). As the sulphate group is labile, every three months unlabelled $E_1S$ was purified of any $E_1$ by toluene extraction. In addition a second clean-up occurred when making the substrate as $^{3H}E_1S$ also underwent removal of any $^{3H}E_1$.

After incubation at 37$^{o}$C, $E_1$ was extracted using liquid-liquid extraction. $E_1S$ is hydrophilic, whereas $E_1$ is hydrophobic and therefore the two can be separated using toluene; 4 mls toluene was added and vortexed for 30 seconds. After this, 1

ml of toluene (top layer) was added to 4 mls scintillin (Fisher Scientific, UK) and counted on a scintillation spectrophotometer. Reactions were performed in duplicate. Positive controls included JEG3 or Caco-2 cells with and without the STS inhibitor STX64. A total count of non-toluene extracted substrate was also counted and this represented maximum conversion and allowed for calculation of hydrolysed $E_1$ concentration. Negative controls were a total count extracted with toluene to assess any potential non-enzymic loss of the $E_1S$ sulphate group, and a PBS blank (background count). The average recovery of $^{14C}E_1$ varied in intact plated cells, most likely due to some SULT1E1 activity. Plated cells in culture would be exposed to the necessary co-factors required for SULT1E1 action whereas these would not be present in the buffer used for lysed cells. The scintillation spectrophotometer software gave an adjusted reading to account for any crossover of $^{3H}E_1$ and $^{14C}E_1$.

$E_1S$ to $E_1$ conversion was corrected for procedural losses using the percentage of 14C recovery ((3H count/ 100) x (100- %14C recovery) + 3H count)). The mass of $E_1S$ hydrolysed was calculated from the $^{3H}E_1$ counts obtained (corrected for dilution, non-enzymatic hydrolysis and recovery of $^{14C}E_1$ added) and the specific activity of the substrate. The results were expressed as nmoles of the product formed/ mg protein/ hour. Each batch of experiments included an incubation without any enzyme (i.e. tissue/ cell lysate) to monitor non-enzymatic hydrolysis of the substrate (blanks).

### 2.8.1 Protein Lysate

Conversion of $E_1S$ to $E_1$ by STS was measured using 60 µg of human colon protein lysate. 0.5 ml of unlabelled $E_1S$, $^{3H}E_1S$, $^{14C}E_1$ in PBS was added to the protein and incubated at 37$^{o}$C for 4 hours in Pyrex tubes (Scientific Laboratory Supplies, Nottingham, UK). Recovery of $^{14C}E_1$ averaged 97.45 % (96.29, 98.60) in human lysate, 91.26% (89.63, 92.90) in mouse colon homogenate, 96.12% ± 3.7 in JEG3 lysate and 95.86% ± 1.01 in Caco-2 lysate.

### 2.8.2 Plated Cells

Cells seeded into 6 well plates (100,000 cells per well) or T25 flasks (250,000 cells per well) had media removed and were washed with PBS. 1 ml of unlabelled $E_1S$, $^{3H}E_1S$, $^{14C}E_1$ in serum free media was added and incubated at 37$^{o}$C overnight (18 hours). This was a longer assay than protein lysate to take into account transport across the cell membrane. To improve accuracy 0.5 ml rather than trying to recover all of the original 1 ml was used for analysis. Protein was harvested and quantified (sections 2.2.2 and 2.2.4) with results expressed as $E_1$ conversion in pmol/mg/hr. Recovery of $^{14C}E_1$ varied between cell lines probably due to SULT1E1 activity e.g. HCT116 $^{14C}E_1$ recovery was 78.96% ± 1.89 and Caco-2 86.44% ± 1.52.

## 2.9 WESTERN BLOTTING

Protein (10-25 µg) with equal volume Laemmli sample buffer (Laemmli Buffer (Bio Rad, Hertfordshire, UK) with a 1 in 20 dilution of β2-Mercaptoethanol (Sigma-Aldrich, Poole, UK)) was denatured at 100$^{o}$C for 5 minutes. Proteins were separated on a 10% SDS-PAGE gel (Protogel, Resolving buffer, Stacking buffer (Geneflow

Ltd, UK), $dH_2O$, TEMED (Sigma-Aldrich, Poole, UK), 10% Ammonium persulfate (Sigma-Aldrich, Poole, UK)). Separated protein was transferred for 1 hour 20 minutes at 360 mA on to a polyvinylidene fluoride (PVDF) membrane (Millipore (UK) Ltd). To prevent non-specific binding membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween (TBST, ($dH_2O$, 50 ml 1 M Tris pH 7.6, 0.625 ml Tween 80 (Sigma) and 20 g NaCl)) for 1 hour at room temperature or overnight at 4$^{o}$C.

Membranes were washed several times in TBST and incubated with primary antibody also made in TBST. Membranes were washed again with TBST before incubation with corresponding HRP conjugated secondary antibody in TBST. Antibodies used and details on incubation length and concentration are shown in Table 2.3.

Antigen antibody complexes were visualised using ECL Western blotting detection reagent (GE Healthcare Life Sciences, UK) and x-ray film (Kodak) which was developed on a Xenograph Compact X4. To allow for detection of multiple proteins a mild stripping buffer (Tris, Glycine, SDS, pH 2.2) was applied twice for 10 minutes at room temperature, followed by further washes of TBST. The membrane was then blocked in 5% non-fat milk again before further antibodies were applied.

Table 2.3 Western blot antibodies used with details on incubation length and concentration.

| Antibody | Antibody Type | Supplier | Primary Concentration | Secondary Concentration |
|---|---|---|---|---|
| **β-actin (A5441)** | Mouse monoclonal | Sigma | 1:5,000 at room temperature for 1 hour | 1:10,000 at room temperature for 1 hour |
| **HSD17β1 (ab51045)** | Rabbit monoclonal | Abcam | 1:2,000 at 4°C over night | 1:5,000 at room temperature for 1 hour |
| **HSD17β2 (10978-1-AP)** | Rabbit polyclonal | Proteintech | 1:1,000 in 1% milk in TBST incubated over night at 4°C | 1:2,000 in 1% milk in TBST at room temperature for 1 hour |
| **HSD17β7 (H00051478-M01)** | Mouse monoclonal | Abnova | 1:1,000 in 1% milk in TBST incubated over night at 4°C | 1:2,000 in 1% milk in TBST at room temperature for 1 hour |
| **HSD17β12 (H00051144-M08)** | Mouse monoclonal | Abnova | 1:1,000 in 1% milk in TBST incubated over night at 4°C | 1:2,000 in 1% milk in TBST at room temperature for 1 hour |
| **PAPSS1 (ab56398)** | Mouse monoclonal | Abcam | 1:1000 in 10% milk in TBST incubated over night at 4°C | 1:1000 in TBST at room temperature for 1 hour |
| **PAPSS2 (ab56393)** | Mouse monoclonal | Abcam | 1:1000 in 10% milk in TBST incubated over night at 4°C | 1:1000 in TBST at room temperature for 1 hour |
| **GPER (sc-48525-R)** | Rabbit polyclonal | Santa Cruz™ | 1:500 in TBST in 1%milk incubated overnight at 4°C | 1:2,000 in TBST incubated at room temperature for 1 hour |
| **Goat anti-mouse (sc-2005)** | Polyclonal | Santa Cruz™ | - | - |
| **Goat anti-rabbit (sc-2004)** | Polyclonal | Santa Cruz™ | - | - |

## 2.10 PLASMID BULK-UP

Plasmids purchased from Origene (Origene Technologies, Inc. Rockville, USA) included human STS (RC210431), and empty vector control (VO, pCMV6-Neo) with

maps displayed in Figure 2.2. The plasmids were reconstituted in water as per Origene instructions; add 10 µl $dH_2O$ (creating a 1 µg/µl solution) and incubate at room temperature for 10 minutes, store at -20°C.

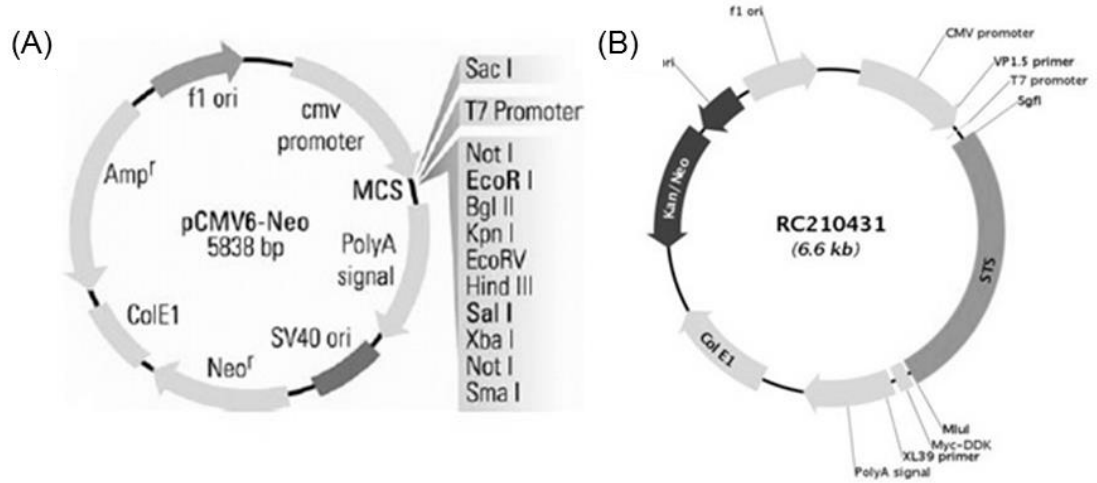



*Figure 2.2 Diagram of plasmids used for transfection into HCT116 cells. Purchased from Origene. (A) empty vector (VO) and (B) STS. Both plasmids when expressed enable resistance to G418 (neomycin).*

### 2.10.1 Bacterial Transformation

Plasmids were diluted to a working solution of 2 ng/µl and 10 ng added to 100 µl of α-select competent cells silver (Bioline Reagent Ltd, London, UK). A positive control provided by the manufacturer was also used. The competent cells and plasmid were incubated on ice for 30 minutes before being heat shocked for 30 seconds at 42°C and then placed back on ice for 2 minutes. The competent cells and plasmid were then added to LB broth (Sigma-Aldrich, Poole, UK) containing 1:1000 (50 µg/µl) ampicillin and incubated for 1 hour at 30°C. The plasmids contained ampicillin resistance therefore only plasmid containing cells grew in the broth. After incubation cells were centrifuged for 3 minutes at 13,000 rpm and supernatant discarded. The

pellet was resuspended in ampicillin LB broth, spread over ampicillin (1:1000) agar (Sigma-Aldrich, Poole, UK) plates and incubated overnight at 37$^{o}$C. Colonies were selected and part transferred to 2 mls of ampicillin LB broth and shaken at 37$^{o}$C for 6-8 hours. This broth cell mix was then transferred to 150 ml and shaken overnight to be used for maxi-prep on the following day.

### 2.10.2 Maxi-Prep

Maxi-prep was performed as per manufacturer instructions (Sigma-Aldrich, Poole, UK). The 150 ml culture (section 2.10.1) was pelleted by centrifugation at 5000 g for 10 minutes and resuspended in resuspension/RNAse A solution. Lysis solution was added and after 3-5 minutes the solution turned clear indicating the need to add chilled neutralising solution. The lysed cell mixture was added to binding solution and poured into a syringe barrel for 5 minutes. The binding column was prepared and lysed cells passed through the syringe into the column followed by centrifugation at 3000 g for 2 minutes. The columns were then washed and plasmid eluted with 3 mls of elution solution. Nano drop (section 2.3.3) confirmed the concentration and then the plasmids were prepared for sequencing.

### 2.10.3 Plasmid Sequencing

AltaBiosciences at The University of Birmingham sequence plasmids. The following mix was supplied:

- 3.2 pmol primer (both forward and reverse provided by Origene)
- 200-500 ng plasmid
- Total to 10 µl $dH_2O$

Sequences were confirmed using Blast®.

## 2.11 PLASMID TRANSFECTION

### 2.11.1 Concentration of Selective Medium

The plasmids used for STS (RC210431) and VO (pCMV6-Neo) contained G418 resistance allowing to select for plasmid containing cells only. For each cell line transfected the optimum concentration of G418 selective media was determined. Cells were seeded at 175,000 cells per well in a 6 well plate (Corning Ltd, UK) and treated in duplicate with G418 concentrations 0 mg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml, 1 mg/ml or 2 mg/ml. Media was refreshed on days 3 and 6. On day 8 wells were examined and the lowest concentration achieving cell death deemed optimum concentration; Colo205 cells 200 µg/ml, Caco-2 cells 500 µg/ml and HCT116 1 mg/ml. Only results from HCT116 cell transfection are discussed in this thesis.

### 2.11.2 Plasmid Transfection

Cells were seeded at a density of 250,000 per well in a 6 well plate. Plasmids (STS 0.4 µg/µl or vector only (VO) 0.148 µg/µl) diluted in Optimem (Life Technologies,

Grand Island, NY, USA) were added to Lipofectamine (Invitrogen, Paisley, UK) also diluted in Optimem and incubated at room temperature for 5 minutes, allowing complexes to form. The lipofectamine-plasmid complexes were then gently dropped onto each well. To ensure any changes in expression were plasmid related Optimem and Lipofectamine only controls were performed alongside. After 24 hours cells from each condition (STS plasmid, VO plasmid, Lipofectamine only and Optimem only) were harvested for protein (section 2.2.2), RNA (section 2.3.1) and transferred into T25 flasks containing the appropriate concentration of G418 selective media.

### 2.11.3 Transfection Validation

To confirm successful transfection and therefore overexpression of STS, real time PCR was performed as described in section 2.6, comparing VO, wild-type (WT) and STS overexpressing cells. Western blotting (section 2.9) was also performed for STS as the plasmids contained a DDK tag and to ensure STS was functional an STS activity assay (section 2.8.2). These stably transfected cells were not grown from a single cell and therefore were a heterogeneous population. This heterogeneous approach was thought to be more representative of colon tumours. Expression and activity of STS were monitored and declined over time despite selective medium. For this reason cells were only cultured for up to 15 passages post transfection.

## 2.12 MASS SPECTROMETRY EXTRACTION

A novel uPLC-MS/MS method for quantifying both oestrogens and their sulphates was created with the help of Dr A. E. Taylor and optimised using Multi-objective Unbiased optimisation of Spectrometry via Closed Loop Experimentation (MUSCLE) software, developed at the University of Birmingham (Viant et al.); see Chapter 3 for full method including validation.

## 2.13 HUMAN TISSUE SAMPLES AND ETHICS

Human CRC samples with matched normal tissue control were provided by the Human Biomaterial Resource Centre (HBRC) and subjected to selection criteria. Samples were adenocarcinomas, females were post-menopausal and those with inflammatory bowel disease or known familial mutations were excluded. Further details can be seen in Appendix I and II. Colorectal tissue was collected from HBRC in accordance to local ethical guidelines. The HBRC is ethically approved by North West 5 Research Ethics Committee, Haydock Park; Ref 09/H1010/75 and authorised to release samples that satisfy the Access Review Panel. All experiments complied with the Human Tissue Act.

## 2.14 STATISTICAL ANALYSIS

The statistical test applied for each experimental question is stated in the relevant figure legend. The majority of experiments involved multiple comparisons and so were analysed using two-way ANOVAs with Bonferroni post-test. A p value $< 0.05$ was deemed significant. Results are expressed as mean ± standard error of the mean unless otherwise stated.

For STS human data the analysis was performed in assistance with Dr Alice Sitch (Lecturer in Medical Statistics, University of Birmingham). Relationships between STS activity and STS mRNA expression were investigated by plotting the data and calculating correlation coefficients. Further analyses used random effects linear regression modelling (with outcomes transformed where appropriate to reduce the impact of outliers) to allow for normal and cancer samples being patient matched. To investigate differences in STS activity, models were fitted with and without adjustment for patient characteristics (sex, age and BMI) and stage (T and Dukes'). Results are shown as 95% confidence interval (CI) of the mean unless otherwise stated. STS activity model required log transformation and the estimates obtained are interpreted as approximate percentage differences.

# CHAPTER 3. DEVELOPMENT, OPTIMISATION AND VALIDATION OF LIQUID CHROMATOGRAPHY, TANDEM MASS SPECTROMETRY METHOD FOR OESTROGEN AND OESTROGEN SULPHATES

## 3.1 INTRODUCTION

Commonly used oestrogen quantification methods include enzyme-linked immunosorbent assays (ELISA) and radioimmunoassay (RIA). However, mass spectrometry (MS) use, especially liquid chromatography-tandem MS (LC-MS/MS), has increased over the last two decades and this trend is expected to continue (Shackleton, 2010). ELISA and RIA are disadvantaged as they can only quantify a single compound at a time and exhibit cross-reactivity from structurally similar molecules and metabolites (both from an endogenous source or drugs). They can also lack specificity which fluctuates between kits (Krasowski et al., 2014). Large variations between commercially available ELISAs for three commonly studied steroids; testosterone, oestradiol and progesterone, can ultimately cause data inaccuracies between studies and centres (Soldin and Soldin, 2009). RIA has the additional drawback of expensive radioactive substrates, risk of user radiation exposure and specialist disposal requirements.

Oestrogen concentration comparisons between pre and postmenopausal women using three methods; immunoassay, RIA and LC-MS/MS showed results were well correlated. However, LC-MS/MS was more accurate for lower concentrations found in postmenopausal women, highlighting one of the main advantages of LC-MS/MS; superior sensitivity (Faupel-Badger et al., 2010). MS is the solution to immunoassay problems as it can overcome cross reactivity and is able to quantify multiple analytes in a single, high throughput and sensitive assay. Therefore, LC-MS/MS was chosen to study oestrogen metabolism in CRC cell lines.

Sensitive and rapid methods were available for $E_1$ and $E_2$, such as a recent publication by Owen et al. (Owen et al., 2014) and for sulphated oestrogens, such as Corona et al. (Corona et al., 2010). However, few methods combined oestrogens and their sulphates in a single assay. No commercial LC-MS/MS kits for measuring oestrogens and their sulphates in a single assay were available.

The aim was to quantify oestrogen metabolites in CRC cell culture medium, including active oestrogens $E_1$ and $E_2$ and their inactive sulphates $E_1S$ and $E_2S$, respectively. There are differences in CRC cell line growth responses to oestrogen which are described in detail in Chapter 5 and 6. It was hypothesised that variable proliferative effects were due to disparities in oestrogen metabolism. A novel LC-MS/MS method that rapidly quantifies these four compounds was required. For high quality data by LC-MS/MS, assays need validating according to industry standards (Taylor et al., 2015); described in this chapter in detail.

## 3.2 MASS SPECTROMETRY PRINCIPLES

Mass spectrometry is an analytical method used to quantify known compounds, identify unknown substances and provide structural and chemical information. It does this by separating gas phase ions according to their mass to charge ratio (m/z) using electrical and/or magnetic fields within a vacuum.

The LC-MS/MS configuration used in these experiments was a Waters uPLC-ESI-triple quadrupole MS. Sample components are initially separated using an ultra performance liquid chromatography (uPLC) column, which retains analytes based

on their polarity. Components are then converted to gas phase ions, such as with Electrospray ionisation (ESI), and enter a mass analyser. In a triple quadrupole configuration, gas phase ions are subsequently fragmented to achieve the selectivity and sensitivity required for quantitative analysis. Each ion is separated by mass to charge ratio (m/z) and the detector gives a signal depending on number of ions detected (Premier Biosoft, 2016, Chromacademy, 2016b). The main components of a MS are shown in Figure 3.1

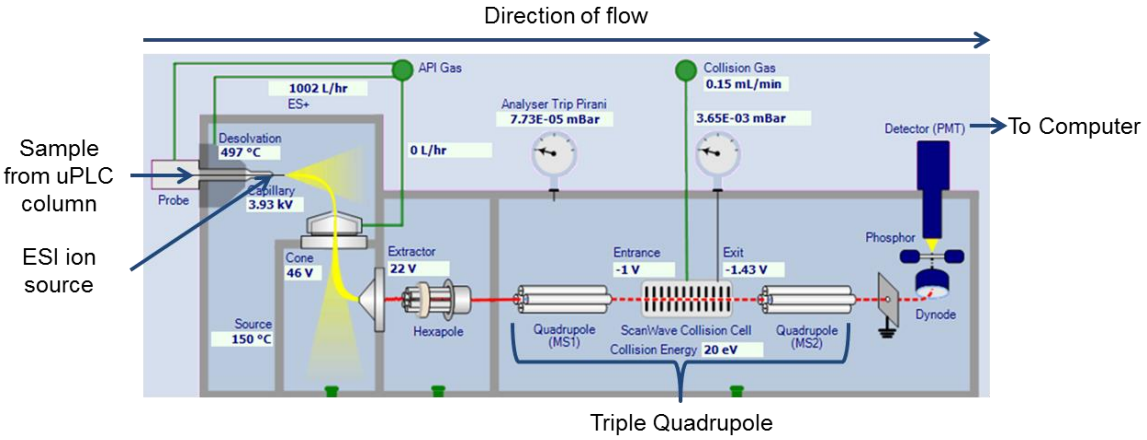



*Figure 3.1 Screenshot from Waters Xevo MS with an Acquity uPLC liquid chromatography system Masslynx software. The main components of a MS are sample introduction system which maintains a vacuum, an ion source to produce the gaseous ions from the analyte (desolvation by ESI), analyser to resolve the ions according to m/z (Triple Quadrupole; MS1, collision cell and MS2), detector to identify the ions and record their relative abundance and a computer to manipulate and display the data.*

The quadrupole was designed by Paul and Steinwegen at Bonn University in 1953 (Paul and Steinwedel, 1953) and is displayed in Figure 3.2. Quadrupole analysers use the stability of trajectories in oscillating electric fields (radio-frequency (RF) and direct current (DC)) to separate ions according to their m/z ratios. Ions travel along a central axis of four parallel circular/ hyperbolic rods. Only ions with a stable, non-

collisional trajectory (relating to a specific m/z ratio) will be detected as all other ions collide with a rod and will discharge (Chromacademy, 2016a).

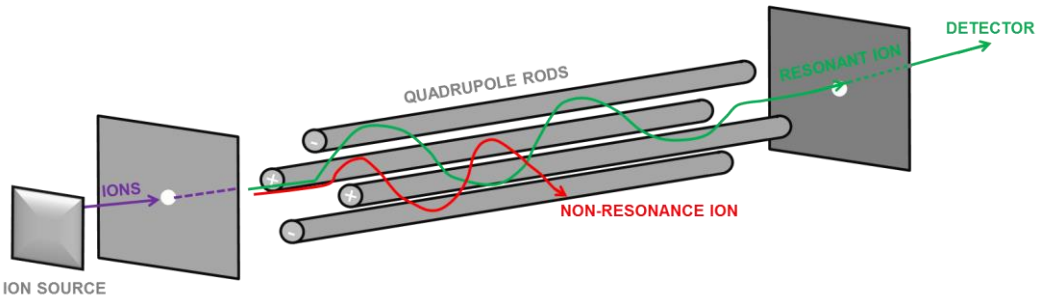



*Figure 3.2 Simplified diagram of a quadrupole. There are four parallel identical rods which form two opposing pairs. One rod pair is connected to a certain direct current (DC) and radio frequency (RF) and the other pair had the opposite applied creating an oscillating electrical field. As ions enter they oscillate; the green ion follows a stable trajectory and is detected and the red ion has an unstable trajectory, collides with a rod and so discharges and is not detected. When only RF is applied to the quadrupole, such as in the collision cell, all ions selected pass through it (Lundanes et al., 2014).*

A triple quadrupole MS, also known as a tandem MS, consists of three quadrupoles arranged in series. The first and last are transmission quadrupoles (MS1 and MS2 in Figure 3.1) and between them is a radio-frequency (RF) only quadrupole which acts as a collision cell. Ions are selected by MS1, fragmented in the collision cell and the resultant product ions analysed by MS2 (Mallet and Down, 2010, Lundanes et al., 2014). Within the collision cell is inert gas (here Argon) and the energy of the gaseous ions can be altered to produce stable product ions fragmented from MS1 ion selection, increasing selectivity and sensitivity.

## 3.3 MASS SPECTROMETRY ASSAY METHOD DEVELOPMENT AND VALIDATION

Multiple steps are needed to develop an LC-MS/MS method, followed by validation to prove the method is robust and reliable. Developing a method is time consuming, subjective and often involves compromising on certain aspects, such as resolution or run time. To develop an oestrogen and oestrogen sulphate method the following steps were completed (National Measurement System, 2014):

1. **Determining ions for quantification**: Direct infusion of $E_1$, $E_2$, $E_1S$ and $E_2S$ into the MS and ascertaining MS parameters, such as ions in positive ($[M]^+$, $[M+H]^+$, $[M+adduct]^+$, $[M-H_2O]^+$, $[M-2H_2O]^+$), or negative ($[M]^-$, $[M+H]^-$, $[M+adduct]^-$, $[M-H_2O]^-$, $[M-2H_2O]^-$) mode.
2. **Cone voltage:** Adjusted to give maximum signal for each ion.
3. **Collision energy**: Ions enter the collision cell and voltage is increased until stable fragments are observed.
4. **Cone voltage:** Adjusted again to give maximum signal for each fragment.
5. **Determination of sensitive MRMs (Multiple reaction monitoring) for quantification**: Each compound is run through a simple gradient and fragments assessed for peak area and shape. Two MRMs are used; largest peak area with good shape is typically used as the quantifier and lower peak area for qualifier.
6. **Optimising chromatography**: Chromatography for combined analytes is optimised to achieve good sample separation using different polar/non polar contributions in the uPLC column (here methanol/water). This step is particularly time consuming, especially with large numbers of analytes.

7. **Internal standards (IS)**: Representative IS are selected and for each analyte and steps one through five are repeated. IS are structurally similar to the compound being measured and a known quantity is added to every sample. Typically they have added deuterium or carbon 13 and should behave in the same manner as the target analytes. This enables a ratio of the IS and analyte ion abundance to be calculated aiding accuracy of quantification.
8. **Method validation**: Measuring specificity, sensitivity, accuracy, precision, extraction efficiency/ recovery and matrix effects. This was completed for each analyte.

Developing an LC-MS/MS method can take weeks to months. This can depend on how easily the compounds ionise and are fragmented into stable MRMs. Development of chromatography is also time consuming with multiple factors such as gradient, run time, column type and mobile phase choice.

## 3.4 MUSCLE SOFTWARE

A novel uPLC MS/MS method for all four oestrogens was developed by building upon a published LC-MS/MS method for $E_1$ and $E_2$ (Owen et al., 2014) and further optimised using MUSCLE (Multi-objective Unbiased Spectrometry via Closed Loop Experimentation) software.

MUSCLE software, developed at The University of Birmingham (Bradbury et al., 2015), is designed to reduce LC and MS method development time. It does this by simultaneously investigating multiple parameters and highlighting optimal solutions,

thus combining steps 2-6 in section 3.3 above. The MS is programmed to independently run multiple experiments while MUSCLE software uses a multi-objective optimisation algorithm to maximise total peak area, total number of peaks and minimise run time (time of last eluting peak). The MS can be run out of hours, without the need for technical staff, improving efficiency and reducing costs.

Parameters can be pre-selected and in addition to run time include maximal separation and signal intensity, methanol gradient, cone voltage, collision energy and column temperature. Objectives are optimised simultaneously, thus a single solution cannot be provided. Instead pareto-efficient solutions are displayed for the user to choose depending on their priorities e.g. shortest run time, best peak resolution or a trade-off between the two. MUSCLE software produces a graphical summary, allowing the analyst to quickly and efficiently review optimisation experiments, comparing experimental conditions (Figure 3.3).

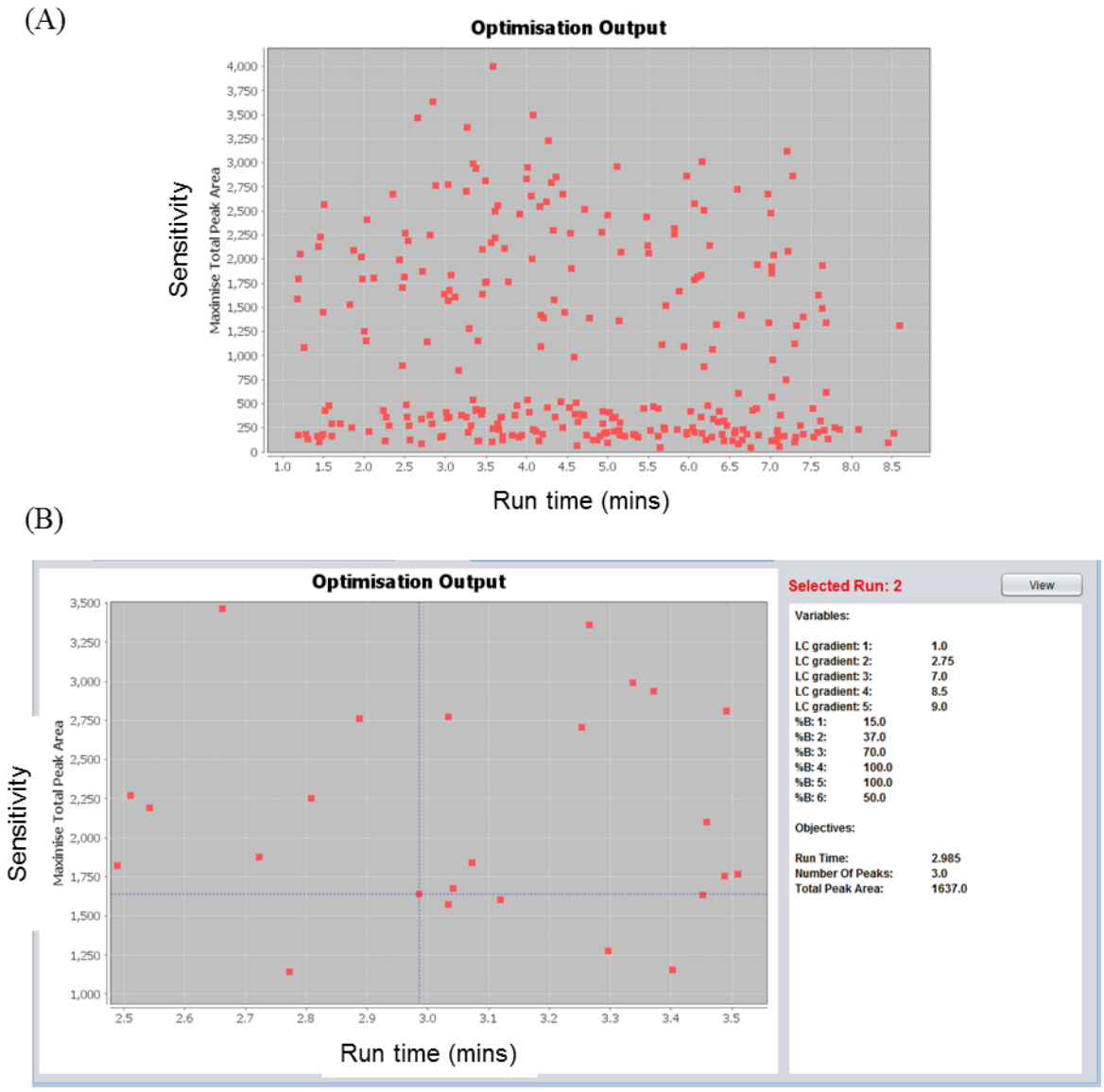



*Figure 3.3 Screenshots from MUSCLE software. (A) Summary of all runs performed by MUSCLE prioritised for run time and sensitivity (peak area). Each red dot represents a method run by MUSCLE and shows its corresponding run time in minutes (affecting throughput) on the x axis and peak area on the y axis (relating to sensitivity). (B) A single run is selected (cross hatch). The method* parameters of a selected point *are displayed on the right hand side, such as methanol and water % gradient, and can be used to further optimise a method if desired.*

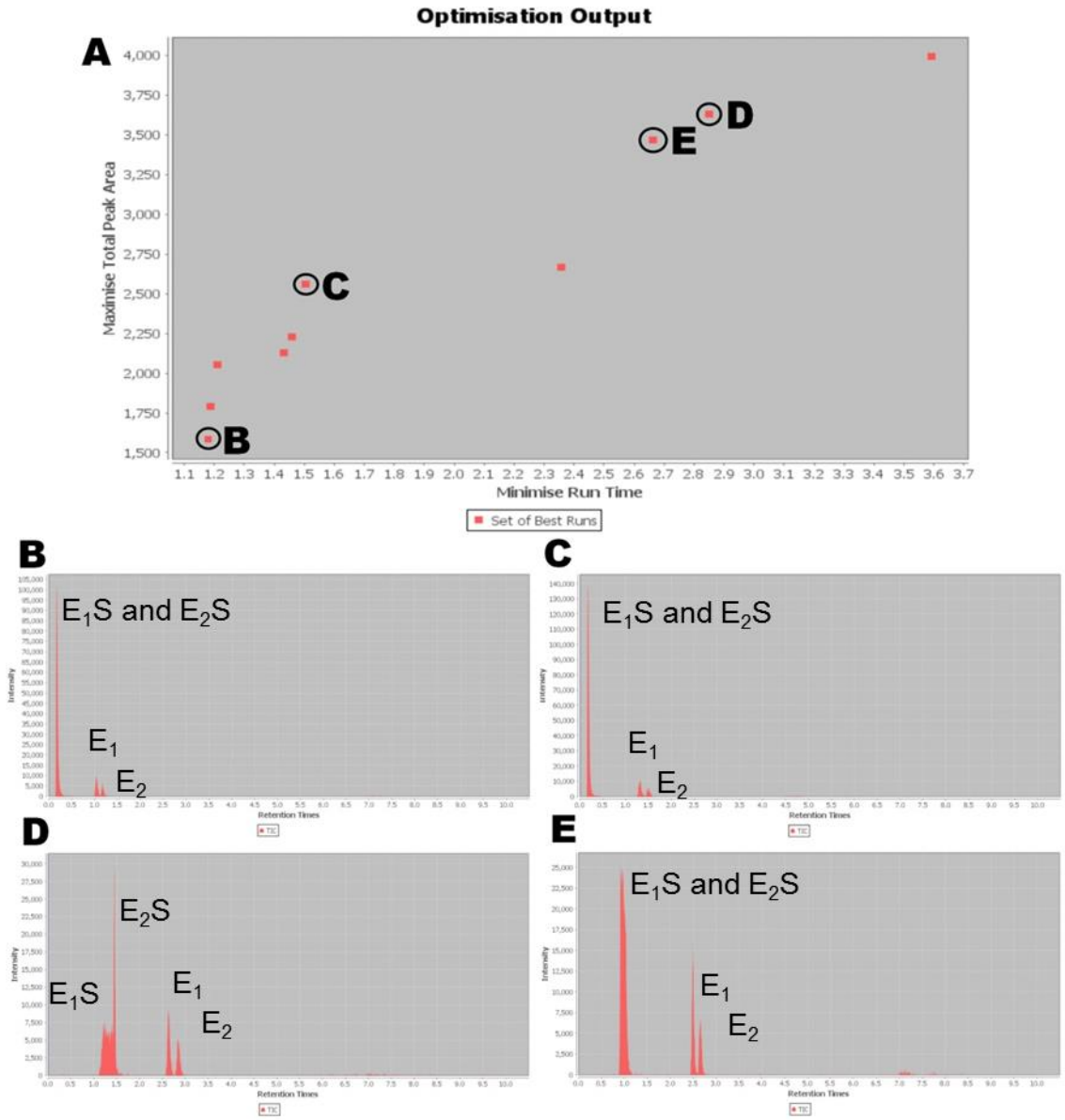



*Figure 3.4 Screenshots of runs selected by MUSCLE. (A) Four runs selected by MUSCLE software based on run time (in minutes) and total peak area (sensitivity). (B-E) Resolution (number of peaks) of four methods.*

For this oestrogen method, optimisation time was reduced from weeks to days with 200 samples run by MUSCLE in 48 hours. Figure 3.4 shows the pareto-optimal methods recommended by MUSCLE based on run time and sensitivity. Methods identifying four chromatographic peaks were examined however these had poor

peak shape (broad with tailing) and intensity (Figure 3.4 D and E). By reviewing methods with shorter run times (Figure 3.4 B and C) an optimum 4 minute method was selected with higher peak intensity that separated $E_1$ and $E_2$, the sulphates co-eluted, but could be mass separated. This method was further optimised with the final method rapidly analysing $E_1S$, $E_2S$, $E_1$ and $E_2$ with good sensitivity and resolution, separating within 5 minutes.

## 3.5 MATERIALS AND METHODS

$E_1$ (Estrone), $E_2$ (17β-Estradiol), $E_1S$ (Estrone 3-sulfate sodium), $E_2S$ (β-Estradiol 3-sulfate sodium), internal standard $^{13}C$-$E_2$ (17β-Estradiol-2, 3, 4-$^{13}C_3$) and ammonium fluoride were all purchased from Sigma-Aldrich, Dorset, UK. Deuterium labelled internal standards for oestrogen sulphates, $E_1S$-d4 (sodium Estrone-2, 4, 16, 16-$d_4$ 3-sulfate) and $E_2S$-d4 (sodium 17β-Estradiol-2, 4, 16, 16-$d_4$ 3-sulfate) were purchased from Cambridge Isotopes Laboratories, Inc., USA. LC-MS grade methanol and water were purchased from Greyhound Scientific, Merseyside, UK.

A Waters Xevo MS with an Acquity uPLC liquid chromatography system was used in these experiments and an electrospray ionisation source was employed in both positive and negative ionisation mode. The validated method separated oestrogens on an Acquity UPLC® HSS C18 SB 1.8 µm, 2.1 mm X 30 mm Column from Waters at 60$^{o}$C using a methanol/ water gradient system with 0.3mM ammonium fluoride added to the aqueous phase. Initial experiments used a C18 and HSS T2 column with formic acid added to the mobile phase. Changing to a shorter column and use

of ammonium fluoride as described by Owen et al. improved $E_1$ and $E_2$ separation and doubled sensitivity (Owen et al., 2014).

Steroid solutions 1 mg/ml solutions in methanol were stored for up to 3 months at -20°C. A working solution of 1000 ng/ml in methanol was infused into the MS. The instrument was manually tuned to each steroid, collision and cone voltage was optimised and mass transitions were determined in both positive and negative mode. Negative mode gave higher intensity ions and thus was used for all further experiments (Table 3.1). A stock solution containing a mix of $E_1$, $E_2$, $E_1S$ and $E_2S$ (1000 ng/ml) was used to produce a calibration series. IS was $E_1S$-d4, $E_2S$-d4, $^{13}C$-$E_2$, $E_2$-d4, (1000 ng/ml), 20 µl of this was added to every sample prior to extraction.

Table 3.1 MRMs for oestrogens, oestrogen sulphates and internal standards determined in negative mode.

| Compound | Abbrev. | MRM transitions | Retention time (min) | Collision energy (eV) | Cone voltage (V) |
|---|---|---|---|---|---|
| Oestrone | $E_1$ | 269.1 > 145.1<br>269.1 > 183.1 | 2.8 | 62<br>62 | 30<br>36 |
| 17β-oestradiol | $E_2$ | 271.2 > 183.1<br>271.2 > 145.1 | 3.07 | 62<br>62 | 36<br>30 |
| Oestrone 3-sulphate | $E_1S$ | 349.1 > 269.1<br>349.1 > 145.0 | 0.7 | 42<br>42 | 32<br>54 |
| β-oestradiol 3-sulphate | $E_2S$ | 351.1 > 271.2<br>351.1 > 145.1 | 0.74 | 48<br>48 | 36<br>56 |
| 17β-oestradiol-2, 3, 4-$^{13}C_3$ | $^{13}C$-$E_2$ | 274.2 > 148.1<br>274.2 > 160.6 | 3 | 92<br>62 | 34<br>32 |
| Oestrone-2, 4, 16, 16-$d_4$ 3-sulphate | $E_1S$-d4 | 353.2 > 273.2<br>353.2 > 147.0 | 0.7 | 44<br>44 | 30<br>54 |
| 17β-oestradiol-2, 4, 16, 16-$d_4$ 3-sulphate | $E_2S$-d4 | 355.2 > 275.2<br>35 5.2 > 147.0 | 0.74 | 48<br>48 | 30<br>54 |

This method was validated following a protocol described by Honour in 2011 (Honour, 2011).

## 3.6 EXTRACTION OPTIMISATION

Desirable MS methods have minimal sample preparation which reduces overall processing time. Two common methods for sample preparation are liquid-liquid extraction (LLE) or solid phase extraction (SPE).

LLE is simple, rapid and has reasonable selectivity (depending on sample type). With aqueous samples, an addition of an immiscible organic solvent, such as methyl tertiary-butyl ether (MTBE), followed by mixing, partitions analytes between the two phases (Chromacademy, 2016c). However, this only works for hydrophobic analytes so could not be used to extract $E_1S$ and $E_2S$.

SPE separates compounds using a column/ solid (stationary phase) and either the desired or undesired components are retained on the column; if retained they can be eluted using a specific solvent or mixture of solvents. ISOLUTE® C18 SPE columns (Biotage, Sweden) were used here. These are retentive, non-polar sorbent suitable for a wide range of analytes from aqueous samples using a non-polar retention mechanism, providing hydrophobic (reversed phase) interactions (Biotage, 2016). In this method hydrophobic $E_1$ and $E_2$ and hydrophilic $E_1S$ and $E_2S$ were extracted simultaneously and retained on the stationary phase. Thus, SPE enabled both sulphated and non-sulphated oestrogens to be extracted in one step. SPE method steps were:

1. Add 20 µl of internal standard solution to 1 ml of sample
2. Activate SPE column with 2 ml methanol
3. Wash SPE column with 2 ml water
4. Add sample to SPE column
5. Add 2 ml methanol to SPE column and collect eluent in sylinised TLC tube
6. Evaporate to dryness under nitrogen at 55$^{o}$C
7. Reconstitute in 200 µl 50/50 methanol/water prior to LC-MS/MS analysis

### 3.6.1 Dansyl Chloride Derivatisation

Dansyl chloride derivatisation (hydroxyl group addition increasing molecular mass by 234) was investigated as many have shown this improves sensitivity (Kushnir et al., 2010, Nelson et al., 2004). After SPE extraction dansyl chloride (1 mg/ml in acetone) was added to samples and heated at 60$^{o}$C for 30 minutes before LC-MS/MS analysis. Dansyl chloride was used initially, aiming to improve ionisation efficiencies and maximise sensitivity. However, in addition to increasing sample preparation time and cost, derivatisation with dansyl chloride was inconsistent leading to poor correlation coefficient values from calibration curves (data not shown). Therefore, it was not used in this method.

## 3.7 RESULTS

### 3.7.1 Run Time

The optimised method parameters are described in Table 3.2 with change in methanol (%B) and water with 0.3mM ammonium fluoride (%A) over time with a flow rate of 0.45 ml per minute.

Table 3.2 Mobile phase elution gradient. Change in water with 0.3mM ammonium fluoride (A) and methanol (B) over time. Gradient profiles 6 represents linear gradient, 11 is the wash step.

| Time (min) | Flow rate (ml/min) | Mobile Phase A (%) | Mobile Phase B (%) | Gradient |
|---|---|---|---|---|
| Initial | 0.45 | 90 | 10 | |
| 2.2 | 0.45 | 50 | 50 | 6 |
| 5.0 | 0.45 | 0 | 100 | 6 |
| 6.5 | 0.45 | 90 | 10 | 11 |

### 3.7.2 Selectivity

Selectivity is the ability to positively identify the compound of interest relative to authentic reference standards.

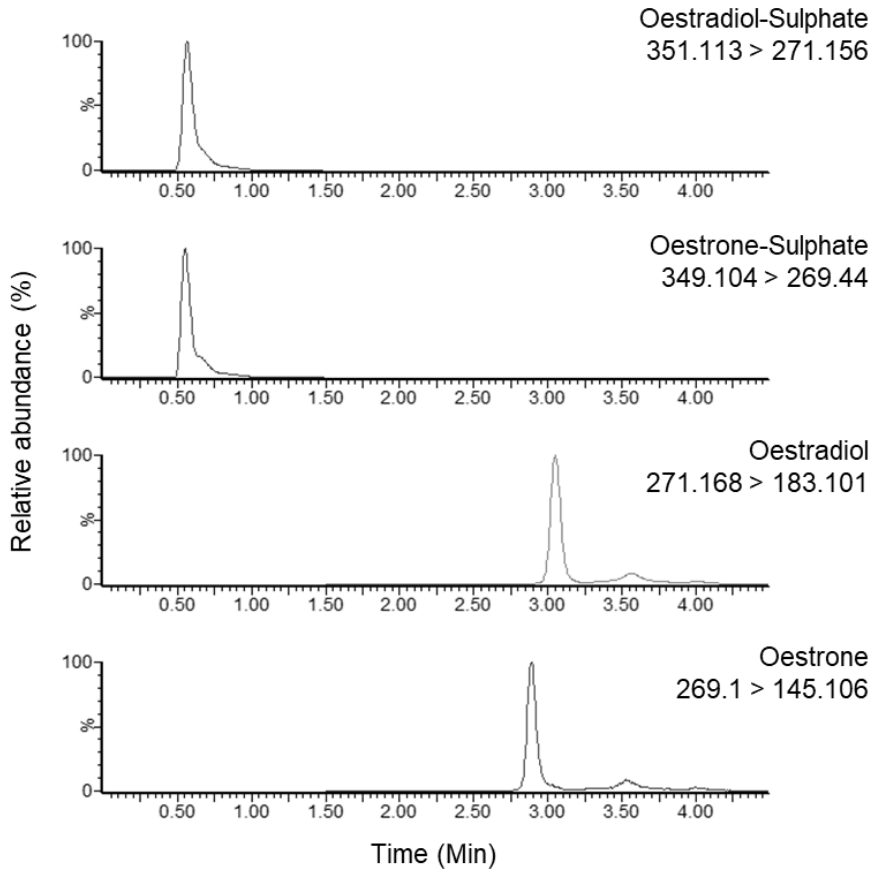



*Figure 3.5 Chromatogram from the optimised MUSCLE method showing time (min) versus relative abundance for oestrone ($E_1$), oestradiol ($E_2$), oestrone-sulphate ($E_1S$) and oestradiol-sulphate ($E_2S$). $E_1$ and $E_2$ peaks are separated chromatographically. Note over column life-time there can be drift of retention times.*

All four analytes could be selectively quantified by mass and/or chromatographic separation. $E_1$ and $E_2$ peaks were well resolved chromatographically. $E_1S$ and $E_2S$ coeluted however due to unique MRMs (Table 3.1) $E_1S$ and $E_2S$ were identified and quantified. Figure 3.5 demonstrates a typical chromatogram.

### 3.7.3 Calibration Curve

By comparison to calibration curves it is possible to accurately quantify steroids within a sample. Thus, calibration curves should cover the expected concentration

range and where possible be made in the same matrix as samples. They should consist of a blank (no added analyte) and six to eight calibrants.

Three sets of calibrations made in phenol red free cell media were extracted using SPE as described in section 3.6. Calibration curves for cell culture covered the range 0, 0.5, 1, 5, 10, 25, 50, 100, 150, 250 and 500 ng/ml for all four steroids. Figure 3.6 shows for each oestrogen three overlaid calibration curves and acceptable linear coefficient of correlation values ranging from 0.9933 to 0.9991 (Honour, 2011).

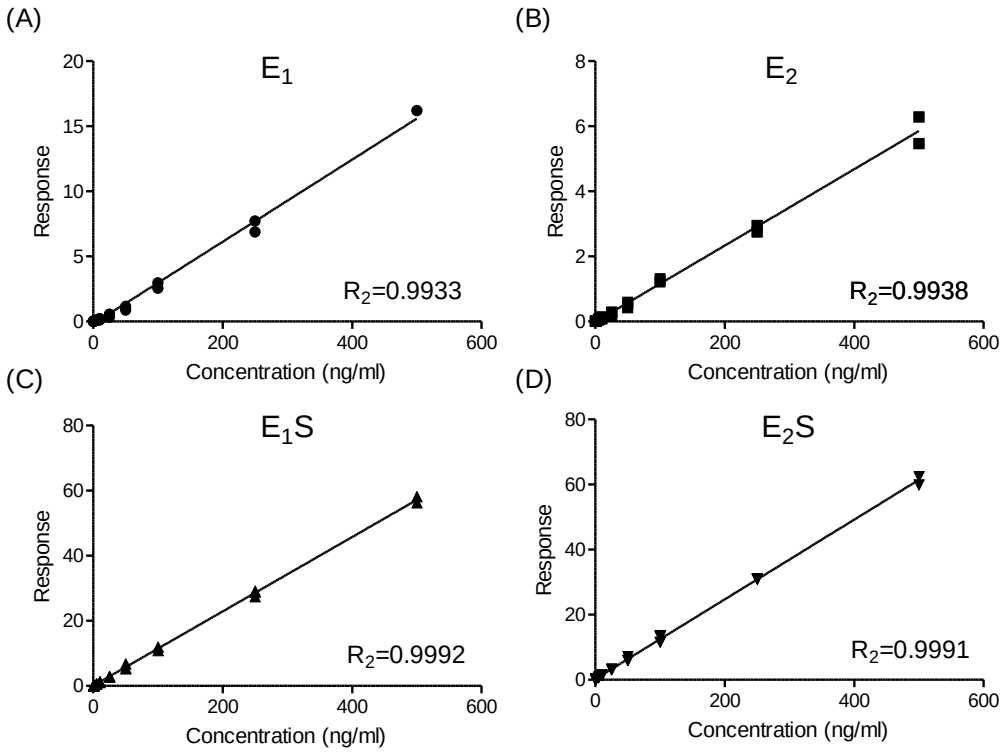



*Figure 3.6 Overlay of three calibration curves across the concentration range 0-500 ng/ml for (A) oestrone, (B) oestradiol, (C) oestrone-sulphate and (D) oestradiol-sulphate.* Response is sample peak area divided by internal standard peak area.

The LLOQ is the lowest concentration with a peak which has a 3:1 signal to noise ratio and provides a reproducible concentration with a precision of 20% RSD (relative standard deviation, ((standard deviation/mean) x100)). LLOQ for each oestrogen are shown in Table 3.3.

Table 3.3 Limit of detection data with signal to noise.

| Oestrogen | LLOQ (ng/ml) | Coefficient variant (%) | Signal:Noise |
|---|---|---|---|
| $E_1$ | 5.0 | 16.2 | 183 |
| $E_2$ | 5.0 | 16.5 | 165 |
| $E_1S$ | 0.5 | 7.6 | 79 |
| $E_2S$ | 0.5 | 8.4 | 59 |

To note over the course of sample analyses there was a build-up on the cone that reduced MS sensitivity. By altering cone gas and angle of ESI probe the reduction in sensitivity was delayed. To ensure no loss of sensitivity the cone was cleaned after each batch, or every 50 samples.

### 3.7.4 Accuracy and Precision

Accuracy is a measure of how close the values obtained are to the true value. Acceptable variation is < 15% for mid and high calibrants and < 20% at the LLOQ. Accuracy was measured with six samples at low (5 ng/ml), medium (20 ng/ml) and high (150 ng/ml) concentration. These were extracted and variability between samples assessed using %RSD in instrument response (peak area) as shown in Table.3.4.

Precision is “the closeness of multiple measurements on the same sample” (Mallet and Down, 2010). Acceptable variation is < 20% for LLOQ and < 15% at higher

levels. Precision was measured by multiple injections of the same sample with %RSD calculated at three concentration levels for each analyte and all fell within acceptable limits.

Table 3.4 shows that $E_1S$ and $E_2S$ had lower precision variability across all ranges than $E_1$ and $E_2$.

Table 3.4 Low (5 ng/ml), medium (20 ng/ml) and high (150 ng/ml) concentration accuracy and precision measured using variability over six injections.

| **Compound** | **Level** | **Concentration (ng/ml)** | **RSD(%)** | |
|---|---|---|---|---|
| | | | Accuracy | Precision |
| $E_1$ | Low | 5 | 11.6 | 16.2 |
| | Medium | 20 | 6.3 | 8.0 |
| | High | 150 | 4.9 | 6.8 |
| $E_2$ | Low | 5 | 3.2 | 16.5 |
| | Medium | 20 | 4.6 | 7.0 |
| | High | 150 | 11.3 | 6.7 |
| $E_1S$ | Low | 5 | 3.0 | 2.7 |
| | Medium | 20 | 5.2 | 2.6 |
| | High | 150 | 11.8 | 3.1 |
| $E_2S$ | Low | 5 | 2.9 | 1.1 |
| | Medium | 20 | 5.2 | 2.1 |
| | High | 150 | 14.5 | 3.8 |

### 3.7.5 Recovery

Recovery measures how efficient the extraction process is by measuring the detected response for the extracted analyte and internal standard compared to a non-extracted pure sample. It is not necessary for recovery to be 100%, but it does need to be reproducible with %RSD < 20%. For this method recovery for all steroids

ranged from 102-105% with low variation in extraction efficiency (%RSD) across the concentration range (6-16%, Table 3.5). Recovery over 100% reflects the variation in measurements within the assay as detailed in section 3.7.4.

Table 3.5 Recovery (%) for $E_1$, $E_2$, $E_1S$ and $E_2S$.

| | **$E_1$** | **$E_2$** | **$E_1S$** | **$E_2S$** |
|---|---|---|---|---|
| 10 (ng/ml) | 114 | 124 | 111 | 108 |
| 25 (ng/ml) | 97 | 98 | 100 | 103 |
| 250 (ng/ml) | 96 | 93 | 95 | 96 |
| Average | 102 | 105 | 102 | 102 |
| SD | 10.4 | 16.6 | 7.8 | 6.1 |
| RSD (%) | 10.1 | 15.8 | 7.6 | 6.9 |

These concentrations were representative of expected values within cell culture experiments.

### 3.7.6 Matrix Effects

Matrix effects are any influence exerted by components of the analyte containing solution which affects the signal, in this case cell media. The presence of other substances within media which elute off the column at the same time as each steroid could potentially interfere with the ionisation process of the analytes leading to ion-suppression or enhancement.

There are various methods that can minimise interference from co-eluting matrix compounds, however there is no standardised strategy for reducing effects and several methods may have to be employed (Trufelli et al., 2011):

- Sample preparation can reduce potential interfering compounds with SPE reported to be the most efficient way of overcoming matrix effects.
- Chromatographic conditions can be modified to shift retention times of target analytes away from interfering matrix compound.
- IS can compensate for signal alteration.
- Potentially use an alternative ionisation source, or ionisation mode which may be less sensitive to matrix effects.

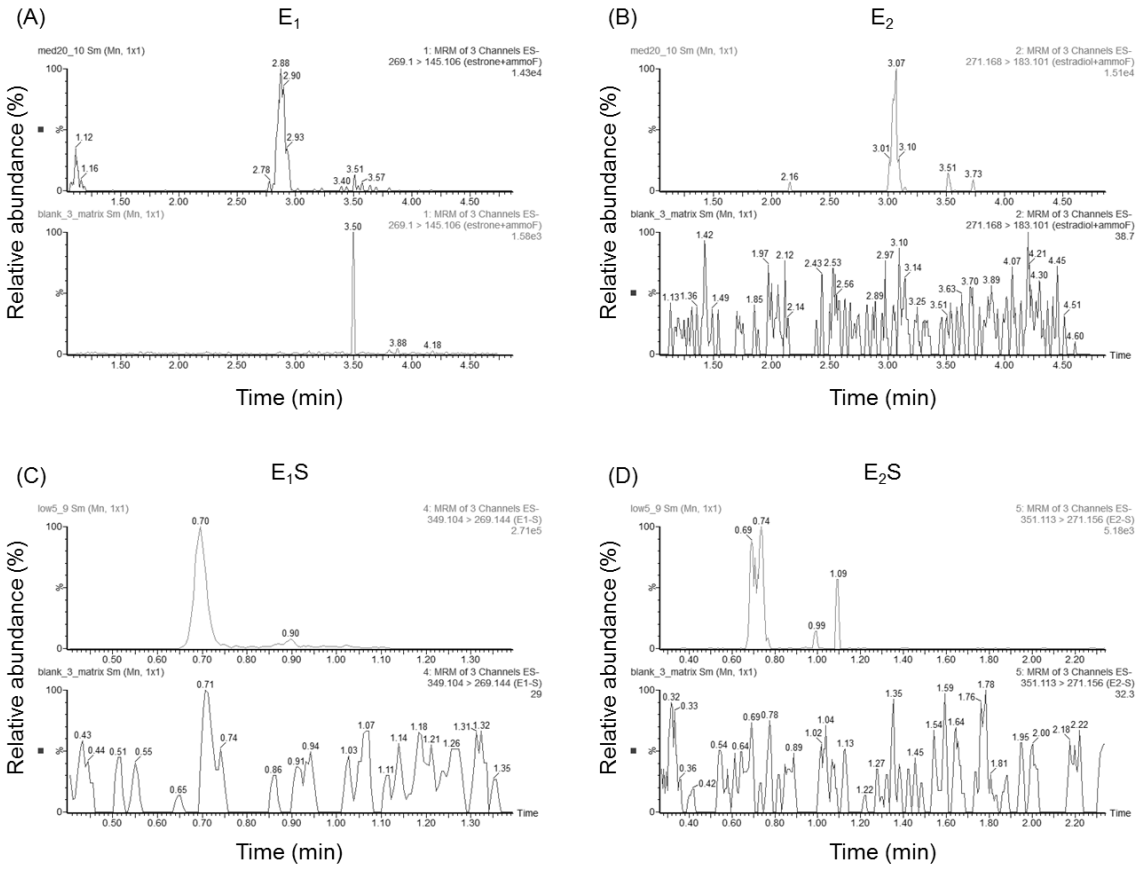



*Figure 3.7 No matrix effects. (A) $E_1$, (B) $E_2$, (C) $E_1S$ and (D) $E_2S$ by injection of water (below) or extracted cell culture medium (above) and continuous post-column infusion of 1000 ng/ml of $E_1$, $E_2$, $E_1S$ and $E_2S$. (A) The peak at 2.8 min represents $E_1$ in the medium sample (B) The peak at 3.07 min is $E_2$. (C) The peak at 0.7 min is $E_1S$. (D) The peak at 0.74 is $E_2S$. There was no loss or enhancement of signal due to components within the matrix.*

Matrix effects were assessed for each of the four oestrogens using the post column infusion method (Taylor, 2005). This is a qualitative assessment whereby each individual analyte (1000 ng/ml for each steroid) is constantly infused, followed by injection of an extracted blank sample (cell culture medium). Variation in signal response of the steroid by co-elution of interfering compounds would indicate ionisation suppression or enhancement. Figure 3.7 demonstrates no matrix effects as there is no peak/trough at retention time of analytes. The top graphs represent cell culture medium injection with peaks corresponding to each steroid from post column infusion and the bottom graphs are baseline. In graph A there is a peak at 3.5 min within the matrix. However, as it has a different retention time this is not an issue. Although in graphs B-D second plots look to have high peaks, the abundance is low with signal < 50 whereas in the top graphs signals are x $10^4$ or higher. Therefore the signals observed at baseline are not interfering components within the matrix.

## 3.8 DISCUSSION

Costs of using MS are falling along with improved sensitivity whilst maintaining specificity and multi-analyte capability (Handelsman and Wartofsky, 2013). However, LC-MS/MS remains expensive and still requires specialist knowledge, therefore alternative methods such as immunoassay and RIA are still commonly used (Krasowski et al., 2014). This may change as a leading journal, The Journal of Clinical Endocrinology and Metabolism, has strongly suggested that manuscripts reporting sex steroid assays as important endpoints should always use MS-based assays (Handelsman and Wartofsky, 2013). However, the use of “well validated

assays" is still acceptable in most studies considering that an immediate change to LC-MS/MS was unrealistic for many groups (Taylor et al., 2015).

This LC-MS/MS method is high throughput, quantifying $E_1$, $E_2$, $E_1S$ and $E_2S$ from the same extract in less than 5 minutes. Oestrogen sulphates are not routinely measured despite their significant role as an active oestrogen reservoir (Reed et al., 2005). Analysing both conjugated and unconjugated steroids is more challenging with many methods opting to quantifying them by dividing extracted samples and performing two separate LC-MS/MS analyses. This can involve both positive and negative ion modes, different ionisation techniques and changing columns (Galuska et al., 2013). This is good for improving sensitivity, but increases sample preparation time and costs, reducing sample throughput. This method has been fully validated to industry standards. LLOQ was 5 ng/ml for $E_1$ and $E_2$ and 0.5 ng/ml for $E_1S$ and $E_2S$ which was acceptable for cell culture experimental models. Owing to instrument limitations these are too high to measure oestrogens in male and postmenopausal female serum as their concentrations are low (Handelsman and Wartofsky, 2013, Huhtaniemi et al., 2012, Lee et al., 2006, Mayo Clinic, 2015). $E_1$ reference ranges in adult males and postmenopausal females are similar at 10-40 pg/ml and 0-40 pg/ml, respectively. $E_2$ ranges are 10-40 pg/ml for adult males and < 10 pg/ml for postmenopausal females (Staros, 2015, Mayo Clinic, 2015). Increasing starting sample volume would increase final concentration of the sample to be injected and so on the column, which could improve sensitivity, but could also potentially increase matrix effects. Additionally, a newer MS, such as the Waters Xevo TQ-S, could achieve limits of detection in the pmol/l range as demonstrated by Owen et

al. 2014 (Owen et al., 2014) and so potentially could be applied to lower concentration samples such as serum.

A major asset for this method development was use of MUSCLE software, vastly increasing the speed and optimisation process. The main advantage of MUSCLE over other programs was the ability to modify and optimise multiple parameters simultaneously. Additionally, MUSCLE, free to download, controlled the LC-MS/MS system and thus was set up to perform overnight and at weekends, maximising productivity whilst reducing cost. Overall MUSCLE reduced optimisation time from weeks to days.

This method was applied to CRC HCT116, Colo205, HT29 and Caco-2 cell lines as described in Chapter 5.

## 3.9 CONCLUSION

A novel high- throughput LC-MS/MS method validated to industry standards capable of quantifying $E_1$, $E_2$, $E_1S$ and $E_2S$ simultaneously in cell culture media was achieved. The optimised extraction and mass spectrometry method allows investigation of oestrogen metabolism in CRC cell lines. In future, expansions of this method to include other oestrogen metabolites would be beneficial to further elucidate oestrogen metabolism in CRC and other malignancies.

# CHAPTER 4. HUMAN COLORECTAL CANCER TISSUE OESTROGEN METABOLISM CHARACTERISATION

## 4.1 INTRODUCTION

Cancer is a heterogeneous disease and classification using stage, grade and cell type, although helpful, does not tailor patient specific treatment and prognosis. Therefore, there are patients who receive unnecessary treatments and side effects with disease recurrence (section 1.2.5). Detailed tumour characterisation opens up the potential for disease classification, targeted treatment and personalised medicine limiting these unnecessary and undesirable side effects.

The associations between oestrogens and CRC risk have been suggested for a number of decades. Nuns are nulliparous women with uninterrupted menstrual cycles and so have a higher lifetime exposure to oestrogen. They have a greater risk of CRC and breast cancer (Fraumeni et al., 1969) and women with higher circulating oestrogens are also at increased risk (Clendenen et al., 2009). Tumour oestrogen metabolism research has shown that increased desulphation (STS) and decreased sulphation (SULT1E1) enzyme expression with higher tumoural oestrogen concentrations reduce CRC survival (Sato et al., 2009). Also, oxidative pathways (HSD17β2 and 4) are reduced in CRC potentially increasing the more potent $E_2$ (English et al., 2000). Thus, tumour oestrogen metabolism enzyme expression and activity offer a credible CRC subclassification as well as treatment targets. No research group has yet looked at the pathway as a whole and with no hormonal treatments currently in use for CRC this is an area that needs further exploration.

Here oestrogen metabolism is examined in human CRC tissue with matched normal colon controls. The focus is on the enzymes within the sulphatase pathway that lead to $E_2$ generation from circulating $E_1S$.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Human Tissue

Selected tissue from HBRC (section 2.13) were all CRC adenocarcinomas and either male or post-menopausal females with no known risk factors such as genetic family history or IBD. Table 4.1 shows a summary of basic characteristics with further detail in Appendices I. These were matched normal and cancerous colon tissue from the same patient.

Table 4.1 Human colorectal tissue samples disease stage and average ages. Further details in Appendices I.

| | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Total | Age |
|---|---|---|---|---|---|---|
| Female | 6 | 13 | 10 | 2 | 31 | 71 (55-87) |
| Male | 5 | 10 | 17 | 0 | 32 | 73 (50-90) |
| Total | 11 | 23 | 27 | 2 | 63 | 72 (50-90) |

### 4.2.2 RNA extraction, cDNA synthesis and Real Time PCR

Human tissue RNA extraction followed by cDNA synthesis and cDNA quantification using Picogreen assay were performed as described in section 2.3 to 2.5. Real-time PCR was used to quantify mRNA expression for key oestrogen metabolism enzymes as described in section 2.6 using the primers and probes for HSD17β1, HSD17β2, HSD17β7, HSD17β12, STS, PAPSS1, PAPSS2 and internal standard gene RPLPO (Table 2.2). Validations of duplex reactions and choice of internal

standard are shown in Appendix II. SULT1E1 was not validated in either singleplex or duplex as shown in Appendix II and so mRNA expression could not be assessed.

### 4.2.3 Protein Extraction and Quantification

Human tissue protein was extracted using RIPA buffer and protein quantified using BCA assay as described in section 2.2.

### 4.2.4 Western Blotting

Western blotting was implemented as per procedure described in section 2.9. Primary and secondary antibodies were optimised and included HSD17β1, 2, 12, PAPSS1 and PAPSS2 with β-actin as loading control (Table 2.3). Despite extensive optimisation the commercial SULT1E1 and STS antibodies were not consistent and so the data was discarded.

### 4.2.5 STS Activity Assay

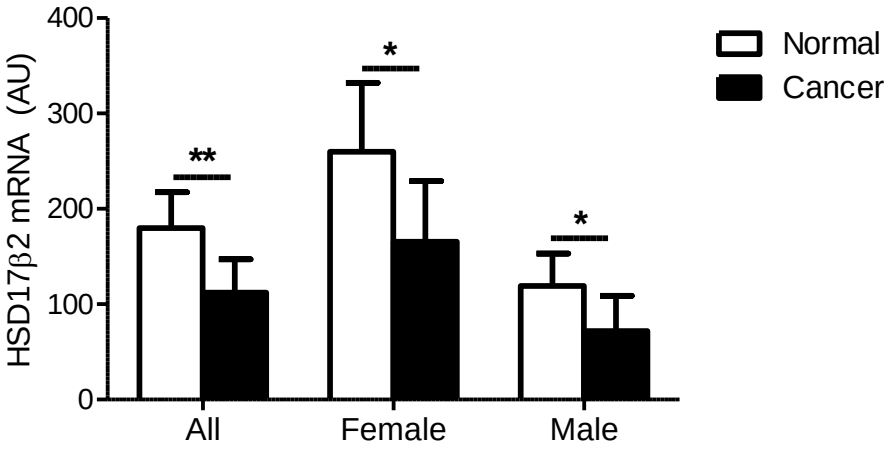
STS activity assay on human tissue was performed as described in section 2.8 using protein lysate.

## 4.3 RESULTS

### 4.3.1 Oxidative HSD17β2 Expression

HSD17β2 enzyme oxidises $E_2$ to $E_1$, reducing oestrogen's efficacy on ERs. HSD17β2 mRNA expression was detected in colon tissue with expression reduced in CRC ($p < 0.01$). When separated by gender HSD17β2 mRNA expression was

reduced in both females and males ($p < 0.05$, (Figure 4.1). This may change the $E_1$ and $E_2$ ratio, leading to an increase in the more potent oestrogen $E_2$ in CRC.




| HSD17β2 mRNA Expression (dCt) | All (n = 44) | Female (n = 19) | Male (n = 25) |
|---|---|---|---|
| Normal | 4.15 [3.33,4.96] | 2.99 [2.06,3.92] | 5.02 [3.84,6.21] |
| Cancer | 6.33 [5.27,7.39] | 5.25 [3.66,6.84] | 7.15 [5.71,8.58] |

*Figure 4.1 HSD17β2 mRNA expression is reduced in human colorectal cancer. HSD17β2 mRNA expression in human colorectal cancer compared to matched normal tissue (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. HSD17β2 mRNA expression reduced overall in colorectal cancer (**p < 0.01) and in females and males (*p < 0.05). Normal and cancer samples were matched and analysed using two-way ANOVA with Bonferroni post-test.*

CRC HSD17β2 protein expression as determined by Western blot did not correspond with mRNA data. HSD17β2 mRNA was consistently reduced in CRC (Figure 4.1) however protein expression was less consistent in both male and female samples (Figure 4.2). In many patients there even appeared to be an increase rather than decrease in expression.

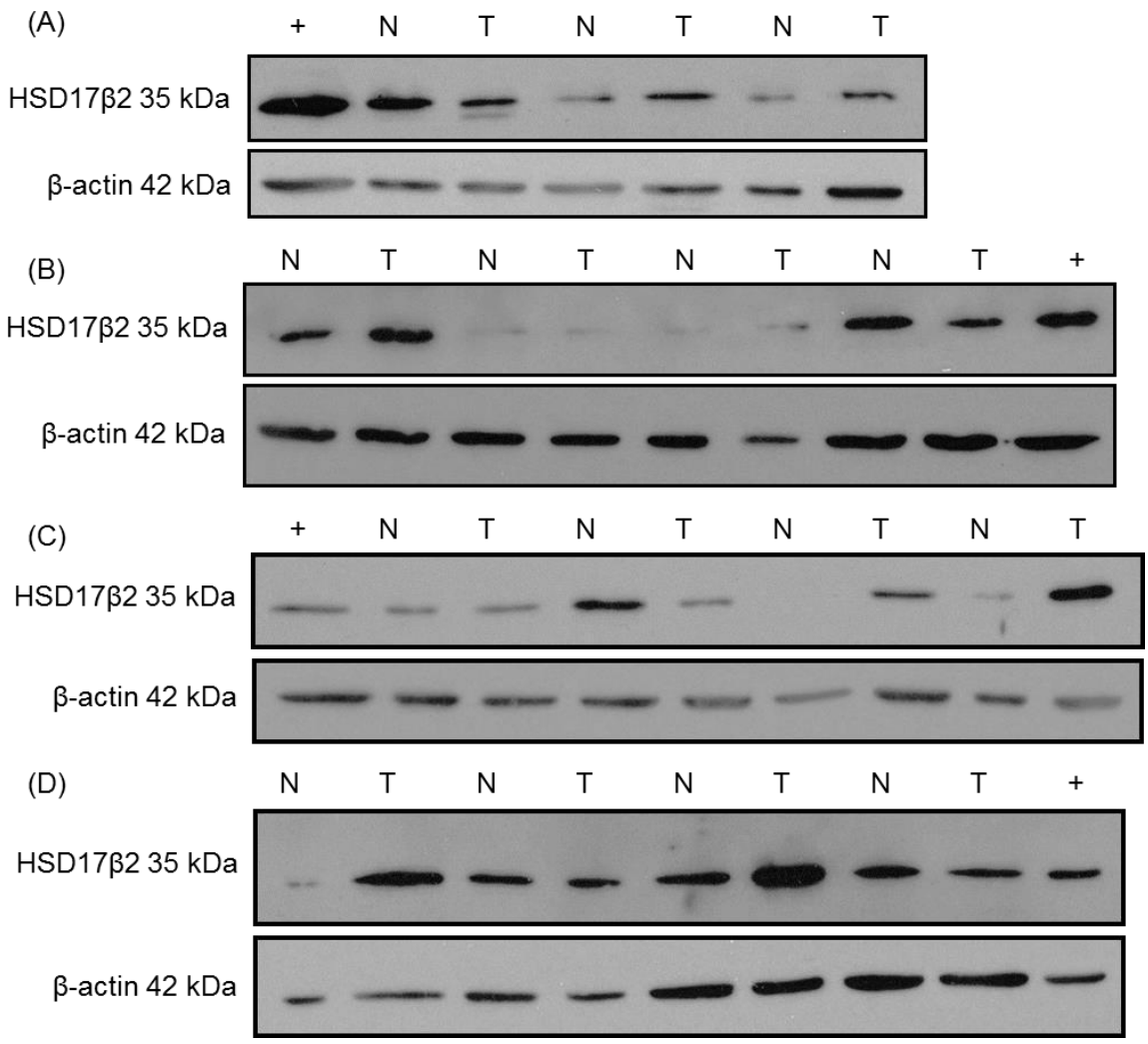



*Figure 4.2 Western blot of HSD17β2 expression in matched colorectal cancer tissue. Western blots of HSD17β2 in female (A and B) and male (C and D) colorectal normal (N) and tumour (T) matched tissue with Caco-2 cells used as positive control (+). No consistent change in protein expression of HSD17β2 between normal and cancerous colon tissue was seen. β-actin was used as loading control and 25 µg protein was loaded into each well, except positive control which was 15 µg.*

### 4.3.2 Reductive HSD17β Expression

#### 4.3.2.1 HSD17β1 Expression

HSD17β1 expression was examined in a selection of matched (CRC and normal tissue from same patient) human samples including seven female and six males. However, HSD17β1 mRNA was not detected in any samples. Western blots on six

of these tissues (three females and three males, Figure 4.3) showed no protein detection in normal or cancerous colon, but can clearly be seen to be expressed in the positive control JEG3 placental cell line.

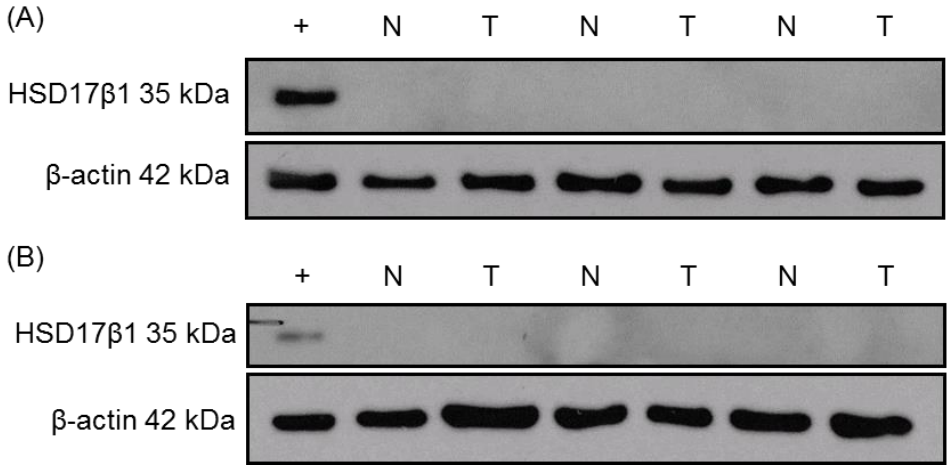
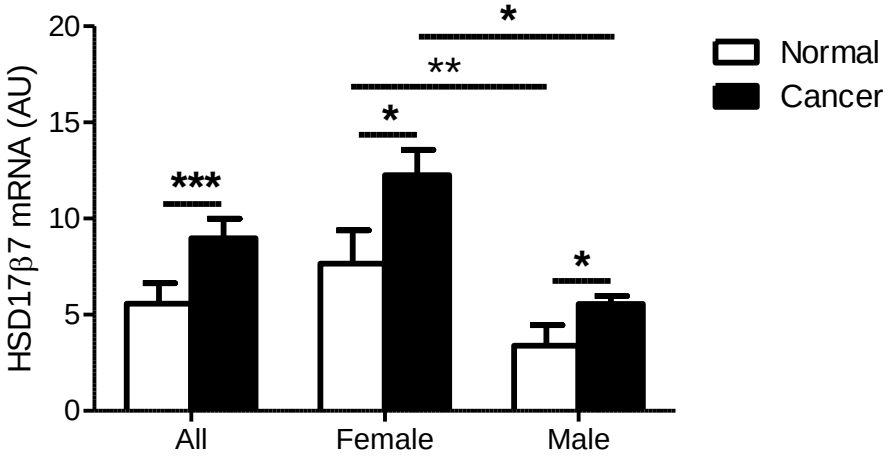



*Figure 4.3 HSD17β1 is not expressed in the colon. Western blot for HSD17β1 with JEG3 cells used as positive control (+). (A) Three female CRC (T) tissue samples with matched normal (N) tissue. (B) Three male samples. No HSD17β1 protein was detected in normal or cancerous colon tissue of both genders. β-actin was used as loading control with 25 µg protein loaded into each well, except positive control, which was 15 µg.*

#### 4.3.2.2 HSD17β7 mRNA Expression

Figure 4.4 shows mRNA expression of enzyme HSD17β7 in paired human CRC and normal tissue. HSD17β7 was identified in the colon and more highly expressed in CRC independent of sex. HSD17β7 was higher in females than males in normal and cancerous colon tissue.



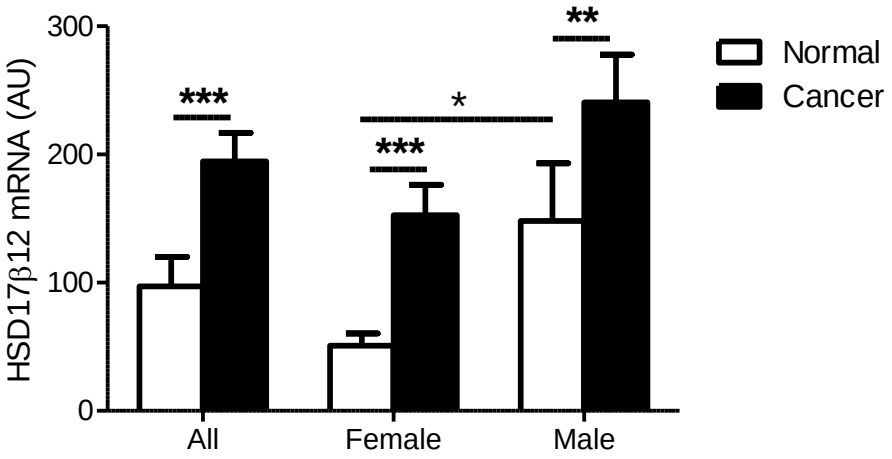

| HSD17β7 mRNA Expression (dCt) | All (n = 41) | Female (n = 21) | Male (n = 20) |
|---|---|---|---|
| Normal | 8.56 [7.88,9.23] | 7.81 [6.86,8.76] | 9.34 [8.42,10.26] |
| Cancer | 7.23 [6.86,7.59] | 6.56 [6.18,6.93] | 7.93 [7.42,8.43] |

*Figure 4.4 HSD17β7 mRNA is increased in colorectal cancer. HSD17β7 mRNA expression in human colorectal cancer compared to matched normal tissue expressed in arbitrary units (AU) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. HSD17β7 was expressed in both normal and cancerous colon tissue with higher mRNA expression in colorectal cancer (***p < 0.001) in both females and males (*p < 0.05). Females have higher expression than males in both normal (**p < 0.01) and cancerous (*p < 0.05) colon. Normal and cancer samples were matched and analysed using two-way ANOVA with Bonferroni post-test.*

Immunoblot with HSD17β7 bands at correct sizing was not possible and so protein expression was not assessed (see example in Figure 5.4).

#### 4.3.2.3 HSD17β12 mRNA Expression

HSD17β12 was expressed in both heathy and cancerous colon tissue (Figure 4.5). HSD17β12 enzyme was more highly expressed in CRC in both males and females. Males also had higher normal HSD17β12 expression than females.




| HSD17β12 mRNA Expression (dCt) | All (n = 40) | Female (n = 21) | Male (n = 19) |
|---|---|---|---|
| Normal | 4.39 [3.84,4.95] | 4.99 [4.26,5.73] | 3.73 [2.94,4.53] |
| Cancer | 2.76 [2.41,3.12] | 3.06 [2.60,3.52] | 2.44 [1.88,2.99] |

*Figure 4.5 HSD17β12 mRNA is increased in colorectal cancer. HSD17β12 mRNA expression in human colorectal cancer compared to matched normal tissue expressed in arbitrary units (AU) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. HSD17β12 is higher in colorectal cancer tissue compared to matched normal control (***p < 0.001) in both females (***p < 0.001) and males (**p < 0.01). Females have lower expression than males in normal colon tissue (*p < 0.05). Normal and cancer samples were matched and analysed using two-way ANOVA with Bonferroni post-test.*

HSD17β12 Westerns for females and males are shown in Figure 4.6 and as with HSD17β2, protein expression did not match mRNA expression and was not detected in all samples.

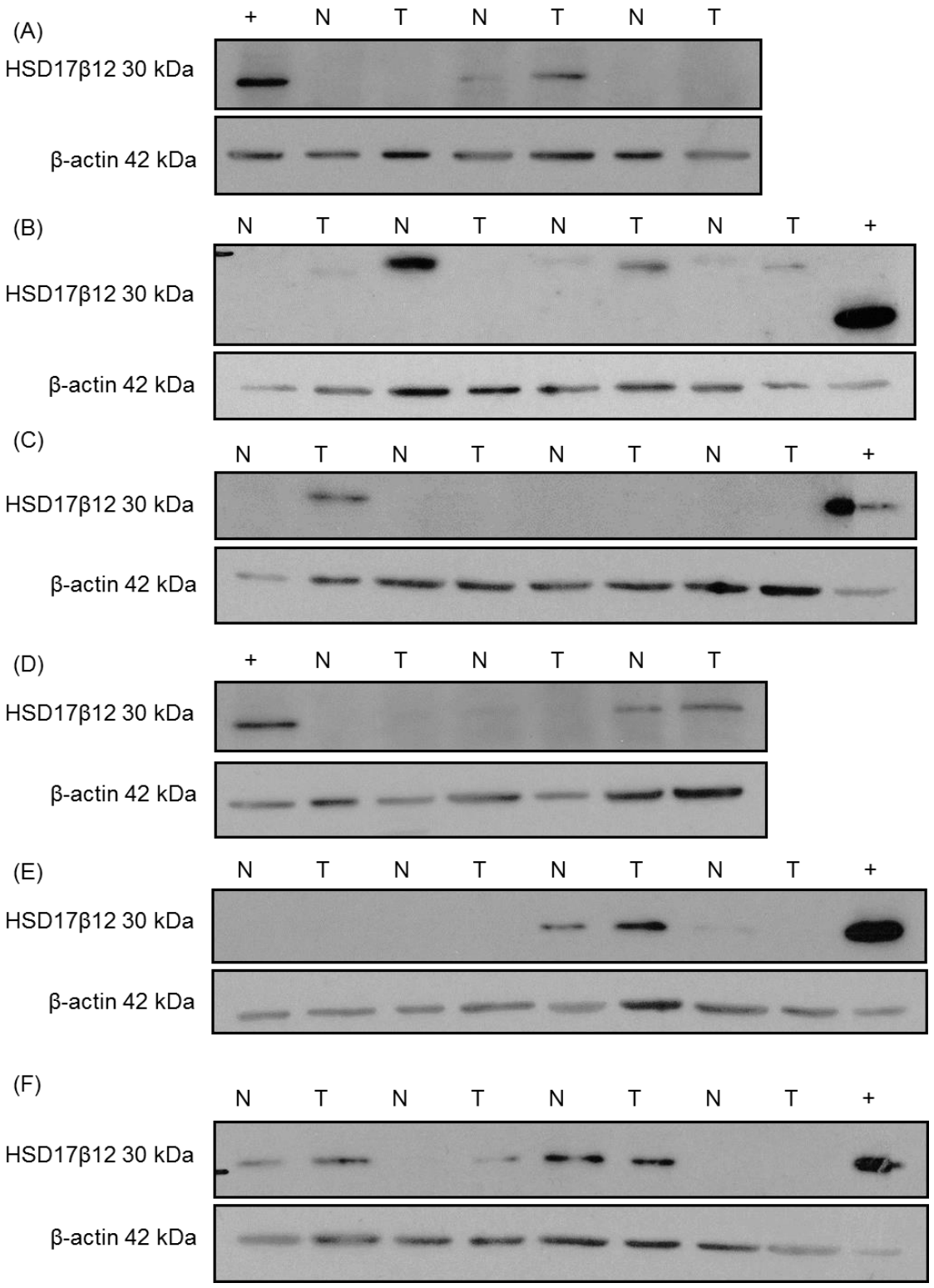
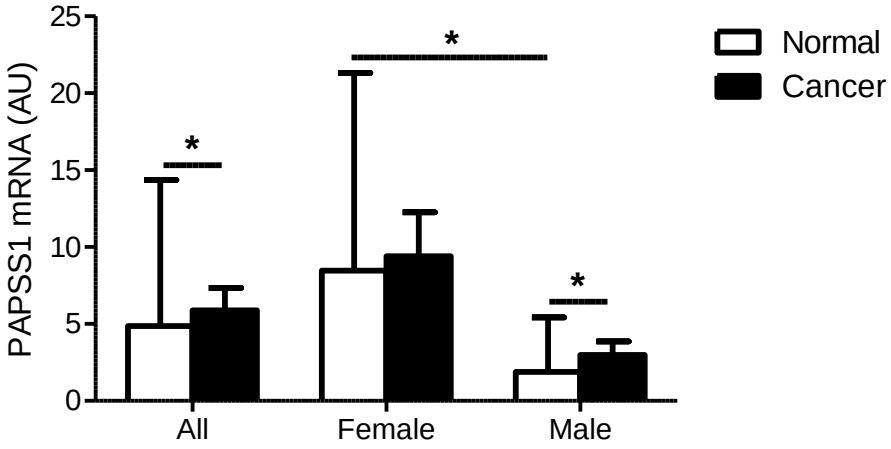



*Figure 4.6 Western blot of HSD17β12 expression in matched colorectal cancer tissue. Female (A-C) and male (D-F) HSD17β12 Western blot of human colorectal cancer tissue (T) with matched normal (N) and HCT116 cells as positive control (+). β-actin is used as loading control and 25 µg of colon tissue protein was loaded into each well and 15 µg of HCT116 protein.*

### 4.3.3 PAPPS1 and PAPSS2 Expression

There was no validated SULT1E1 mRNA primer and probe or consistent commercial antibody for protein detection thus expression could not be determined. Attempts were made to develop a SULT1E1 activity assay in extracted protein. However, problems arose with PAPS purity and it was hypothesised that the RIPA buffer used may have affected SULT1E1 structure and function.



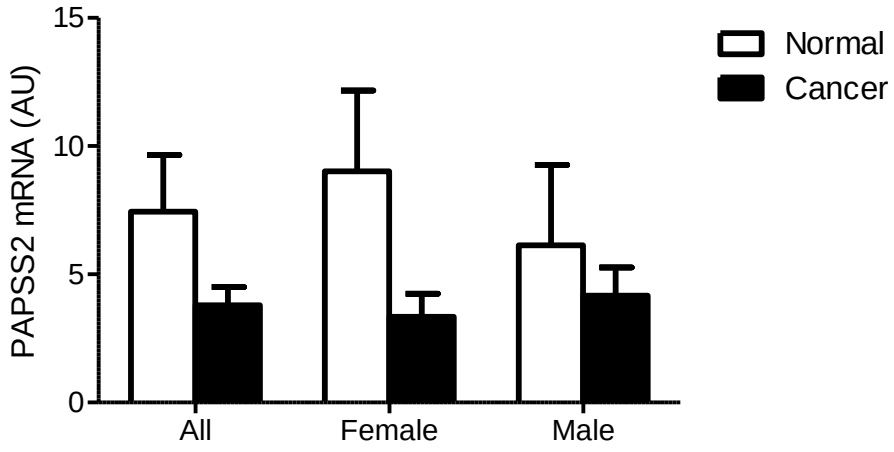

| PAPSS1 mRNA Expression (dCt) | All (n = 42) | Female (n = 19) | Male (n = 23) |
|---|---|---|---|
| Normal | 9.89 [8.85,10.92] | 8.73 [7.04,10.41] | 10.84 [9.60,12.09] |
| Cancer | 8.79 [8.16,9.42] | 7.91 [6.96,8.86] | 9.51 [8.73,10.28] |

Figure 4.7 PAPPS1 mRNA expression in matched colorectal cancer tissue. PAPPS1 mRNA expression in human colorectal cancer compared to matched normal tissue (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. PAPPS1 was detected in both normal and cancerous colon tissue. There was large expression variability with significant increases in male CRC (*$p < 0.05$) and expression in normal female colon was higher than normal male colon (*$p < 0.05$). Normal and cancer samples were matched and analysed using two-way ANOVA with Bonferroni post-test.

PAPSS is required to generate the universal sulphate donor (PAPS) for SULT1E1 action and impacts on SULT1E1 regulation (section 1.6.2). Therefore, PAPSS expression was investigated as a possible marker of sulphation activity within CRC.

PAPSS1 and 2 expression were explored, though it should be noted they are not a validated substitute for measurement of SULT1E1. PAPSS1 (Figure 4.7) mRNA was statistically different in CRC in all samples and males. However, the actual difference was small questioning the physiological impact.

PAPSS2 mRNA expression appeared to be reduced in CRC however this trend was not significant (Figure 4.8).




| PAPSS2 mRNA Expression (dCt) | All (n = 44) | Female (n = 20) | Male (n = 24) |
|---|---|---|---|
| Normal | 9.34 [8.46,10.23] | 8.93 [7.49,10.37] | 9.69 [8.52,10.87] |
| Cancer | 9.30 [8.57,10.04] | 9.41 [8.22,10.60] | 9.21 [8.22,10.20] |

Figure 4.8 PAPPS2 mRNA expression in matched colorectal cancer tissue. PAPPS2 mRNA expression in human colorectal cancer compared to matched normal tissue (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. PAPPS2 was detected in both normal and cancerous colon tissue however there were no significant differences between normal tissue and cancerous tissue.

PAPSS1 and PAPSS2 protein expression was assessed by WB and samples followed similar trends to their mRNA expression (Figure 4.9).

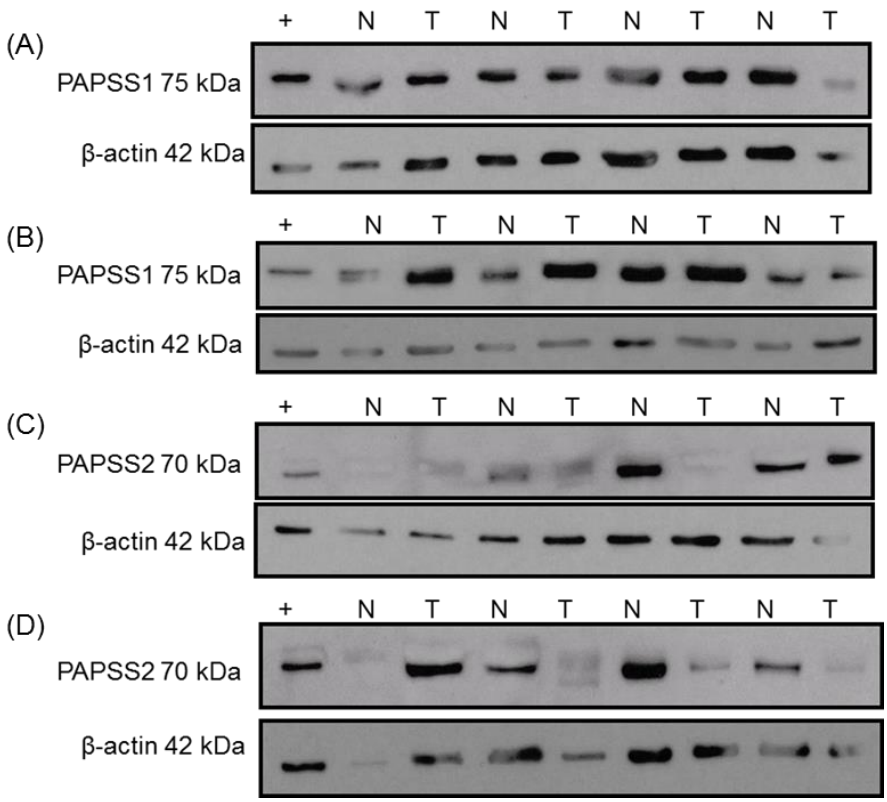
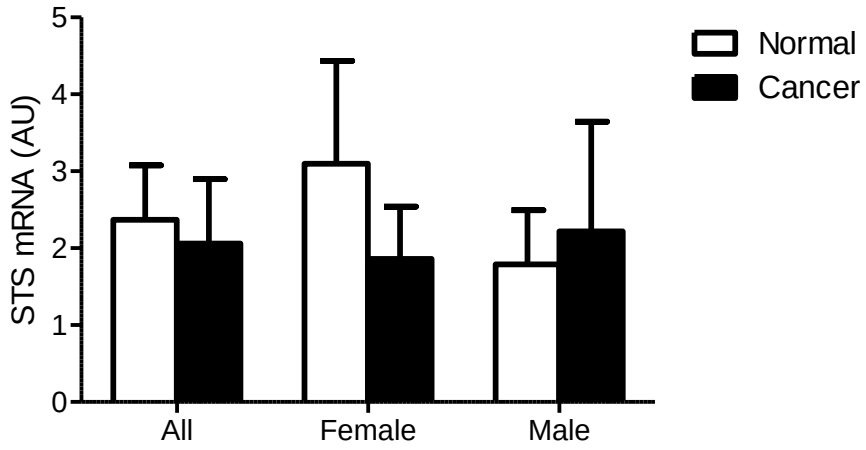



*Figure 4.9 Western blot of PAPSS1 and 2 expression in colorectal cancer tissue (T) with matched normal (N) and HT29 cells used as positive control (+). β-actin is used as loading control and 25 µg of colon tissue protein was loaded into each well and 15 µg of HT29 protein. Western blot of PAPSS1 female (A), male (B) and PAPSS2 female (C) and male (D) protein expression.*

### 4.3.4 STS Expression and Activity

STS mRNA expression was identified in the colon however no significant changes in expression were observed between normal and cancerous matched colon tissue in either males or females (Figure 4.10).

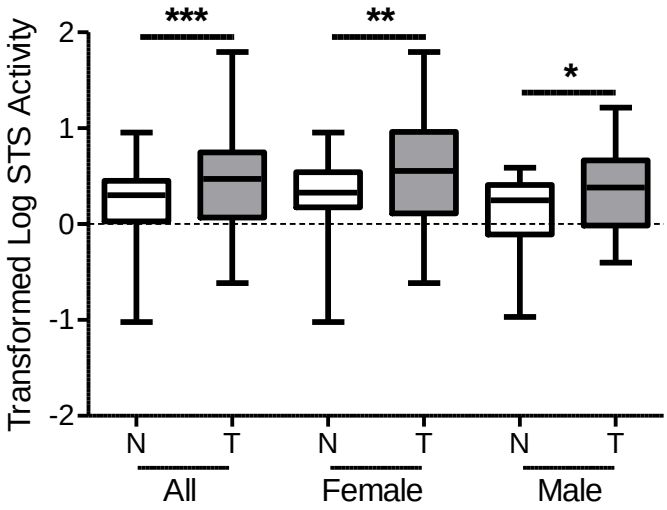



| STS mRNA Expression (dCt) | All (n = 45) | Female (n = 20) | Male (n = 25) |
|---|---|---|---|
| Normal | 11.51 [10.57,12.45] | 11.09 [9.53,12.65] | 11.85 [10.62,13.09] |
| Cancer | 11.24 [10.50,11.98] | 10.71 [9.67,11.75] | 11.67 [10.60,12.74] |

*Figure 4.10 STS mRNA expression is unchanged in colorectal cancer. STS mRNA expression in human colorectal cancer compared to matched normal tissue (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. STS was detected in both normal and cancerous colon tissue. There were no significant differences between normal tissue and cancerous tissue expression in either males or females.*

STS mRNA could not be compared to STS protein expression using Western blotting as no consistent commercial antibody was available. This was despite optimisation attempts using antibodies from different manufacturers (Abcam, Abnova). STS can undergo post-translational modification, impacting on activity. Therefore, mRNA and protein expression may not necessarily coincide with activity (section 1.5.1). STS activity measured using a reliable $^{3H}E_1S$ assay was the preferred method to quantify functional differences in STS in CRC. Figure 4.11 demonstrates a significant increase in STS activity in CRC compared to normal

matched controls. This shows the disparity in expression and activity and highlights why multiple analysis methods are required before conclusions can be made.




| $E_1S$ to $E_1$ (nmol/mg/hr) | All (n = 61) | Female (n = 29) | Male (n = 32) |
|---|---|---|---|
| Normal | 1.30 [1.20,1.40] | 1.35 [1.20, 1.51] | 1.25 [1.11,1.39] |
| Cancer | 1.56 [1.36, 1.75] | 1.72 [1.37,2.07] | 1.42 [1.22,1.62] |

*Figure 4.11 STS activity is increased in colorectal cancer. Homogenised human colorectal tissue was subjected to $^{3H}E_1S$ assay and conversion to $E_1$ measured in nmol/mg/hour with mean and 95% CI displayed in brackets in the table. As STS activity was not normally distributed, further analyses used random effects linear regression modelling (with outcomes transformed where appropriate to reduce the impact of outliers) to allow for normal and cancer samples being patient matched. Results were further log transformed and the estimates obtained are interpreted as approximate percentage differences. STS activity was increased in colorectal cancer in both females and males. Two-tailed paired t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, error bars are SD.*

#### 4.3.4.1 STS Activity, HSD17β and PAPSS Expression Correlations

Changes seen in CRC included decreased HSD17β2, increased HSD17β7 and 12 and increased STS activity with no changes in STS mRNA. To assess if any changes in CRC were likely to occur together correlations between these enzymes

were examined. HSD17β7 and 12 perform the same reductive $E_1$ to $E_2$ function and mRNA expression for both was increased in CRC. When HSD17β7 was plotted against HSD17β712 it can be seen that mRNA expression correlated in normal but not in cancerous tissue (Figure 4.12 (C)). Therefore, in cancer both do not increase simultaneously. HSD17β2 mRNA is decreased in CRC and was plotted against HSD17β7 and 12 mRNA. These also did not correlate in either normal or cancerous colon tissue (Figure 4.12 (A and B)). STS mRNA expression was unchanged between normal and cancerous colon however STS activity was found to be increased. It was therefore hypothesised that due to post-translational modifications there would be no correlation between STS mRNA expression and STS activity. Figure 4.12 (H) shows no correlation between mRNA and activity for STS in either normal or cancerous colonic tissue. STS action can lead to generation of active oestrogens through desulphation and has been shown to be involved in feedback mechanisms to regulate oestrogen concentrations within tissues. HSD17β enzymes affect the potency of local oestrogens and may also be subject to feedback loops and so correlations were investigated between STS activity and each of the HSD17β enzymes. However no correlations were identified (Figure 4.12 (D to F)). PAPSS1 and 2 mRNA were also compared to see if there was any correlation between expression in normal and cancerous colon tissue. In normal healthy colon PAPSS1 and 2 expression did correlate ($p = 0.03$) however this was not the case in CRC (Figure 4.12 (G)). Taken together there were no correlations in normal or cancerous colon between these oestrogen enzymes.

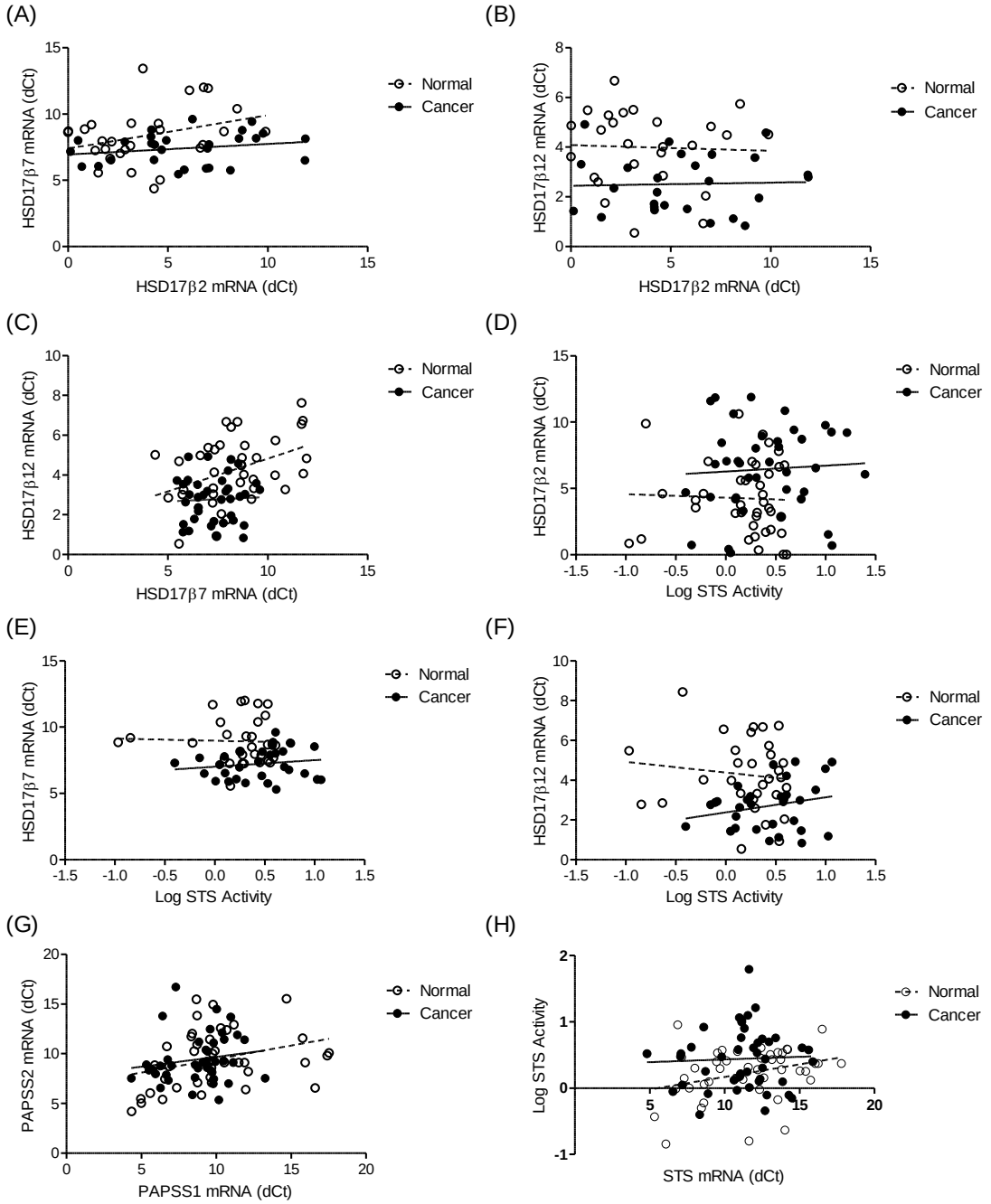



*Figure 4.12 No correlations between CRC oestrogen enzymes. HSD17β7 (A) and 12 (B) mRNA plotted against HSD17β2 mRNA. Expression did not correlate in either normal or cancerous colon tissue (n = 28 and 26, respectively). (C) HSD17β7 and 12 mRNA. Expression correlated in normal ($R^2$ = 0.161, *p = 0.01) but not in cancerous colon tissue ($R^2$ = 0.002, p = 0.79) (n = 38). Log STS activity was compared to HSD17β2 (D), HSD17β7 (E) and HSD17β12 (F) but no correlation was seen. (G) PAPSS1 and PAPSS2 mRNA. Expression correlated in normal ($R^2$ = 0.0997, *p = 0.03) but not in cancerous colon tissue ($R^2$ = 0.02768, p = 0.12). (H) STS activity did not correlate with STS expression (dCT) in normal or cancerous colon tissue.*

As mRNA expression of oxidative HSD17β2 was decreased and reductive HSD17β7 and 12 were increased, changes in the ratio of the mRNA of these enzymes was assessed. Although there appeared to be an increase in the ratio of reductive/ oxidative mRNA, this was not significant (Figure 4.13).

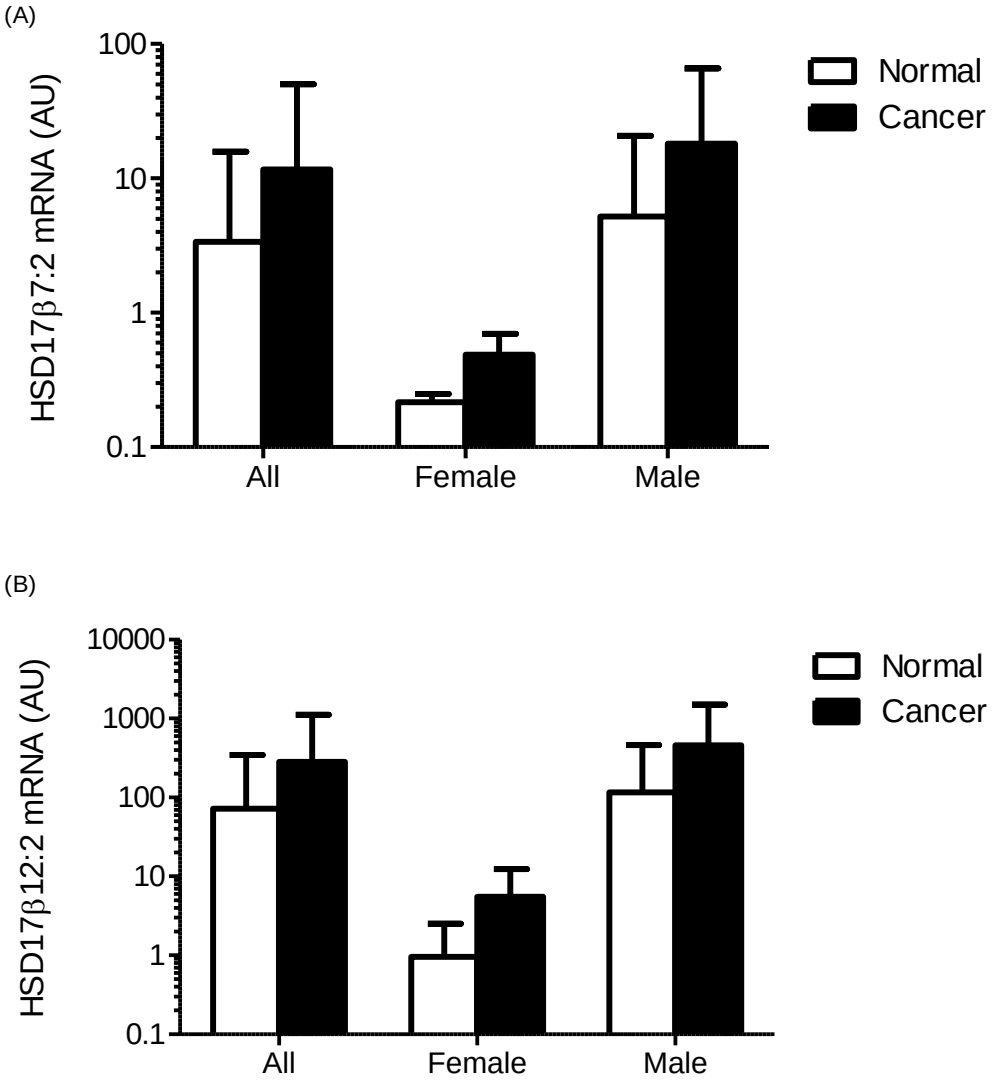


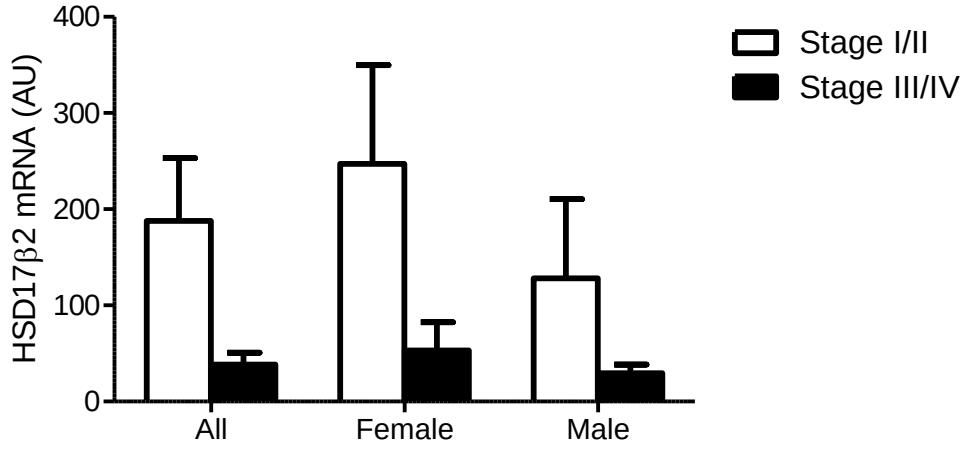

*Figure 4.13 Ratio of HSD17β7:HSD17β2 and HSD17β12:HSD17β2 mRNA expression changes in colorectal cancer. (A) HSD17β7 and (B) HSD17β12 over HSD17β2 mRNA (arbitrary unites (AU) on log scale, error bars are SEM).*

### 4.3.5 HSD17β Expression and STS Activity by Disease Stage

To assess if HSD17β2, HSD17β7 and 12 mRNA and STS activity in CRC were related to TNM stage patients were divided into two groups; TNM stage I and II, or III and IV.

#### 4.3.5.1 HSD17β2

Later stage disease was associated with lower expression of HSD17β2 mRNA in both males and females however this trend was not significant (Figure 4.14).

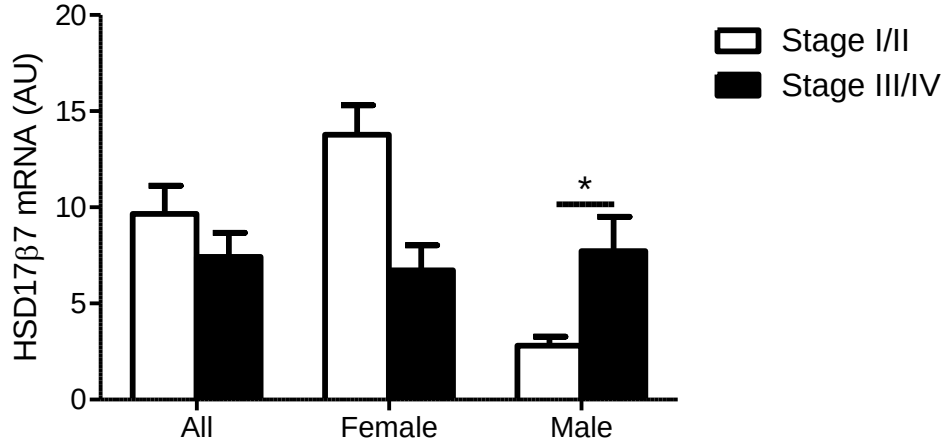



| HSD17β2 mRNA Expression (dCt) | All | Female | Male |
|---|---|---|---|
| Stage I/II | 5.36 [3.82,6.90] | 4.31 [2.19,6.42] | 6.57 [4.39, 8.74] |
| Stage III/IV | 6.88 [5.42,8.33] | 6.55 [3.84,9.26] | 7.08 [5.10,9.06] |

*Figure 4.14 HSD17β2 mRNA expression is reduced in later stage colorectal cancer. HSD17β2 mRNA expression (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below for CRC TNM stage. Expression is lower in latter colorectal cancer disease stage; however this trend was not significant. Stage I/II n = 11 for females and n = 11 males. Stage III/IV n = 8 for females and n = 13 for males.*

#### 4.3.5.2 HSD17β7

HSD17β7 mRNA expression in all CRC patients appeared similar in earlier and later disease stage. However, when divided into gender males had increased HSD17β7 in stage III/IV compared to stage I/II (Figure 4.15).



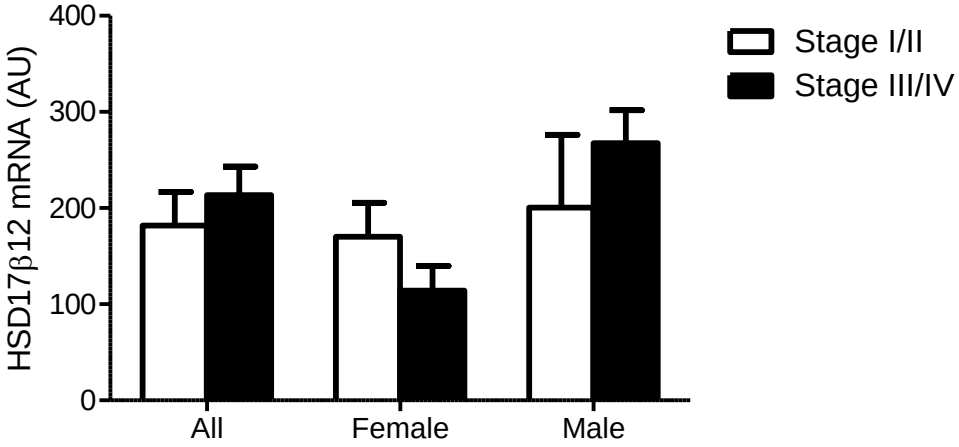

| HSD17β7 mRNA Expression (dCt) | All | Female | Male |
|---|---|---|---|
| Stage I/II | 7.19 [6.63,7.75] | 6.34 [5.92,6.76] | 8.62 [8.10,9.13] |
| Stage III/IV | 7.35 [6.87,7.83] | 7.32 [6.54,8.11] | 7.36 [6.67,8.06] |

*Figure 4.15 HSD17β7 mRNA expression is different in males and females in later stages of colorectal cancer. HSD17β7 mRNA expression (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below for CRC TNM stage. Expression was increased in males in later stage disease (*p < 0.05). Two-way ANOVA with Bonferroni post-test analysis. Stage I/II n = 15 for females and n = 9 males. Stage III/IV n = 5 for females and n = 11 for males.*

#### 4.3.5.3 HSD17β12

HSD17β12 mRNA expression was unchanged across CRC disease stages. This enzyme also showed some gender disparity with HSD17β12 reduced in later

disease stages for females and increased in males (Figure 4.16), though this did not reach significance.

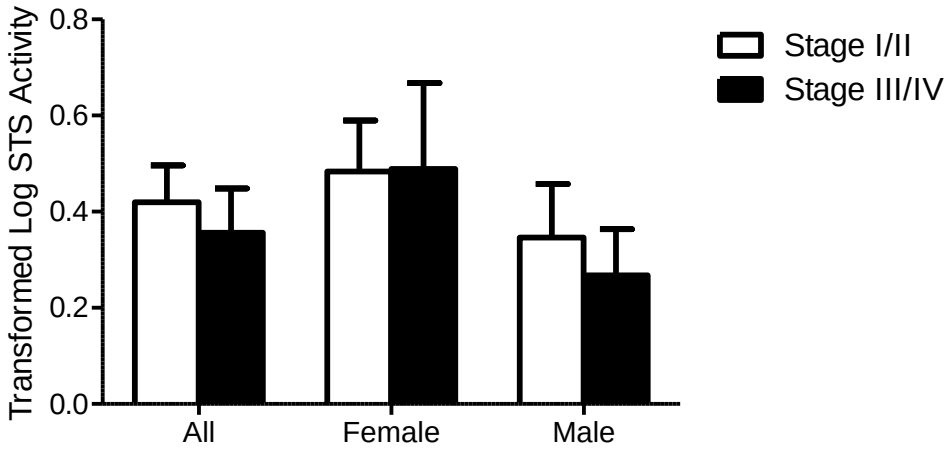



| HSD17β12 mRNA Expression (dCt) | All | Female | Male |
|---|---|---|---|
| Stage I/II | 2.94 [2.40,3.48] | 2.91 [2.27,3.55] | 2.99 [1.76,4.21] |
| Stage III/IV | 2.51 [1.99,3.03] | 3.37 [2.33, 4.42] | 2.04 [1.58,2.49] |

*Figure 4.16 HSD17β12 mRNA expression is similar across colorectal cancer disease stages in males and females. HSD17β12 mRNA expression (arbitrary units (AU)) (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below for CRC TNM stage. Stage I/II n = 13 for females and n = 8 males. Stage III/IV n = 6 for females and n = 11 for males.*

#### 4.3.5.4 STS

The relationship between CRC TNM stage and STS activity was also explored and results displayed in Figure 4.17. There were no significant differences between the groups.




| $E_1S$ to $E_1$ (nmol/mg/hr) | All | Female | Male |
|---|---|---|---|
| Stage I/II | 1.61 [1.35,1.87] | 1.69 [1.27,2.11] | 1.52 [1.18,1.85] |
| Stage III/IV | 1.58 [1.26,1.91] | 1.87 [1.13,2.60] | 1.40 [1.10,1.67] |

*Figure 4.17 STS activity is similar across colorectal cancer disease stages in males and females. As STS activity was not normally distributed, further analyses used random effects linear regression modelling (with outcomes transformed where appropriate to reduce the impact of outliers) to allow for normal and cancer samples being patient matched. Results were further log transformed and the estimates obtained are interpreted as approximate percentage differences. Error bars are SEM. The table below shows the mean STS activity ($E_1S$ to $E_1$) in nmol/mg/hr for CRC TNM stage with 95% CI in brackets. Stage I/II n = 15 for females and n = 13 males. Stage III/IV n = 10 for females and n = 15 for males.*

## 4.4 DISCUSSION

### 4.4.1 Oestrogen Oxidoreductive Pathways in Colorectal Cancer

Here HSD17β1, 2, 7 and 12 have been examined in human CRC. There is a reduction in HSD17β2 mRNA expression suggesting $E_2$ to $E_1$ oxidation is down-regulated in CRC (Figure 4.1). Normal female colon tissue had higher HSD17β2 mRNA expression compared to normal male tissue, though this was not statistically significant. It is well documented that many females experience bowel changes

during their menstrual cycle with oestrogen frequently causing constipation (Bernstein et al., 2014, Oh et al., 2013). Gender differences may be due to a higher lifetime exposure to circulating oestrogen concentrations in women compared to men. The colon may have higher oxidative capacity to reduce oestrogen potency in an attempt to maintain homeostasis. These changes may then continue to persist after the menopause. Oxidoreductive pathways have previously been examined in CRC (English et al., 2001) and this data supports published findings of decreased HSD17β2 expression in CRC. However, here the dataset is larger and shows novel gender disparities.

Ratios of HSD17β7 and 12 to HSD17β2 mRNA showed a shift towards reductive HSD17β expression, though this was not significant (Figure 4.13). Additionally, this was mRNA only and may not relate to activity of these enzymes. In addition to measuring activity it would be beneficial to expand mRNA and protein expression of this sample set to include other HSD17β types that may alter $E_1/E_2$ metabolism. HSD17β4 is potentially able to oxidise $E_2$ to $E_1$ but *in vivo* evidence suggests its main role is in peroxisomal fatty acid β-oxidation (Breitling et al., 2001, Qin et al., 1997, Day et al., 2008b) with only a minor role in steroid metabolism (Miettinen et al., 1999). Nevertheless, HSD17β4 has also been shown to be down-regulated in CRC (English et al., 2000). Thus, a collective decrease in $E_2$ oxidation via HSD17β2, 4 would potentially increase the ratio of $E_2$ to $E_1$ in CRC. There is also evidence that HSD17β8 inactivates $E_2$ however it also has reductive activity (Fomitcheva et al., 1998). HSD17β8 is located in mitochondria and suspected to have a larger role in fatty acid metabolism (Chen et al., 2009) thus the role in steroidogenesis is currently

unclear. A proteomic study did find HSD17β8 to be increased in late stage CRC (Luque-García et al., 2010), but due to its dual activity it is unclear what effect this would have on the $E_1/E_2$ ratio.

HSD17β2 protein expression as assessed semi-quantitatively by Western blot did not follow the same expression pattern as mRNA. Protein was detected in the majority of samples tested (Figure 4.2), but some CRC samples had increased and others decreased HSD17β2 compared to matched normal tissue. This is in keeping with published data (English et al., 2000). It is not unusual to find discrepancies between mRNA and protein; mRNA is not a surrogate for corresponding protein levels or activity without further verification (Kendrick, 2014). There are many biological and technical factors as to why this occurs such as translation efficiency and protein half-life, additionally post-translational modifications and co-factor availability are important for enzymatic activity (Guo et al., 2008, Maier et al., 2009). In breast cancer cell lines activity assays of HSD17β1 and 2 have shown high association with mRNA expression (Day et al., 2006). Additionally, colon oxidative enzymatic activity has been shown to more closely follow mRNA expression; healthy colon favours $E_2$ to $E_1$ metabolism which was reduced in CRC (English et al., 2000). Unfortunately without activity data it is not possible to conclude that HSD17β2 activity is reduced in CRC.

HSD17β1 is the principal enzyme for $E_1$ to $E_2$ conversion (Martel et al., 1992, Peltoketo et al., 1996) and is found in hormonal and reproductive tissues such as breast, endometrium and cervix (Setiawan et al., 2004, Tomaszewska et al., 2015).

Reductive activity of $E_1$ to $E_2$ has previously been measured in the colon (English et al., 1999). Some papers have noted HSD17β1 expression in the colon with lower expression in CRC (Rawluszko et al., 2011). However, mRNA and protein expression were not detected in the present study (Figure 4.3). Alternative $E_1$ to $E_2$ HSD17β enzymes that may be expressed in the colon were explored including HSD17β7 and 12 (Torn et al., 2003). Here detection of HSD17β7 and 12 is shown for the first time in the normal and cancerous colon (Figure 4.4 to Figure 4.6). Additionally, mRNA was increased in CRC for both enzymes. HSD17β12 was detected using WB, but as with HSD17β2 did not follow the same pattern as mRNA. HSD17β7 mRNA expression was lower than HSD17β12 and not detected by WB. It is possible that HSD17β7 protein is not expressed in the colon, or alternatively the problems with detection could be antibody related or due to the protein extraction method used i.e. RIPA buffer.

At the time of this thesis methods pertaining to HSD17β enzyme activity in fresh or homogenised human colon tissue were not available to confirm $E_2/E_1$ ratio changes suggested by mRNA expression. Although there were clear oxidoreductive HSD17β enzymes mRNA changes in CRC, protein expression was highly variable and unfortunately it was impossible to deduce enzyme activity from current available data.

### 4.4.2 Sulphatase Pathway in Colorectal Cancer

STS mRNA, protein and activity have been shown to increase in many hormonal cancers such as breast and prostate (Reed et al., 2005, McNamara et al., 2013).

Previously STS upregulation and SULT1E1 downregulation in the colon has been associated with a poorer prognosis and the reverse was also shown; low STS and high SULT1E1 correlated with improved survival (Sato et al., 2009). Here the dataset was smaller but also identified STS mRNA in the colon but with no change in expression in CRC compared to matched normal tissue (Figure 4.10). STS protein would have been assessed using WB but there was no usable commercial WB antibody, however post-translational modification of the STS enzyme can significantly alter activity (section 1.5.1). Thus, protein quantification would not accurately determine functional desulphation changes in CRC and consequential oestrogen activation. Instead STS activity was measured using $^{3H}E_1S$. STS activity was increased in CRC (Figure 4.11) supporting previous research (Sato et al., 2009). As mRNA had remained unchanged in CRC it was unsurprising that STS mRNA and activity did not correlate (Figure 4.12 (H)). Therefore, factors other than increased transcription or $E_1S$ availability must affect STS activity in CRC. For example as discussed in section 1.5.1 the STS enzyme can undergo post-translational glycosylation impacting on activity (Stengel et al., 2008). Additionally, C75 to FGly occurs at the active site and is regulated by SUMF1 and 2 availability (Thomas and Potter, 2013, Schmidt et al., 1995).

### 4.4.3 SULT1E1 and PAPSS in Colorectal Cancer

A limitation of this study was the inability to measure SULT1E1 either mRNA, protein or activity. Despite careful optimisation, real time primers and probes could not be validated and Western Blots revealed no consistent bands. As PAPS is a universal sulphate donor and can affect SULT1E1 activity (section 1.6.2) PAPSS1 and

PAPSS2 expression was explored. Changes in PAPSS may have a knock-on effect with SULT1E1 but are not a validated substitute or indirect measurement. PAPSS1 was significantly increased in male CRC compared to matched normal tissue (Figure 4.7) and WB followed mRNA expression (Figure 4.9). However, the actual increase was small so the physiological impact is unclear.

### 4.4.4 Oestrogen Enzyme Correlations

The pattern emerging from mRNA and STS activity data implied in human CRC there was an increase in active $E_2$ synthesis compared to normal colon; reduced HSD17β2, increased HSD17β7 and 12 and increased STS activity. To assess if there was a relationship between these changes correlations were explored. There were positive correlations in normal colon between HSD17β7 and 12 and PAPSS1 and PAPSS2 (Figure 4.12 (C and G, respectively)) but none were found in CRC. This suggests that whereas these enzymes are regulated and subject to feedback mechanisms under normal healthy conditions this is not the case in CRC where dysregulation occurs. It also implies that in CRC expression of HSD17β and activity of STS are not co-ordinated and so patients may present with a mix of enzyme changes. This would fit with CRC being a heterogeneous disease. Thus, if these pathways were to be targeted therapeutically it would be essential to determine which patients would benefit from which treatment e.g. STS inhibitors and/or HSD17β inhibitors, to avoid unnecessary side-effects.

### 4.4.5 Colorectal Cancer Stage and Oestrogen Metabolism

There was no change in STS activity across CRC disease stage (Stage I and II versus III and IV) suggesting that higher activity is not associated with advanced CRC (Figure 4.17) and instead may be an earlier stage event. It is not clear where it would sit in the accruing mutation hypothesis (Fearon and Vogelstein, 1990). STS can be regulated by inflammatory modulators such as TNFα and IL-6. Pre-cancerous adenomas are associated with inflammation (Bilinski et al., 2012) and through increased STS activity could lead to an early local active oestrogen concentration rise. Additionally, this STS pathway may also be active in IBD, particularly when severe or poorly controlled. Disease severity is linked with higher risk of CRC (Barral et al., 2015), whether STS activity contributes to this is currently unknown. Additionally, the menstrual cycle and oestrogen use such as contraceptives can affect IBD symptoms; some women have worsening symptoms and others improved (Nee and Feuerstein, 2015). The sulphatase pathway may also be inhibited by anti-inflammatories, reducing CRC risk (Smalley and DuBois, 1997). TNFα and IL-6 regulation of STS is discussed in Chapter 7.

Decrease in HSD17β2 mRNA in CRC appeared to occur more commonly in latter stage disease (Figure 4.14) however, this trend was not significant. Increased HSD17β12 expression also did not change across disease stages (Figure 4.16). HSD17β7 however was increased in males at more advanced CRC (Figure 4.15). HSD17β12 showed a similar trend but was not significant. This emphasises the gender disparities in CRC and the importance of stratification of disease to deliver personalised medicine.

### 4.4.6 Summary of Human Colorectal Cancer Oestrogen Enzyme Changes

Figure 4.18 shows the summary of changes in CRC in the entire dataset. These data suggest that STS activity is frequently raised in CRC and as an early event if STS activity is found to be linked with tumour proliferation it would be an attractive treatment target for all cancer stages. HSD17β2 mRNA is often downregulated at latter disease stages and HSD17β7 and 12 mRNA are increased in CRC. Therefore, if $E_2$ increases CRC proliferation HSD17β7 and 12 are also potential treatment targets. Although both males and females followed the same trends in oestrogen enzyme expression/ activity overall there were gender differences within CRC disease staging. In later stages HSD17β7 mRNA was lower in female disease but higher in males and a similar pattern was seen for HSD17β12 mRNA. This may reflect some of the differences in survival between men and women.

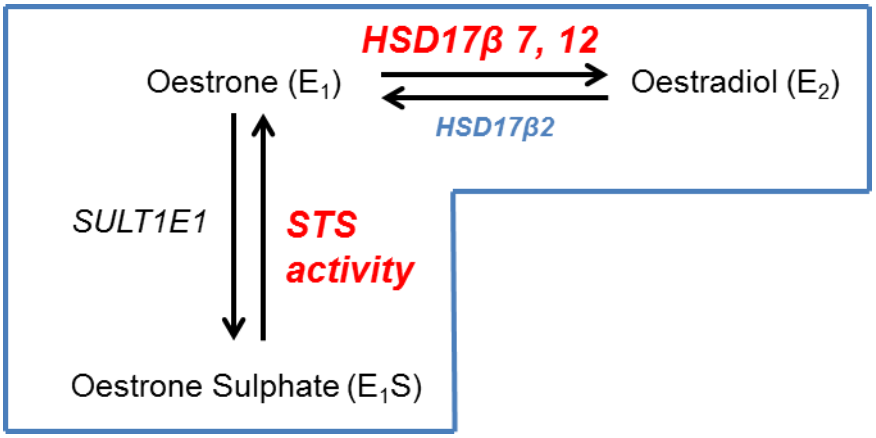



*Figure 4.18 Summary of oestrogen metabolism changes in colorectal cancer. In colorectal cancer there was increased STS activity, increased HSD17β7 and 12 and reduced HSD17β2 mRNA.*

Evidence suggests that higher STS expression reduces survival (Sato et al., 2009) implying active oestrogens are detrimental in CRC. However, Oduwole showed that

reduced HSD17β2 and potentially higher $E_2/E_1$ ratio was associated with improved prognosis in females (Oduwole et al., 2003). This is contradicted by English and colleagues, where $E_2$ was suggested to be the 'bad' oestrogen and $E_1$ 'good'. However, rather than the direct effect of oestrogens the improved survival witnessed by Oduwole et al. may instead reflect more on the tumour's response to therapy. Oestrogen responsive tumours might be associated with less aggressive, lower grade tumours and more sensitive to current chemotherapeutics. Further studies would be needed to investigate this. These conflicting results may also be because in each of these studies only part of the oestrogen metabolism pathway was examined and results were not categorised by gender. It is necessary to evaluate the entire oestrogen pathway as changes depend on gender and disease stage. It is not clear how these oestrogen metabolism changes influence prognosis and recurrence, but this would be an interesting advancement of this thesis.

## 4.5 CONCLUSION

In human CRC there is a frequent rise in STS activity, reduction in HSD17β2 mRNA expression and increase in HSD17β7 and 12 mRNA expression. Increased desulphation via STS would increase $E_1$ concentrations locally as $E_1S$ circulates at high concentrations. Combined with a loss of HSD17β2 and increased HSD17β7 and 12 there may be a shift in the $E_2/E_1$ ratio in favour of $E_2$. $E_2$ could then bind to ERs with greater affinity, potentiating oestrogen action in CRC.

# CHAPTER 5. COLORECTAL CELL LINE CHARACTERISATION AND PROLIFERATION

## 5.1 INTRODUCTION

To examine CRC behaviour *in vitro* there are multiple immortalised cell lines. By characterising matched human CRC tissue (Chapter 4) and CRC cell lines important commonalities between them should be ascertained, increasing understanding of CRC behaviour and aiding identification of potential therapeutics. However, there are published inconsistencies for CRC cell line oestrogen proliferation responses *in vitro* such as $E_2$ both inhibiting (Hsu et al., 2014) and stimulating (Santolla et al., 2012) LoVo cells. $E_2$ also increased proliferation in Caco-2 cells (Di Domenico et al., 1996) whereas $E_1$ decreased proliferation in SW620 cells (English et al., 1999). Thus, CRC cell lines respond differently to oestrogens and are hypothesised to have variable oestrogen enzyme profiles, possibly reflecting subtypes of colorectal adenocarcinomas. Currently it is unclear how oestrogen is metabolised, how it exerts its effects in the colon and if it is beneficial or unfavourable in colon carcinogenesis. The aim of this chapter was to characterise oestrogen metabolism in commonly used CRC cell lines and assess how oestrogen impacts on their proliferation.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Cell Culture

Cells selected were cultured as described in section 2.1. These CRC cell lines are all adenocarcinomas and originate from both men and women (Table 2.1). Two breast cancer cell lines (MCF7 and MDA-MB-231) and a placental cell line (JEG3) were chosen to use as positive controls for mRNA and protein expression. Additionally, protein from HEK (human embryonic kidney) cells overexpressing

PAPSS1 or PAPSS2 was kindly donated from Dr. Jan Idkowiak (University of Birmingham) and used as positive control for WB.

FBS is usually added to cell culture medium however it contains oestrogens (Dang and Lowik, 2005, Aakvaag et al., 1990). Phenol red, a cell media constituent is commonly used as a media pH indicator and also binds to ERs (Berthois et al., 1986). To enable accurate oestrogen proliferation responses cells were cultured 72 hours prior to experiments in charcoal stripped FBS (SFBS, Sigma-Aldrich, Dorset, UK) phenol red free media. Dextran treated charcoal selectively removes hormones including steroids such as oestrogens ensuring only oestrogens added to the media are responsible for the effects seen. To note other components are depleted in SFBS including vitamins, thyroid hormones and electrolytes (Cao et al., 2009).

### 5.2.2 RNA Extraction, cDNA Synthesis and Real Time PCR

Cell line RNA extraction followed by cDNA synthesis and cDNA quantification using Picogreen assay were performed as described in section 2.3 to 2.5. Real-time PCR was used to quantify mRNA expression for key oestrogen metabolism enzymes as described in section 2.6 using the primers and probes for HSD17β1, HSD17β2, HSD17β7, HSD17β12, STS, PAPSS1, PAPSS2 and internal standard gene RPLPO (Table 2.2). Validations of duplex reactions and choice of internal standard are shown in Appendix II. SULT1E1 was not able to be validated in either singleplex or duplex as shown in Appendix II and so mRNA expression could not be assessed.

### 5.2.3 Protein Extraction and Quantification

Cell line protein was extracted using RIPA buffer and protein quantified using BCA assay as described in section 2.2.

### 5.2.4 Proliferation Assay

Proliferation was assessed using CyQUANT or BrdU as described in section 2.7. The method used is stated for each experiment. Cells were treated with $E_1$, $E_2$, $E_1S$ or $E_2S$ in SFBS phenol red free media across 0-500 nM range for 48 hours with media refreshed daily to maintain oestrogen concentrations.

### 5.2.5 Western Blotting

Western blotting was implemented as per procedure described in section 2.9. Primary and secondary antibodies were optimised and included HSD17β1, 2, 12, PAPSS1 and PAPSS2 with β-actin as loading control (Table 2.3). SULT1E1 and STS antibody were not assessed as previously described as there were no consistent commercial antibodies.

### 5.2.6 STS Activity Assay

STS activity assay was performed as described in section 2.8 with 100,000 cells seeded per well of a 6 well plate. Cells were also treated with STX64 (1 µM), a selective, potent, irreversible and non-toxic STS inhibitor (Malini et al., 2000, Stanway et al., 2006).

### 5.2.7 Stable Overexpressing STS HCT116 Cells

HCT116 cell lines overexpressing STS ($HCT116_{STS}$) or VO ($HCT116_{VO}$) were developed as described in sections 2.10 and 2.11. STS and VO plasmids contained G418 resistance to select out transfected cells (Figure 2.2) however G418 was not used during experimental conditions owing to potential off-target effects.

### 5.2.8 LC-MS/MS Method

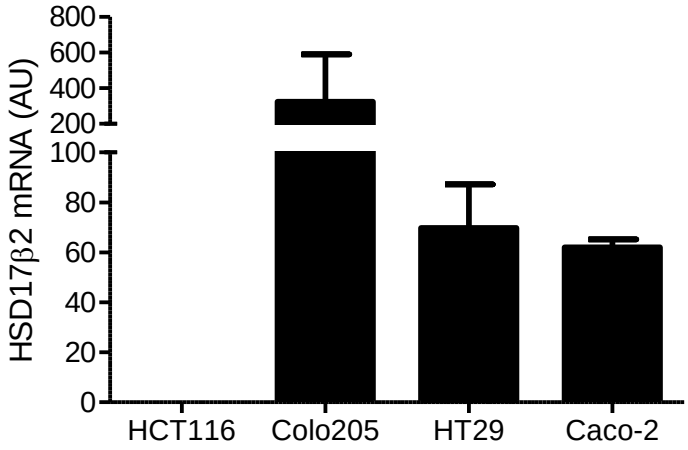
Cells were seeded at 250,000 per well into 6 well plates. Once the cells were adhered the media was removed and cells washed twice with PBS. Cells were then treated with SFBS phenol red free media containing $E_1$ 100 nmol/l, $E_2$ 100 nmol/l or $E_1S$ 500 nmol/l for 24 hours. Media was collected and frozen at -20$^{o}$C until extraction and analysis using LC-MS/MS as described in Chapter 3. Cell protein was also harvested with concentration determined by BCA assay (section 2.2).

## 5.3 RESULTS

### 5.3.1 Colorectal Cell Line Characterisation

#### 5.3.1.1 Oxidative HSD17β2 Expression

HSD17β2 mRNA expression was detected in three of the four CRC cell lines examined. HCT116 cells did not express HSD17β2 mRNA and Colo205 cells had the highest mRNA expression (Figure 5.1). Protein expression as determined by WB (Figure 5.2) revealed HSD17β2 protein did not correlate with mRNA detection of HSD17β2 in all cell lines. Colo205 had the lowest protein expression and HCT116 did express HSD17β2.




| Cell Line | HCT116 | Colo205 | HT29 | Caco-2 |
|---|---|---|---|---|
| dCt ± SEM | 18.64 ± 0.66 | 1.78 ± 1.03 | 3.74 ± 0.43 | 4.19 ± 0.18 |

*Figure 5.1 HSD17β2 mRNA expression in colorectal cancer cells. HSD17β2 mRNA expression (arbitrary units (AU)) in Colo205, HT29 and Caco-2 (n = 3) cells, but not seen in HCT116 (n = 2) cells. The table below shows dCt values ± SEM.*

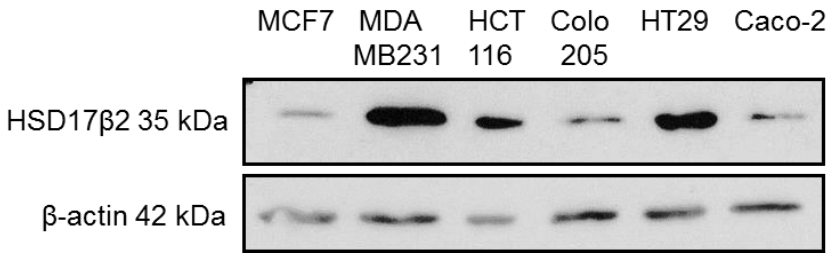


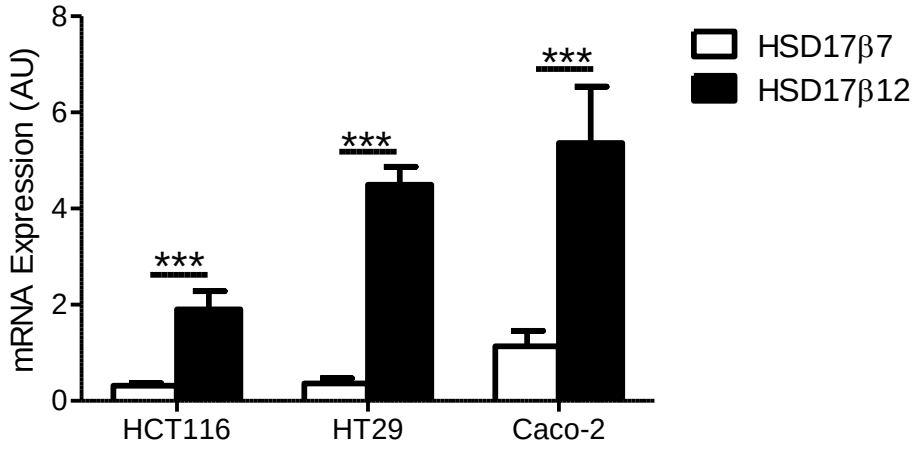

*Figure 5.2 Protein expression of HSD17β2 in colorectal cancer cells does not follow same pattern as mRNA. Western blot of HSD17β2 in colorectal cancer cell lines HCT116, Colo205, HT29 and Caco-2. Breast cancer cell lines MCF7 and MDA-MB-231 were used as controls. β- actin was used as loading control with 15 µg protein loaded into each well*

#### 5.3.1.2 Reductive HSD17β Expression

CRC cell lines had either very low or no HSD17β1 mRNA and protein expression (data not shown) which was consistent with human colon tissue (Chapter 4). Thus,

any $E_1$ reductive activity would be via an alternative enzyme such as HSD17β7 or 12.

HSD17β7 and 12 mRNA were expressed in CRC cells as shown in Figure 5.3. As in human colon tissue (section 4.3.2) HSD17β12 was more highly expressed than HSD17β7 in all CRC cells tested.




| Cell Line | HCT116 | HT29 | Caco-2 |
|---|---|---|---|
| HSD17β7 | 11.61 ± 0.28 | 11.65 ± 0.51 | 9.98 ± 0.37 |
| HSD17β12 | 9.15 ± 0.34 | 7.77 ± 0.13 | 7.45 ± 0.30 |

*Figure 5.3 HSD17β12 mRNA expression is higher than HSD17β7 in colorectal cancer cell lines. HSD17β7 and 12 mRNA expression in untreated colorectal cancer cell lines expressed in arbitrary units (AU) (n = 3). The table below shows dCt values ± SEM. *** p < 0.001, two-way ANOVA with Bonferroni post-test.*

HSD17β7 protein was not clearly detected by Western blot in any CRC or breast cancer cell lines despite strategic optimisation and technical assistance from company manufacturer. Figure 5.4 shows a typical immunoblot with no bands at predicted size.

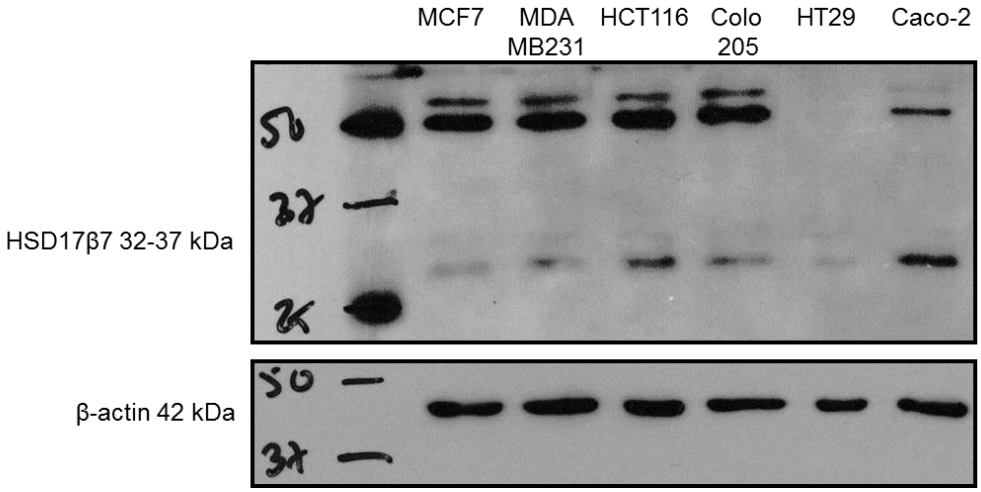



*Figure 5.4 HSD17β7 protein expression in colorectal cancer cells. Western blot of HSD17β7 in breast cancer cell lines MCF7 and MDAMB231 and colorectal cancer cell lines HCT116, Colo205, HT29 and Caco-2. β-actin is used as loading control and 15 µg of protein was loaded into each well. Note no bands appeared at the predicted size of 32-37 kDa.*

As with colon tissue HSD17β12 mRNA and protein did not correlate. Protein was expressed in HCT116 and HT29 cells with low expression in Caco-2 whereas Caco-2 had the highest mRNA expression of these cell lines (Figure 5.5).

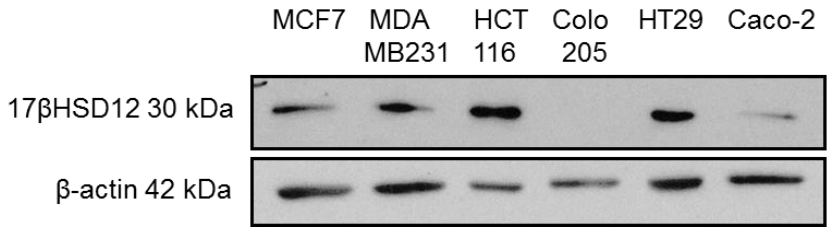
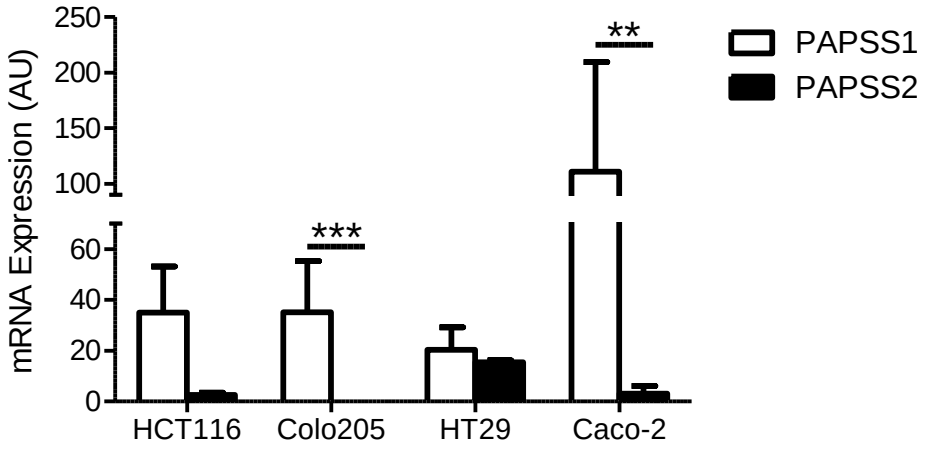



*Figure 5.5 Protein expression of HSD17β12 in colorectal cancer cells does not follow same pattern as mRNA. Western blot of HSD17β12 in breast cancer cell lines MCF7 and MDAMB231 and colorectal cancer cell lines HCT116, Colo205, HT29 and Caco-2. β-actin is used as loading control and 15 µg of protein was loaded into each well.*

### 5.3.1.3 PAPSS1 and PAPSS2

SULT1E1 could not be examined in cell lines so PAPSS was used as a surrogate marker for sulphation activity (also see section 4.3.3). All cell lines expressed PAPSS1 mRNA and PAPSS1 was more highly expressed than PAPSS2 in Colo205 and Caco-2 cells (Figure 5.6). Protein expression as assessed by WB showed similar findings to mRNA with PAPSS1 clearly expressed in all cell lines. PAPSS2 was not present in Colo205 cells and had only minimal detection in Caco-2 cells (Figure 5.7).




| Cell Line | HCT116 | Colo205 | HT29 | Caco-2 |
|---|---|---|---|---|
| PAPSS1 | 5.32 ± 0.64 | 5.66 ± 0.93 | 6.66 ± 1.30 | 4.78 ± 1.55 |
| PAPSS2 | 8.86 ± 0.50 | 14.79 ± 0.63 | 6.02 ± 0.07 | 11.59 ± 1.88 |

*Figure 5.6 PAPSS1 mRNA expression is higher than PAPSS2 in colorectal cancer cell lines. PAPSS1 and PAPSS2 mRNA expression (arbitrary units (AU)) in HCT116, Colo205, HT29 and Caco-2 cells (n = 3-4). The table below shows dCt values ± SEM. PAPSS1 expression was significantly higher than PAPSS2 in Colo205 and Caco-2 cells (**p < 0.01, ***p < 0.001, two-way ANOVA with Bonferroni post-test).*

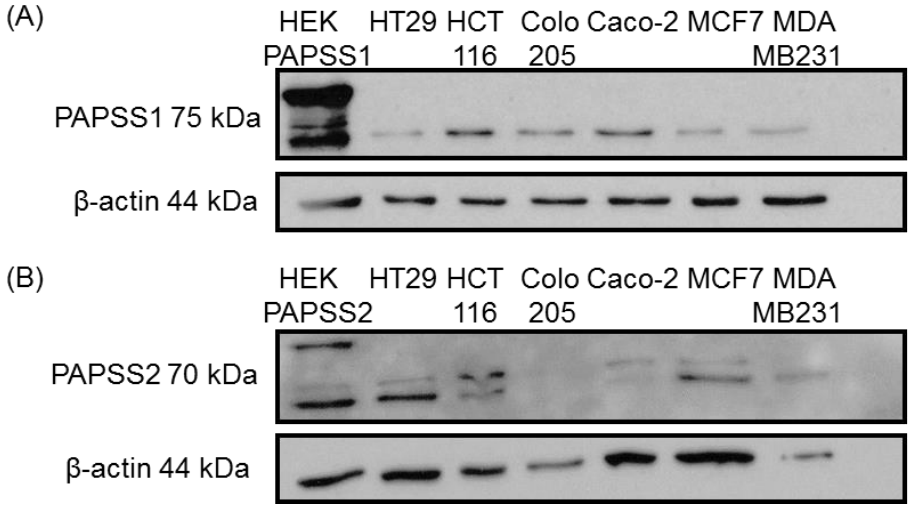
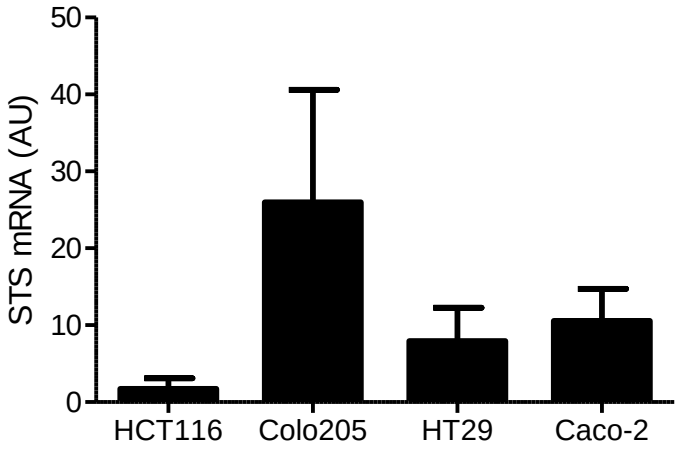



*Figure 5.7 PAPSS1 and PAPSS2 protein expression in colorectal cancer cell lines. Western blot of PAPSS1 and PAPSS2 expression in CRC cancer cells. (A) PAPSS1 is expressed in all CRC cells tested and breast cancer cell lines MCF7 and MDAMB231 with HEK cells overexpressing PAPSS1 used as positive control and β-actin as loading control. (B) PAPSS2 is expressed in HT29 and HCT116 cells but not in Colo205 or Caco-2 cells. There is some expression in breast cancer cell lines MCF7 and MDAMB231. HEK cells overexpressing PAPSS2 were used as positive control and β-actin as loading control.*

#### 5.3.1.4 STS Expression and Activity in Colorectal Cancer Cell Lines

STS mRNA was expressed in all CRC cell lines with highest expression in Colo205 cells, though this was not statistically significant (Figure 5.8).




| Cell Line | HCT116 | Colo205 | HT29 | Caco-2 |
|---|---|---|---|---|
| dCt ± SEM | 11.27 ± 1.74 | 7.56 ± 1.68 | 8.03 ± 1.56 | 7.79 ± 0.74 |

*Figure 5.8 STS mRNA in CRC cell lines. STS mRNA expression (arbitrary units (AU)) in HCT116 (n = 4), Colo205 (n = 6), HT29 (n = 3) and Caco-2 (n = 9) cells. All CRC cell lines expressed STS mRNA with the highest expression found in Colo205 cells. The table below shows dCt values ± SEM.*

STS activity varied across the CRC cell lines and did not follow the same pattern as STS mRNA. Highest activity was seen in Caco-2 cells whereas it was Colo205 cells that had the most abundant mRNA. When treated with STS inhibitor, STX64, STS activity reduced (Figure 5.9).

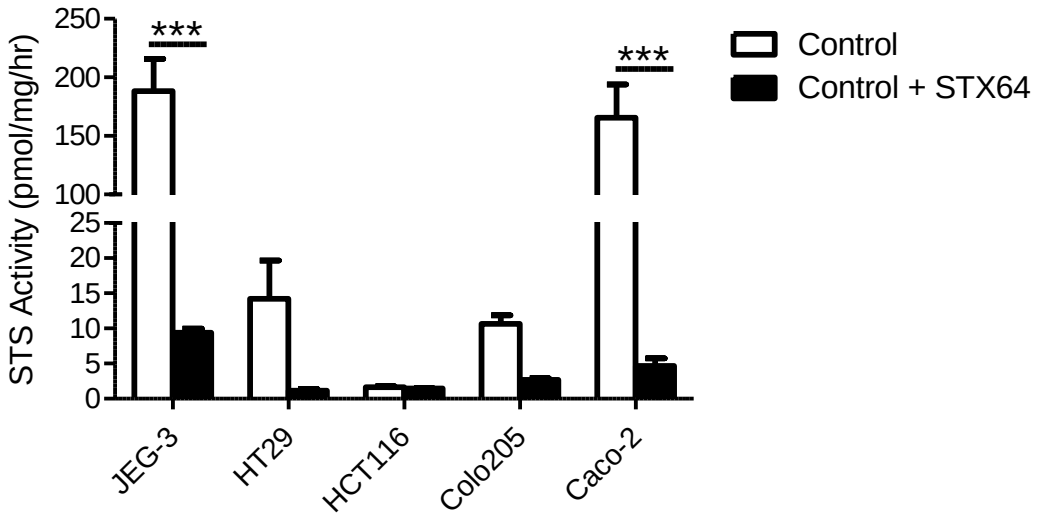



*Figure 5.9 STS activity is highest in Caco-2 cells. STS activity in CRC cells lines measured using $^{3H}E_1S$ assay with Jeg3 cells as a positive control. Caco-2 cells have the highest activity of CRC cells tested. When cells were treated with potent STS inhibitor, STX64 (1 µM), activity was inhibited (n = 3). *** p < 0.001, two-way ANOVA with Bonferroni post- test.*

### 5.3.2 Generation of Stable STS Overexpression in HCT116 cells

Human CRC had increased STS activity compared to normal colon tissue (section 4.2.5). Previously, STS expression has been linked with tumoural oestrogen concentrations and survival (Sato et al., 2009). To explore if STS influences proliferation in CRC HCT116 cells were transfected to stably overexpress STS. These cells were chosen as they had the lowest STS expression and activity of the CRC cell lines and so an effect on proliferation would be more easily identifiable.

Wild-type HCT116 cells ($HCT116_{WT}$) and $HCT116_{VO}$ cells have low STS mRNA expression and when transfected with STS plasmid ($HCT116_{STS}$) expression increased (Figure 5.10 (A)). Transfected STS plasmid was functional as activity increased from 5.42 ± 0.85 pmol/mg/hr in $HCT116_{VO}$ cells to 85.35 ± 7.35

pmol/mg/hr in HCT116$_{STS}$ cells (Figure 5.10 (B)). To ensure transfection was maintained STS activity was periodically monitored and remained stable for up to 15 passages (data not shown).

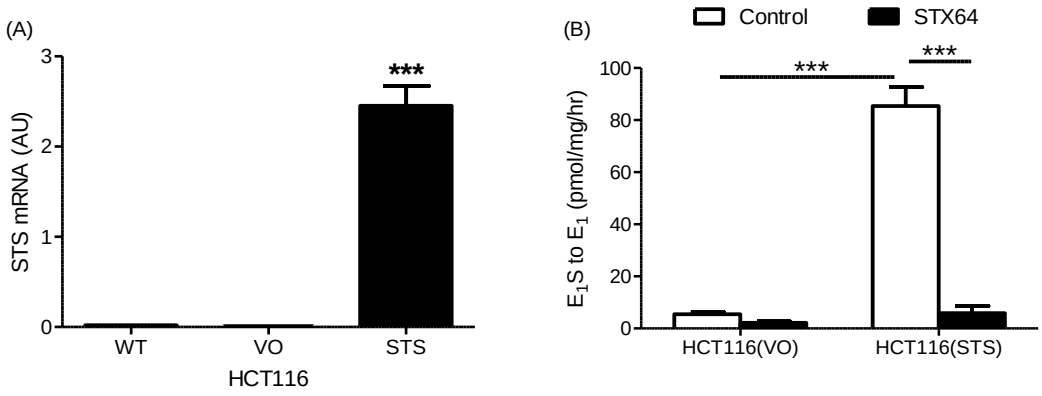



*Figure 5.10 Stable overexpression of STS in HCT116 cells. (A) Transfection of STS into HCT116 cells increased STS mRNA expression (one-way ANOVA with Bonferroni post-test). (B) STS activity increase in HCT116$_{STS}$ cells compared to HCT116$_{VO}$ (n = 3) and inhibition by STS inhibitor, STX64 (1 µM) (two-way ANOVA with Bonferroni post-test. Wild-type (WT), vector only (VO), over expression of steroid sulphatase (STS). ***p < 0.001.*

Proliferation of HCT116$_{STS}$ and HCT116$_{VO}$ cultured in complete media was measured using CyQUANT assay. HCT116$_{STS}$ cells proliferated more than HCT116$_{VO}$ cells and this was attenuated by STS inhibitor STX64 (Figure 5.11). This experiment lasted 5 days and supports previous studies showing STX64 is non-toxic (Stanway et al., 2006).

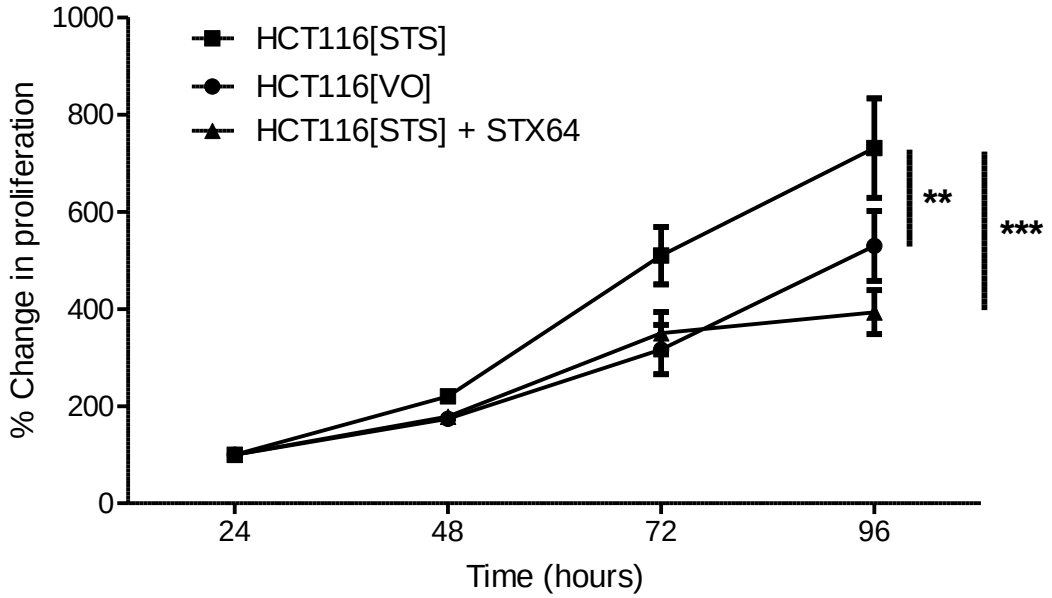



*Figure 5.11 HCT116$_{STS}$ cells proliferate faster than HCT116$_{VO}$. Growth of HCT116 cells overexpressing STS or VO in complete media over 4 days expressed as mean ± SD. HCT116$_{STS}$ cells have an increased proliferation rate which is reduced by STS inhibitor, STX64 (1 µM) (n = 3). Two-way ANOVA, **p < 0.01, ***p < 0.001.*

### 5.3.3 STS Overexpression Increases Proliferation in HCT116 Cells

Stable HCT116$_{STS}$ cells proliferated more than WT and VO HCT116 cell types in complete media. To assess if STS increased proliferation without oestrogen in the media SFBS was used and proliferation measured by BrdU assay. HCT116$_{STS}$ cells proliferated more than HCT116$_{WT}$ or HCT116$_{VO}$ in SFBS media and this effect was abolished by STX64. Growth was greater in all HCT116 cell types treated with $E_2S$ (100 nmol/l) but more pronounced in HCT116$_{STS}$ cells. $E_2S$ growth effect was also abrogated in all cell types by STX64 (Figure 5.12).

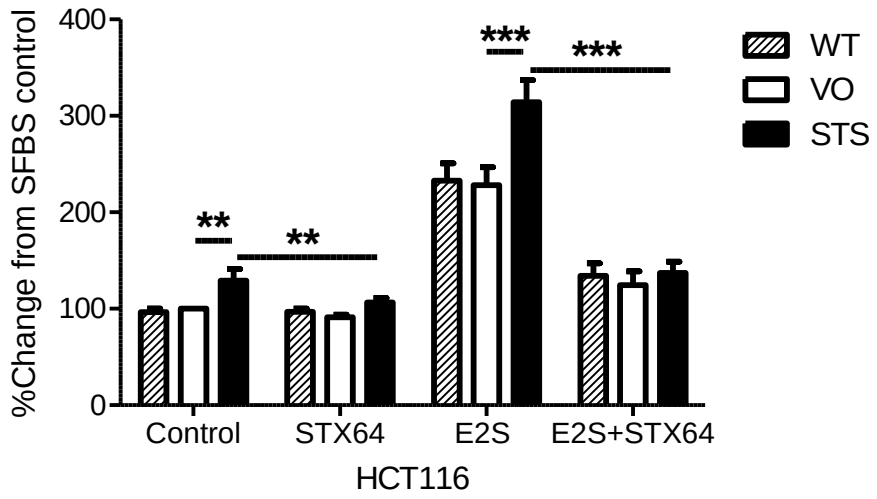



*Figure 5.12 HCT116$_{STS}$ cells have increased proliferation with oestradiol sulphate treatment compared to HCT116$_{VO}$. Proliferation measured using BrdU incorporation assay. Cells were treated for 48 hours with $E_2S$ (100 nmol/l) and/or STX64 (1 µM) and compared to SFBS HCT116$_{VO}$. HCT116$_{STS}$ cells had increased proliferation (**p < 0.01) inhibited by STS inhibitor (STX64, **p < 0.01). $E_2S$ increased proliferation in all cells (***p < 0.001) with highest rates in HCT116$_{STS}$ cells (***p < 0.001). The increase in proliferation due to $E_2S$ was inhibited by STX64 (***p < 0.001). Two-way ANOVA with Bonferroni post-test. These experiments were performed with intercalating medical student Habibur Rahman.*

### 5.3.4 Oestrogen Stimulates Proliferation in HCT116 and HT29 Cells

$E_2S$ increased proliferation in HCT116$_{WT}$ cells despite low STS activity. Therefore, only small oestrogen changes may be required to have an effect on colon growth. The effect of active oestrogen ($E_1$ and $E_2$) bypassing the need for STS and inactive oestrogen ($E_1S$) requiring STS action on proliferation in CRC cell lines HCT116, HT29 and Caco-2 cells was assessed and they had different responses.

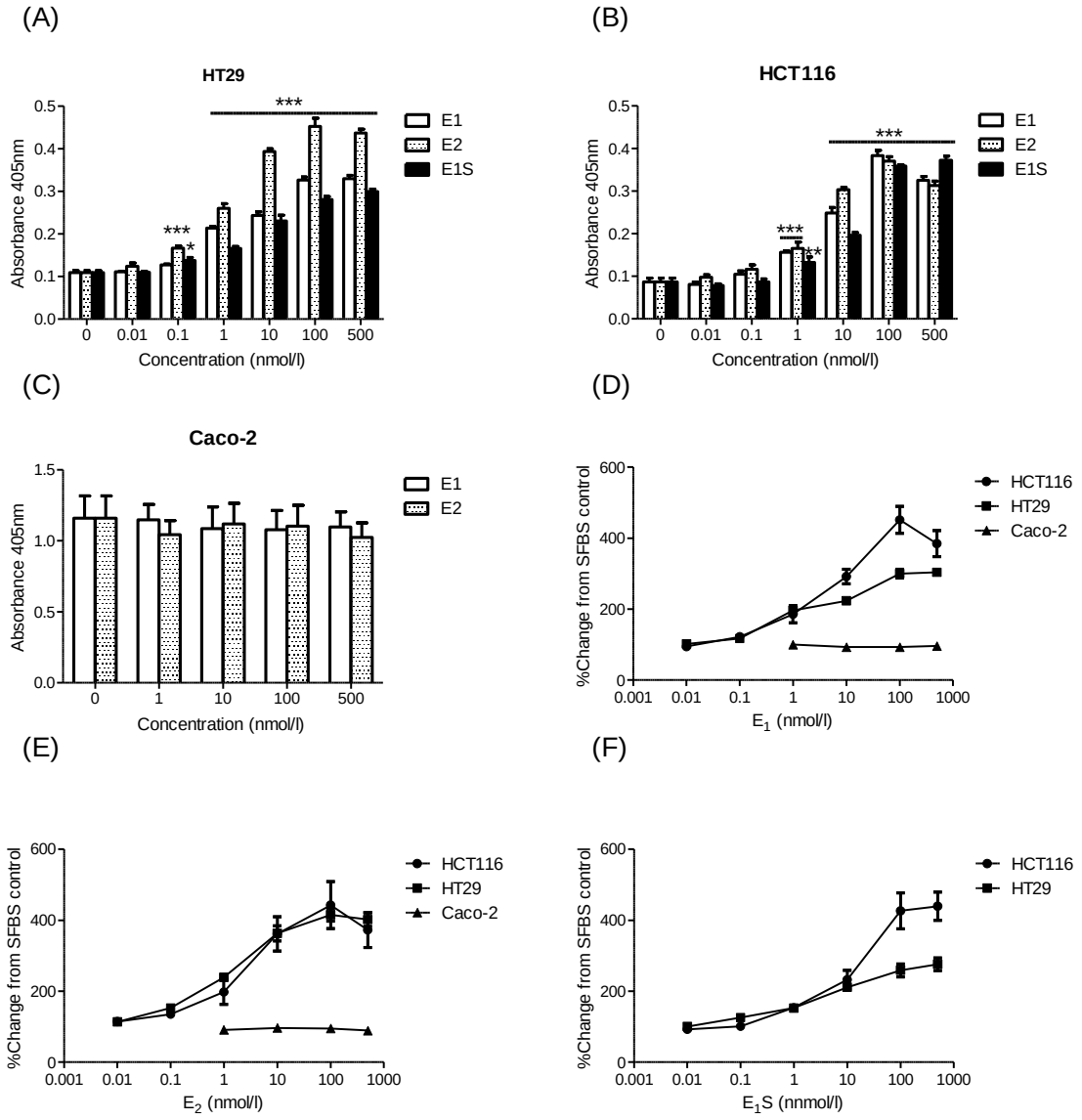



*Figure 5.13 Oestrogen increases proliferation in HCT116 and HT29 cells but not Caco-2 cells. HT29, HCT116 and Caco-2 cells cultured in SFBS for 72 hours were treated with $E_1$, $E_2$ or $E_1S$ 0.01-500 nmol/l for 48 hours. Proliferation was measured using BrdU assay. HT29 (A) and HCT116 (B) proliferated in a dose dependent manner in response to treatment, whereas Caco-2 growth (C) remained static (n = 3). D-F show proliferation as percentage change from SFBS controls; note the slower growth rate of Caco-2 cells and the lack of proliferative response to $E_1$ (D) and $E_2$ (E). HCT116 and HT29 also proliferated in response to $E_1S$ (F). Experiments performed in conjunction with intercalating medical student Habibur Rahman.Two-way ANOVA with Bonferroni post-test *p < 0.05, **p < 0.01, ***p < 0.001.*

HT29 cells have low STS activity and proliferation increased with all oestrogen treatments in a dose dependent manner with $E_2$ having the greatest effect (Figure 5.13 (A)). HCT116 cells ((Figure 5.13 (B)) had a similar response to HT29 cells. Significant growth responses in HT29 and HCT116 cells occurred at 0.1 and 1 nmol/l respectively. EC50 values for HT29 cells were 2.5, 1.4 and 5.3 nmol/l for $E_1$, $E_2$ and $E_1S$, respectively. EC50 for HCT116 cells were 4.8, 2.4 and 16.2 nmol/l for $E_1$, $E_2$ and $E_1S$, respectively. Oestrogen had no impact on Caco-2 cell growth ((Figure 5.13 (C)). Oestrogen proliferation responses for these cell lines were compared using percent change from SFBS for $E_1$ ((Figure 5.13 (D)), $E_2$ ((Figure 5.13 (E)) and $E_1S$ ((Figure 5.13 (F)).

### 5.3.5 Oestrogen Metabolism in CRC Cell Lines

As HCT116 and HT29 had similar proliferation responses to oestrogen (Figure 5.13) it was predicted that they metabolised oestrogen similarly. As Caco-2 cells did not proliferate in oestrogen media it was hypothesised they metabolised oestrogen differently to HCT116 and HT29 cells. HCT116, HT29 and Caco-2 cell metabolism of $E_1$, $E_2$ and $E_1S$ was assessed using extracted media collected from oestrogen treated cells and analysed by LC-MS/MS. Due to LLOQ and LLOD on the LC-MS/MS method concentrations below 14 nmol/l for $E_1$ and $E_2$ and 18 nmol/l for $E_1S$ and $E_2S$ could not be determined. The percentage of $E_1$, $E_2$ and $E_1S$ remaining in cell media after 24 hours of treatment in HCT116, HT29 and Caco-2 cells is shown in Figure 5.14 (A).

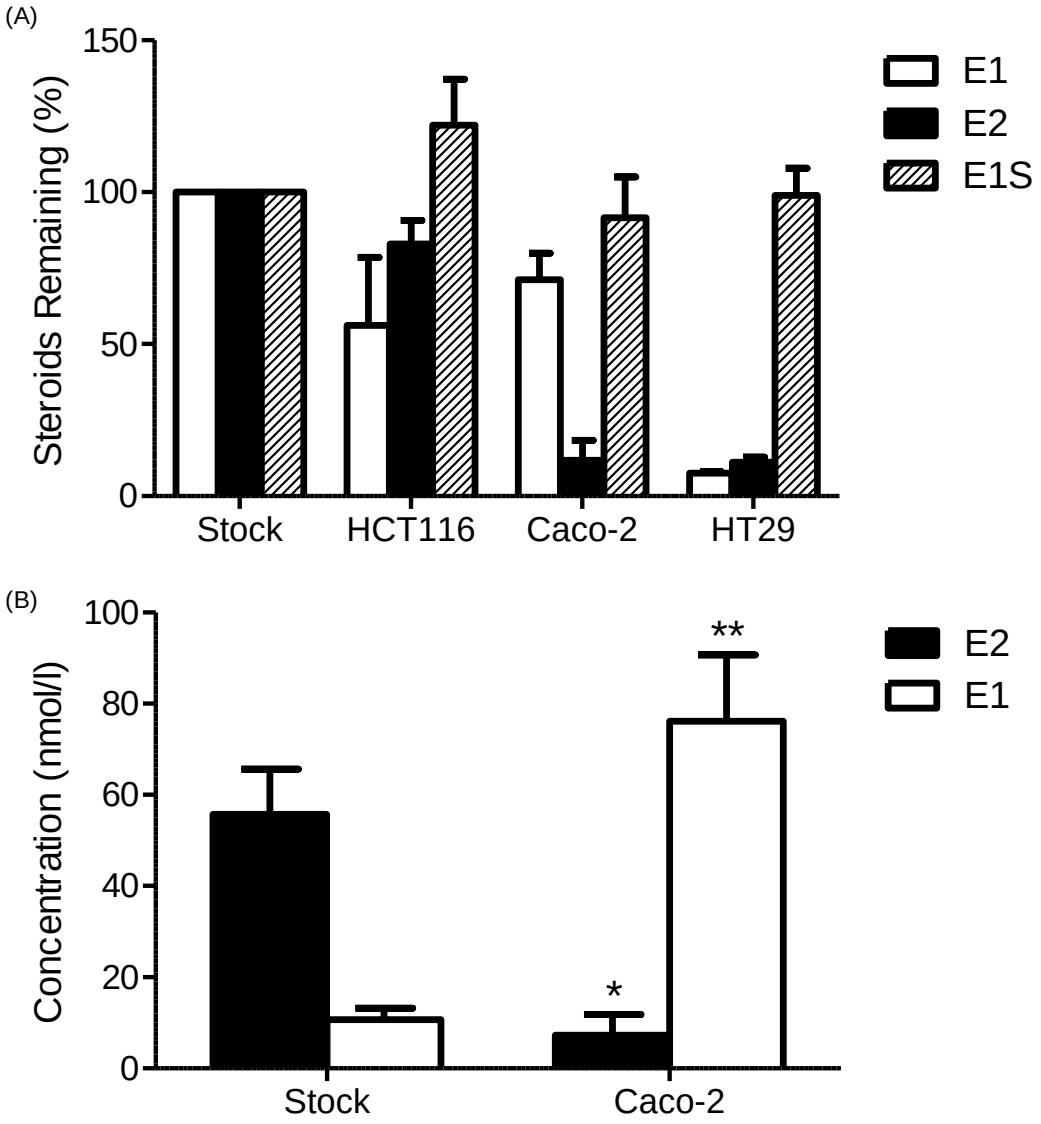



*Figure 5.14 Colorectal cancer cell lines do not metabolise oestrogen equally. (A) HCT116, Caco-2 and HT29 cells were treated with $E_1$, $E_2$ (100 nmol/l) $E_1S$ (500 nmol/l) or SFBS for 24 hours and media collected for LC-MS/MS analysis. The percentage change in media of $E_1$, $E_2$ or $E_1S$ concentration was calculated in comparison to non-metabolised 100% solution (stock). The remaining $E_1$, $E_2$ or $E_1S$ in cell media (%) is displayed. After 24 hours of treatment, Caco-2 cell media appeared to have less $E_2$ than starting stock concentration and HT29 cell media appeared to have less $E_1$ and $E_2$ compared to stock concentration. (B) Caco-2 cells were treated with $E_2$ for 24 hours and cell media subjected to LC-MS/MS analysis. $E_1$, $E_2$, $E_1S$ and $E_2S$ were quantified with concentrations of $E_1$ and $E_2$ in stock solution and Caco-2 cell media displayed. $E_2$ Stock had some $E_1$ contamination. After 24 hours Caco-2 cell media had less $E_2$ and more $E_1$ than original stock treatment, suggesting oxidative activity. Two-way ANOVA with Bonferroni post-test *p < 0.05, **p < 0.01, ***p < 0.001, n=3.*

HCT116 cells had minimal changes in $E_1$ and $E_2$ concentration and Caco-2 cells seemed to metabolise $E_2$ but not $E_1$. HT29 cells appeared to metabolise $E_1$ and $E_2$. Neither cell line metabolised $E_1S$.

HT29 cells metabolism of $E_1$ and $E_2$ were to steroids uncharacterised in these experiments as there was no increase in the oestrogens detectable by this LC-MS/MS method ($E_1$, $E_2$, $E_1S$ and $E_2S$). However, Caco-2 cells metabolised $E_2$ to $E_1$ (Figure 5.14 (B)). $E_2$ treatment solution (stock) consisted mainly of $E_2$ with minor $E_1$ contamination and Caco-2 cell media collected 24 hours after $E_2$ treatment is comprised mainly of $E_1$ with low $E_2$ concentration. Thus, Caco-2 cells oxidised the majority of $E_2$ to $E_1$ implying HSD17β2 activity.

## 5.4 DISCUSSION

### 5.4.1 Colorectal Cancer Cell Line Comparison to Colon Tissue

There were some similarities between these authenticated CRC cell lines and the human colon cancer tissue samples in Chapter 4. HSD17β1 mRNA was not detected in colon tissue or CRC cell lines. As an alternative, enzymes HSD17β7 and 12 mRNA were explored and as with colon tissue were shown here for the first time in CRC cell lines. Additionally, HSD17β12 mRNA was more highly expressed than HSD17β7 in colon tissue and CRC cell lines (Figure 5.3). Mirroring human samples HSD17β2 and 12 mRNA did not match protein expression on Western blot. For example, Caco-2 cells had the highest HSD17β12 mRNA expression but the lowest protein expression on WB (Figure 5.5). HSD17β2 mRNA was not detected in HCT116 cells (Figure 5.1) but protein was detected by WB. Colo205 cells had the

highest expression of HSD17β2 mRNA at a similar expression level to healthy human colon tissue.

These cells all expressed STS mRNA and had STS activity. HCT116 displayed the lowest STS activity of 1.66 ± 0.14 pmol/mg/hr and Caco-2 cells the highest at 165.48 ± 28.51 pmol/mg/hr. However, STS activity was not as high as in colon tissue (normal 1.30 [1.20,1.40] nmol/mg/hr and cancer 1.56 [1.36,1.75] nmol/mg/hr). It should be noted though that colon tissue was a lysate whereas CRC cell lines in these experiments were intact and so direct comparison is not possible. Not being able to measure STS protein by WB due to poor antibody did not limit this study as STS function through an activity assay gave more reliable data.

The large variability seen with this small selection of commonly used CRC cell lines shows the difficulty in applying *in vitro* findings to *in vivo* situations. Although, there are similarities between these cells and human colon tissue, no cell line reflected what was measured in human CRC. Human CRC is heterogeneous, influenced by genetics and environment, with one therapy unlikely to apply to all. Unlike immortalised CRC cell lines, homogenised human tissue is not a pure cell population and in addition to colon cells may contain other cell types such as from inflammatory infiltrate. Medicine is becoming more personalised; however individualised therapy is, at least currently, impractical and too expensive especially for NHS services. If large CRC subtype groups that predict response to therapy can be identified, for example through oestrogen enzyme expression, patients can be

stratified by disease type allowing for tailored treatment whilst crucially remaining affordable.

### 5.4.2 Proliferative Effect of Oestrogen in the Colon

HCT116 and HT29 both proliferated in response to $E_1$, $E_2$ and $E_1S$ in a dose dependent manner. Conversely, Caco-2 cell growth remained static (Figure 5.13). This is different to published findings whereby Caco-2 cells proliferated in response to $E_2$, but not $E_1$ (Di Domenico et al., 1996). These discrepancies may be due to method differences. Here these authenticated cell lines were treated with higher oestrogen concentrations; 1-500 nmol/l compared to 0.5-10 nmol/l of $E_2$. Also, Domenico and colleagues measured proliferation using cell counting rather than a BrdU colourimetric assay which have different sensitivities. Here BrdU assay was performed with six replicates with cells seeded after counting using a haemocytometer to maximise seeding uniformity.

These three CRC cell lines had differing responses to oestrogen demonstrating CRC heterogeneity and the potential importance of individualised medicine stratification. It was predicted that oestrogen proliferation differences were due to how oestrogen was metabolised. $E_1S$ and $E_1$ have low affinity for ERs (Zhu et al., 2006) and therefore it is unlikely to be their direct action increasing proliferation in HCT116 and HT29 cells. Despite HCT116 and HT29 displaying similar proliferative responses, oestrogen metabolism varied across all three CRC cell lines (Figure 5.14). With the LC-MS/MS method limitations of LLOQ and LLOD meant $E_1$ and $E_2$ were unquantifiable below 14 nmol/l. Therefore low level oxidation and reduction

could not be determined (Chapter 3). However, HCT116 cells had minimal oestrogen metabolism, whereas HT29 cells appeared to metabolise both $E_1$ and $E_2$ to metabolites not detectable by this LC-MS/MS method. Thus, despite similar proliferative responses to oestrogen HCT116 and HT29 cells have very different oestrogen profiles and so activated receptors and down-stream signalling may also differ. It would be beneficial to extend this LC-MS/MS method to include additional oestrogen metabolites such as 2-methoxy-estradiol and oestriol to understand further how oestrogen is metabolised.

Caco-2 cells oxidised the majority of $E_2$ to the much less potent $E_1$ (Figure 5.14), implying they had HSD17β2 activity and agreeing with the HSD17β2 mRNA expression data (Figure 5.1 and Figure 5.2). $E_1$ binds to ERα with 10% affinity of $E_2$, 2% for ERβ (Zhu et al., 2006) and just 0.1% for GPER (Thomas et al., 2005). Reduction of oestrogen potency may explain why in this study proliferation was unchanged in Caco-2 cells. $E_1$ to $E_2$ metabolism was not demonstrated in any of these cell lines but may occur at concentrations lower than detectable by this LC-MS/MS method.

Caco-2 cells have the highest STS activity of these three cell lines and HCT116 cells the least (Figure 5.9). $E_1S$ has negligible binding affinity for ERα or ERβ (Zhu et al., 2006) however the sulphate group is unstable. This is partly counteracted by the addition of Tris stabiliser but the sulphate group can still dissociate and thus become $E_1$ without the need for STS action. To minimise this effect media was refreshed daily and $E_1S$ stored at -20$^{o}$C. However, this cannot fully explain how $E_1S$

increased proliferation in HCT116 and HT29 cells as LC-MS/MS analysis showed no sulphate loss. The maintained $E_1S$ concentration may instead be through SULT1E1 activity or there may be desulphation activity below limits of detection of this method with only very low concentrations of $E_2$ required to increase growth. These CRC cell lines did express PAPSS which is required for sulphation action. They all had higher PAPSS1 than PAPSS2 expression both in mRNA and protein (Figure 5.6 and Figure 5.7). However, despite multiple attempts and optimisation it was unfortunate and limiting not to be able to asses SULT1E1 mRNA, protein or activity in these cell lines.

HCT116 cells were transfected to stably overexpress STS as they had the least baseline activity. Predictably this resulted in increased STS mRNA expression and activity (Figure 5.10) that remained for at least 15 passages. Raising desulphation ability increased proliferation of HCT116 cells in both complete and starved media (Figure 5.11 and 5.12) which was abrogated by the potent STS inhibitor STX64 (Figure 5.11). As STS increased proliferation in oestrogen stripped SFBS media this suggests that the effect is not just active oestrogen related. To assess if driving oestrogen desulphation also increased proliferation all HCT116 cell types were treated with $E_2S$. This required only one enzyme step to procure potent $E_2$ and not rely on additional HSD17β action. $E_2S$ increased growth in all HCT116 cell types but more so in overexpressing STS cells. This effect was inhibited by STX64 implying desulphation of $E_2S$ to $E_2$ via STS action also increases HCT116 cell growth and is not off target plasmid effect.

In these authenticated cell lines ERα and ERβ were not detected by Western blot in HCT116 or HT29s and only low expression in Caco-2s (Appendix III). Some groups have shown ERβ expression in HCT116 (Fiorelli et al., 1999) Caco-2 and HT29 cells (Campbell-Thompson et al., 2001). Additionally, studies have shown that in CRC cell lines $E_2$ induces apoptosis. This is thought to be through ERβ action (Qiu et al., 2002, Fiorelli et al., 1999). It is currently unclear how oestrogen acts to induce proliferation in the HCT116 and HT29 cells used in this thesis as they do not express ERs. As an alternative how oestrogen increases proliferation through GPER is explored in Chapter 6.

## 5.5 CONCLUSION

CRC cell lines have some oestrogen metabolism similarities to human cancerous colon tissue. However, there are differences such as lower STS activity in CRC cells. These cell lines also had differing proliferation responses to oestrogen which is partly explained by their oestrogen metabolism profiles. Active oestrogens generated through STS desulphation action increased proliferation in HCT116 cells, which may be due to efficacious receptor binding. As STS activity is increased in human colon cancer tissue this is an attractive treatment target. However, with low or undetectable ERα and ERβ expression in CRC cells and colon tissue how oestrogen increases proliferation is yet unclear.

# CHAPTER 6. GPER EXPRESSION AND EFFECT OF ACTIVATION ON COLON CANCER PROLIFERATION

## 6.1 INTRODUCTION

Oestrogen metabolism is altered in CRC with a drive towards active $E_2$ generation (Chapter 4). Previous research has shown that raised tumoural oestrogen concentrations are associated with poorer prognosis (Sato et al., 2009). Chapter 5 shows *in vitro* oestrogens increase proliferation in HCT116 and HT29 CRC cells and overexpressing STS in HCT116 cells ($HCT116_{STS}$) also increases proliferation. It is currently unclear and controversial how oestrogen exerts proliferative effects in the colon. It is generally accepted that in the healthy colon pro-apoptotic ERβ is predominant and assists in normal colon cell turnover (Caiazza et al., 2015). In CRC ERβ is reduced or lost (Niv, 2015) leading to loss of differentiation and decreased apoptosis (Elbanna et al., 2012). Pro-mitogenic ERα if present in the colon is only at low concentrations (Campbell-Thompson et al., 2001) thus unlikely to account for unregulated proliferation. A relatively new ER, the G protein- coupled oestrogen receptor (GPER), has been detected in the colon myenteric plexus where it is thought to impact on peristalsis and be responsible for change in bowel frequency seen by many women over their menstrual cycle (Li et al., 2015). Although there is minimal research on GPER in CRC, GPER stimulation is associated with proliferation in breast (Pandey et al., 2009), endometrial (Vivacqua et al., 2006b) and ovarian (Albanito et al., 2007) cancer cell lines.

Oestrogen initially regulates cell-turn over in the colon through ERβ and this regulation is lost in CRC due to reduced ERβ expression. Here is it hypothesised that increased active oestrogen generation through STS activity instead stimulates GPER promoting colorectal tumourigenesis. This may explain the apparent dual

role of oestrogen in the colon with evidence for both protection against and increasing CRC risk (Rossouw et al., 2013).

The aims of this chapter were to assess GPER stimulation on CRC cell line proliferation *in vitro* and *in vivo*.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Real Time PCR

RNA extraction from cells and colon tissue followed by cDNA synthesis and cDNA quantification using Picogreen were undertaken as described in section 2.6 with primers and probes for GPER and RPLPO (Table 2.2).

### 6.2.2 Western blotting

Western blotting was performed as described in section 2.9 using GPER and β-actin antibodies as described in Table 2.3.

### 6.2.3 BrdU

Proliferation was quantified using BrdU assay as described in section 2.7.2. After 72 hours grown in SFBS phenol red free media cells were treated with GPER agonist, G1 (0-500 nM) or antagonist, G15 (1 μM) for 48 hours. The G15 dose used was chosen as it was 10 fold that of effective G1 (100 nM) to make sure receptors were saturated.

### 6.2.4 Xenograft Models

All experiments were performed under conditions complying with UK Home Office Animals (Scientific Procedures) Act 1986 and local institutional ethical guidelines. Athymic nude mice were purchased from Harlan (Bicester, Oxfordshire, UK) at five weeks old and allowed to acclimatise for one week. They had a 12 hour light and dark cycle with free access to normal chow and water. $5 \times 10^6$ $HCT116_{STS}$ cells were inoculated into the right flank of 20 mice. After one week mice were randomly divided into two groups; 10 received GPER antagonist G15 (50 µg/kg i.p three times per week in 100 µl aqueous vehicle (0.9% NaCl with 0.1% albumin and 0.1% Tween-20) and 10 vehicle (100 µl aqueous vehicle). These concentrations and medium were chosen from *in vivo* literature published on G15 (Dennis et al., 2009). Throughout the study, mice were weighed and tumour volumes (length x $width^2/2$) calculated using electronic callipers. For ethical reasons once tumours exceeded 1.4 cm diameter the study was terminated and mice euthanised by cervical dislocation. Xenograft tumours were harvested and frozen in liquid nitrogen for future study.

## 6.3 RESULTS

### 6.3.1 GPER Expression in CRC Cell Lines

GPER mRNA expression was low in CRC cell lines HCT116 and HT29 and undetectable in Caco-2 cells (data not shown). However, protein expression was detected in all CRC cell lines (Figure 6.1). HCT116 cells had the lowest protein expression and Caco-2 cells the highest. Breast cancer cell lines MCF7 and MDA-MB231 cells both showed GPER protein expression.

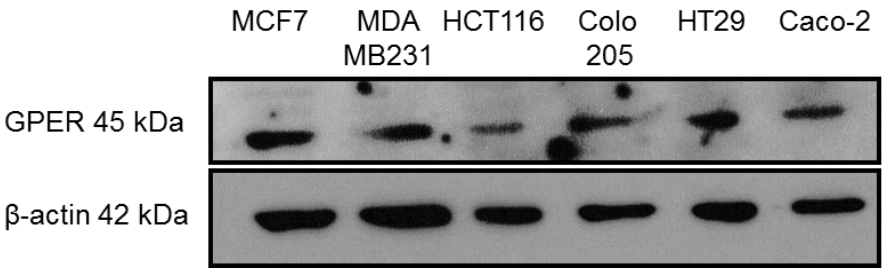



*Figure 6.1 GPER is expressed in colorectal cancer cell lines. Typical Western blot of GPER in breast cancer cell lines MCF7 and MDAMB231 and colorectal cancer cell lines HCT116, Colo205, HT29 and Caco-2. β-actin used as a loading control with 15 µg loaded into each well.*

### 6.3.2 GPER Stimulation Increases Proliferation in CRC Cell Lines

Directly stimulating GPER through agonist G1 instead of through a ligand such as $E_2$ bypassed the possibility of the ligand being metabolised to a non-stimulatory substrate such as $E_2S$. GPER agonist G1 increased proliferation in CRC cell lines HCT116, HT29 and Caco-2 after 48 hours as assessed using BrdU. Absorbance values over G1 concentration range for all three cell lines are shown in Figure 6.2 (A) with (B) displaying percentage change compared to SFBS control. Unlike with $E_1$ and $E_2$ treatment whereby HCT116 and HT29 cells proliferated but Caco-2 cells did not (Figure 5.13) all three cell lines responded to G1. Caco-2 cells have higher overall absorbance values meaning they incorporated more BrdU during the 4 hour incubation (see section 2.7.2), but their response to G1 was not as pronounced compared to HCT116 and HT29 cells. Instead G1 EC50 was 0.11, 3.56 and ~12.78 nmol/l for HCT116, HT29 and Caco-2 cells, respectively.

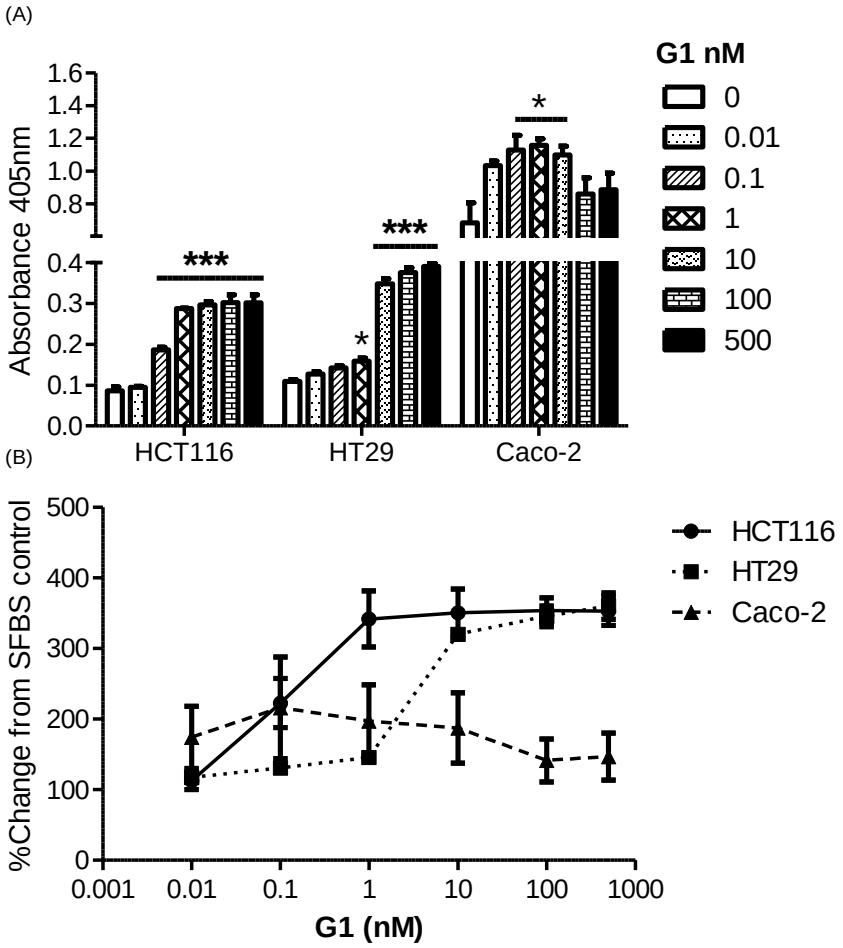



*Figure 6.2 GPER stimulation increased proliferation in colorectal cancer cell lines. Cells lines were treated with G1 between 0-500 nmol/l for 48 hours and proliferation measured using BrdU assay. (A) Absorbance values and (B) percentage change from SFBS. Proliferation increased in all cell lines. G1 EC50 was 0.11, 3.56 and ~12.78 nmol/l for HCT116, HT29 and Caco-2 cells, respectively. (A) One-way ANOVA with Bonferroni post-test *p < 0.05, **p < 0.01, ***p < 0.001, (n = 3-4). (B) The response of HCT116 and HT29 cells to G1 was greater than Caco-2 cells (***p < 0.001, two-way ANOVA with Bonferroni post-test). Experiments performed with medical student Habibur Rahman.*

In complete media, SFBS media and SFBS media supplemented with $E_2S$ $HCT116_{STS}$ have higher proliferation rates than $HCT116_{WT}$ or $HCT116_{VO}$ cells and this effect is lost in each case with STS inhibitor STX64 (Figure 5.11). To test if $E_2$ generated by STS desulphation acts through GPER HCT116 cells were treated with GPER antagonist G15 and $E_2$ (Figure 6.3). $E_2$ treatment increased proliferation in

all HCT116 cell types and this was inhibited by G15, suggesting $E_2$ acting via GPER increases proliferation in HCT116 cells.

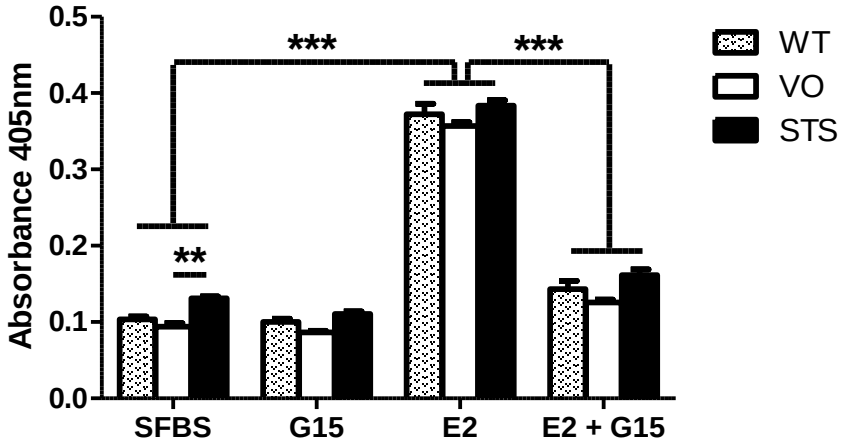
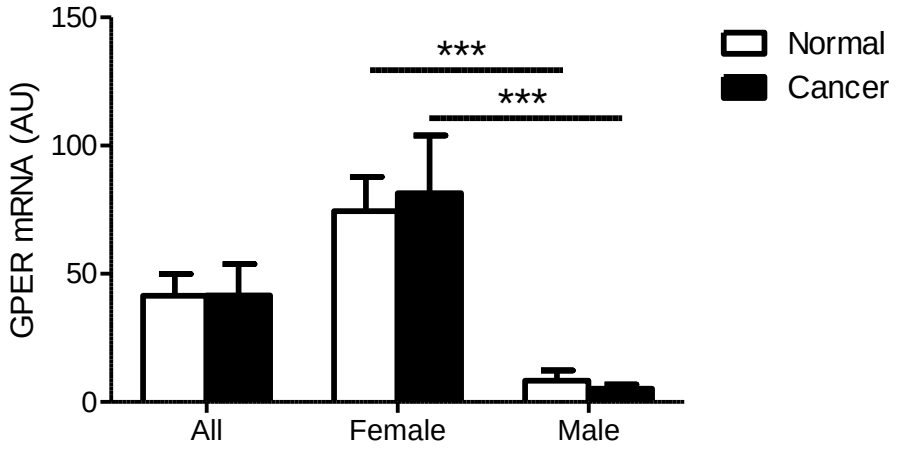



*Figure 6.3 GPER inhibition reduces proliferation in oestrogen stimulated HCT116 cells. $HCT116_{WT}$, $HCT116_{VO}$ and $HCT116_{STS}$ cells were treated with SFBS, GPER antagonist, G15 (1 μM), $E_2$ (100 nM) or $E_2$ plus G15 for 48 hours. Proliferation was measured by BrdU incorporation assay. G15 inhibited all cell types when treated with proliferation promoting $E_2$. These n = 3 experiments were performed with intercalating medical student Habibur Rahman. Two-way ANOVA with Bonferroni post-test *p < 0.05, **p < 0.01, ***p < 0.001.*

### 6.3.3 GPER Expression in Human Colon Tissue

GPER has previously been demonstrated in the myenteric plexus of human colon (Li et al., 2015). Here, GPER mRNA expression was detected in normal and cancerous colon tissue with no significant expression change between matched normal and cancerous tissue (Figure 6.4). However, there was a significant difference in expression between female and male colon with higher GPER expression in both normal and diseased female colon tissue samples.




| GPER mRNA Expression (dCt) | All (n = 37) | Female (n = 18) | Male (n = 19) |
|---|---|---|---|
| Normal | 7.33 [6.10,8.55] | 4.57 [3.84,5.29] | 9.94 [8.38,11.49] |
| Cancer | 7.10 [5.89,8.31] | 4.22 [3.76,4.68] | 9.83 [8.36,11.29] |

*Figure 6.4 GPER is expressed in healthy and cancerous human colon. GPER mRNA expression (arbitrary units (AU)) in human colorectal cancer tissue with matched normal controls (error bars are SEM). Mean dCt values and 95% CI displayed in brackets in the table below. Significantly higher expression of GPER was seen in females compared to males in both normal and tumour tissue. Two-way ANOVA with Bonferroni post-test ***p < 0.001.*

WB showed GPER protein was expressed in female and male human colon. GPER was detected in all samples tested but expression varied between patients with no consistent changes between healthy and cancerous colon (Figure 6.5).

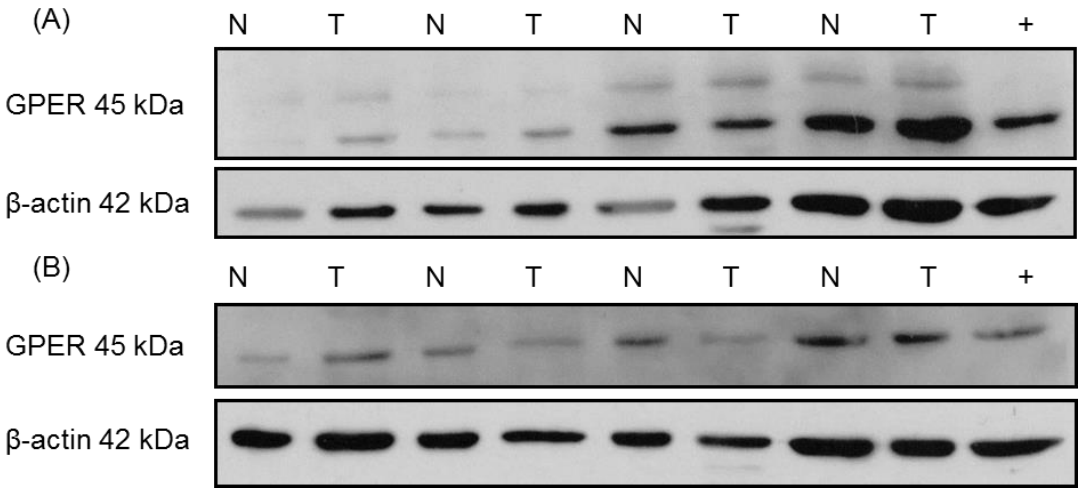



*Figure 6.5 GPER protein expression in match human colorectal cancer. Western blot of GPER in human female (A) and male (B) colorectal cancer tissue (T) with matched normal (N) control. MCF7 cells are used as a positive control (+). β-actin is used as loading control and 25 µg of colon tissue protein was loaded into each well and 15 µg of MCF7 protein.*

### 6.3.4 In vivo

*In vitro* overexpression of STS and $E_2$ treatment in HCT116 cells increased proliferation. These effects were inhibited by GPER antagonist G15 (Figure 6.3). Directly stimulating GPER with agonist G1 also increased proliferation *in vitro* (Figure 6.2). *In vivo* research within the group has supported these *in vitro* findings. $HCT116_{STS}$ cell xenografts in intact female athymic nude mice proliferated faster than $HCT116_{VO}$ cells and this effect was abolished by STS inhibitor STX64 (unpublished, Appendix III). This suggests that STS desulphation or oestrogen increases proliferation both *in vitro* and *in vivo*.

To assess if the increase in proliferation caused by STS activity *in vivo* was due to GPER action mice bearing $HCT116_{STS}$ xenografts were treated with G15. $HCT116_{STS}$ cells were implanted into the right flanks of athymic nude mice. Mice

were treated three times per week with G15 (50 µg/kg) or vehicle control and tumour sizes ($mm^3$) monitored. After 19 days the experiment was terminated due to large tumour size. G15 treatment inhibited tumour growth in $HCT116_{STS}$ xenografts (406.7 ± 51.4$mm^3$) compared to aqueous vehicle control (580.7 ± 91.5$mm^3$, p = 0.007, Figure 6.6).

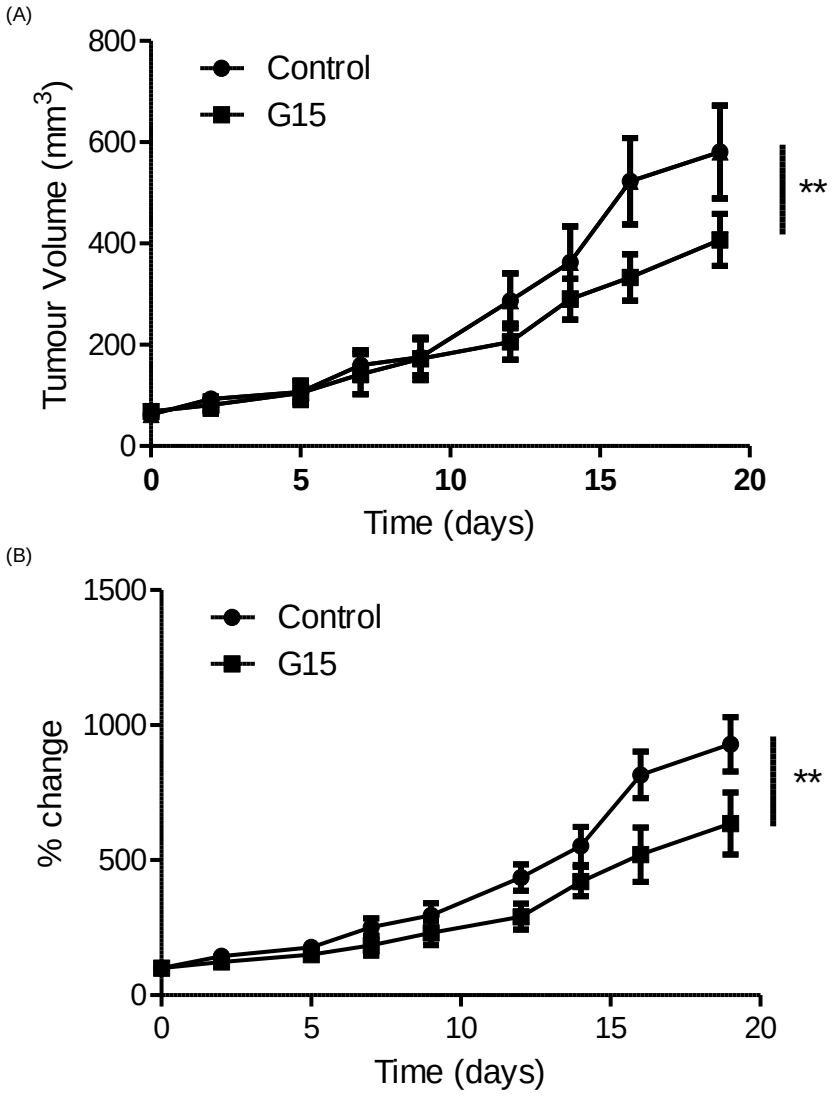



*Figure 6.6 G15 inhibits $HCT116_{STS}$ cell growth in vivo. Mice treated with G15 (50 µg/kg) three times per week i.p had reduced growth of $HCT116_{STS}$ xenograft tumours compared to aqueous vehicle (two-way ANOVA **p = 0.007). (A) $HCT116_{STS}$ tumour volume ($mm^3$) change over time. (B) Percentage change in tumour volume ($mm^3$) compared to day 0. Control n = 10 and G15 treated n = 9.*

## 6.4 DISCUSSION

### 6.4.1 GPER Expression and Function in the Colon *in vitro*

GPER appears to have multiple functions with roles in reproduction and fertility, vasculature, kidney, brain, adipocyte function and cancers (Barton, 2016). It has been located within intracellular membranes and on the cell surface undergoing endocytosis (Gaudet et al., 2015). Within the colon GPER has only been shown in the myenteric plexus and is thought to have a role in changes in GI motility throughout the menstrual cycle (Li et al., 2015). Here, GPER expression is shown in both normal and cancerous colon homogenate (Figure 6.4) and CRC cell lines (Figure 6.1). Colon tissue homogenate will contain a mix of cell types and thus cannot distinguish which cells express functional GPER. However, its presence in both healthy colon and in cancer is important as it offers a potential receptor for oestrogen stimulated growth.

HCT116 and HT29 CRC cells did not have detectable ERα or ERβ however they did express GPER (Figure 6.1). This receptor could account for their increased proliferation in response to oestrogen (Chapter 5, Figure 5.13). Despite GPER expression Caco-2 cell growth remained static in response to oestrogens. This could be explained by their oestrogen metabolism profile showing $E_1$ generation when given $E_2$, probably through HSD17β2 activity. $E_1$ has less than 0.1% affinity for GPER compared to $E_2$ (Thomas et al., 2005) and so may not lead to the oestrogen growth effects seen in HCT116 and HT29 cells.

Both HCT116 and HT29 cells proliferated in a dose-dependent manner with oestrogen treatment ($E_1$, $E_2$ and $E_1S$) (Figure 6.2). However, although Caco-2 cells did not have a growth response to $E_1$ or $E_2$ (Figure 5.13) G1 did increase their growth. Caco-2 cell response to G1 was not as large as HCT116 and HT29 cells despite having a prominent GPER band on Western blot (Figure 6.1). This suggests that GPER stimulation may only play a limited role in proliferation in Caco-2 cells.

GPER has been identified both on the plasma membrane and intracellularly (Gaudet et al., 2015) and it is not currently clear how cellular location affects GPER action. In breast cancer GPER expression has been linked with tumour proliferation (Marjon et al., 2014). Also, in breast cancer the cellular location of GPER has been associated with prognosis with predominately cytoplasmic localisation correlating with a better outcome (Samartzis et al., 2014). Further studies investigating GPER location and intracellular trafficking in these CRC cell lines may reveal a difference and explain the smaller proliferative response in Caco-2 cells.

Other studies have shown different findings to those presented here. They found Caco-2 cell growth increased with $E_2$ and was inhibited by GPER agonists tamoxifen and ICI 182,780 (Di Domenico et al., 1996, Thomas et al., 2005). However, Domenico and colleagues demonstrated ER expression in Caco-2 cells. The cells presented here have been authenticated (DNA Diagnostics Centre, 2015) and ERα and ERβ were only minimally detected in Caco-2 cells and were not expressed in HCT116 and HT29 cells (Appendix III). Although low, ERβ expression may account for the reduced oestrogen proliferative response of Caco-2 cells compared to

HCT116 and HT29 as ERβ action may induce apoptosis. Low or non-existent ER expression is in keeping with human CRC where the usually dominant ERβ is down-regulated (Campbell-Thompson et al., 2001, Elbanna et al., 2012, Niv, 2015).

$HCT116_{STS}$ cells proliferated more than $HCT116_{VO}$ and $HCT116_{WT}$ and this effect was inhibited by STS inhibitor STX64 (Chapter 5, Figure 5.12) and GPER antagonist G15 (Figure 6.3). $E_2$ stimulated growth in these three HCT116 cell types was also inhibited by G15 (Figure 6.3). Together these results suggest desulphation of oestrogens by STS leads to GPER activation and proliferation *in vitro*.

### 6.4.2 GPER Expression and Function in the Colon *in vivo*

To investigate GPER action in CRC *in vivo* $HCT116_{STS}$ cell xenografts were injected into the right flank of athymic nude mice resulting in rapidly growing tumours. Proliferation was slower in $HCT116_{STS}$ tumours treated with G15 indicating GPER inhibition can reduce tumour growth *in vivo* (Figure 6.6) as well as *in vitro*. Previous research (unpublished, Appendix III) has shown that overexpressing STS in HCT116 cells increases tumour proliferation *in vivo* compared to $HCT116_{VO}$ and this effect is inhibited by STX64. Together this demonstrates that STS increases proliferation in CRC and this effect can be inhibited by GPER antagonist. Thus, a potential novel pathway has been demonstrated. Active oestrogens generated through increased STS desulphation and loss of pro-apoptotic ERβ leads to GPER action and increased CRC proliferation (Figure 6.7).

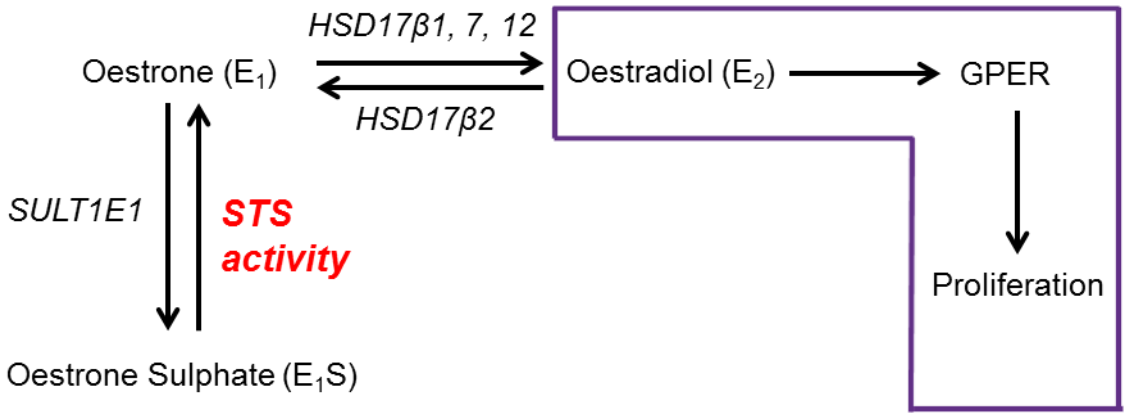



*Figure 6.7 Oestrogen metabolism pathway in colorectal cancer. Locally generated oestradiol acts on GPER to stimulate proliferation.*

## 6.5 CONCLUSION

Oestrogen receptor GPER is expressed in both normal and cancerous colon tissue and is pro-mitogenic *in vitro*. GPER antagonist G15 can inhibit oestrogen proliferative effects *in vitro* and *in vivo* demonstrating a novel pathway for which locally produced $E_2$ can cause CRC proliferation, potentially offering new treatment targets.

# CHAPTER 7. REGULATION OF STS BY TNFα AND IL-6

## 7.1 INTRODUCTION

Chronic inflammation is an acquired tumour cell trait necessary for growth, survival and metastasis (Hanahan and Weinberg, 2000, Colotta et al., 2009, Hanahan and Weinberg, 2011). IBD and obesity are two inflammatory states that raise CRC risk and so chronic inflammation may also initiate and promote tumour growth (Flores et al., 2012, Flores et al., 2015, Barral et al., 2015). Oestrogens influence inflammatory processes, but the relationship is not straightforward with evidence for both pro and anti-inflammatory effects (Straub, 2007).

Inflammation around a solid malignancy such as CRC accumulates a variety of inflammatory cells that produce an assortment of inflammatory mediators, including TNFα and IL-6 (Peddareddigari et al., 2010). TNFα and IL-6 can regulate STS leading to increased expression and/or activity in breast and prostate cancer cell lines (Newman et al., 2000, Suh et al., 2011). Chapter 5 demonstrates immortalised CRC cell lines display diversity in STS activity, oestrogen enzyme mRNA expression and protein expression. Chapter 4 also shows patients had increased STS activity in tumour tissue without changes to STS mRNA expression implying post translational modification of STS. Thus, TNFα and IL-6 within the tumour microenvironment could be responsible for STS dysregulation and ultimately alter oestrogen metabolism.

A link between STS activity and inflammation has been demonstrated previously. Elevated STS activity, commonly found in human CRC (see Chapter 4), increases CRC proliferation. The aim of this chapter was to investigate the effects of TNFα

and IL-6 on STS activity in the colon *in vitro* and *in vivo*. TNFα receptors have been shown to be expressed in the colon and CRC cells (Hamilton et al., 2011, Mizoguchi et al., 2002, Chopra et al., 2013, Peng et al., 2012, Naude et al., 2011).

## 7.2 MATERIALS AND METHODS

### 7.2.1 Cell Culture

HCT116, Colo205 and Caco-2 cells were cultured as described in section 2.1.

### 7.2.2 STS Activity Assay

#### 7.2.2.1 TNFα and IL-6 Treatment

HCT116, Colo205 and Caco-2 cells were seeded into T25 flasks at 250,000 cells per flask and treated with 40 ng/ml TNFα (PeproTech), 10 ng/ml IL-6 (PeproTech), both TNFα and IL-6 or PBS as a control. After 48 hours media was removed and STS assay performed as described in section 2.8.2 with results expressed as $E_1S$ to $E_1$ conversion in pmol/mg/hr. The concentrations of TNFα and 48 hour time point were chosen as a result of experiments by technician Anne-Marie Hewitt and BMedSci student Alice Ross (Appendix III). IL-6 concentration was chosen at a dose that other research groups had used in STS activity and expression studies for prostate and breast cancer cell lines *in vitro* (Im et al., 2012, Honma et al., 2002, Speirs et al., 1993). Further optimisation of IL-6 and TNFα concentrations and time points would be beneficial.

#### 7.2.2.2 TNFα mice

Mice that globally overexpress human TNFα (Tg-TNF) are used in inflammatory arthritis studies (Keffer et al., 1991, Hardy et al., 2016). Once culled these 9 week old Tg-TNF mice were kindly donated along with age-matched WT controls from Dr Rowan Hardy at The University of Birmingham. Liver, lung, kidney, heart, large bowel, spleen, adipose tissue and skin were harvested and snap frozen in liquid nitrogen before being stored at -80$^{o}$C. Tissues were then homogenised to collect both RNA and protein as described in sections 2.2 and 2.3. Protein was used to assess STS activity as described in section 2.8.1.

### 7.2.3 RT-PCR

RNA extraction, cDNA synthesis, cDNA quantification followed by real-time PCR were performed as described in section 2.3- 2.6 using STS, HSD17β2, HSD17β7, HSD17β12 and RPLPO genes in CRC cell lines. Additionally, il-6, TNFα, sts, hsd17β2 and gapdh were measured in mouse tissue (Table 2.2).

## 7.3 RESULTS

### 7.3.1 TNFα and IL-6 Increase STS Activity in CRC Cell Lines

The effect of TNFα and IL-6 on STS activity in Colo205, Caco-2 and HCT116 cells was assessed by treating cells with TNFα and IL-6 for 48 hours followed by STS activity assay. Figure 7.1 shows all three CRC cell lines had similar response trends e.g. STS activity increased with TNFα and TNFα plus IL-6 treatment. STS activity was significantly higher in treated Colo205 and Caco-2 cells whereas the upwards

trend in HCT116 cells was not significant. STS mRNA expression remained unchanged (data not shown).

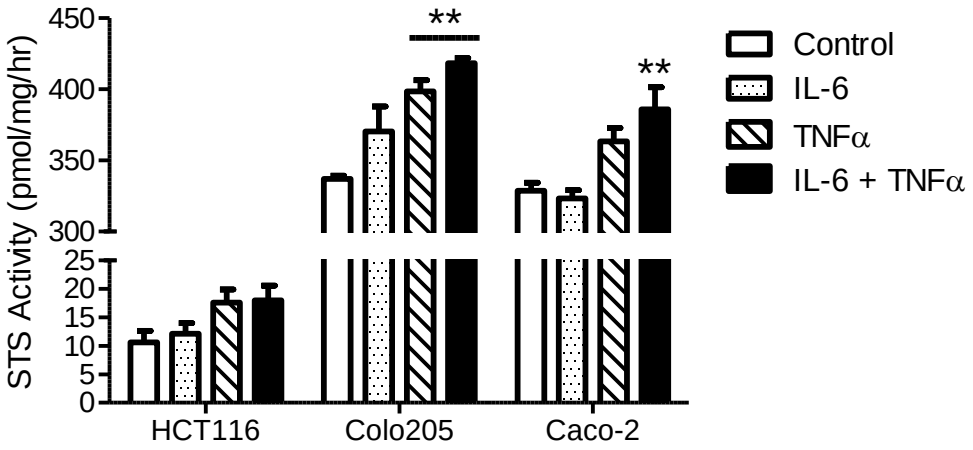



*Figure 7.1 TNFα and IL-6 increase STS activity in colorectal cancer cell lines. STS activity significantly increased in Colo205 and Caco-2 cells treated with inflammatory mediators TNFα (40 ng/ml) and IL-6 (10 ng/ml) for 48 hours (n = 3). HCT116 cells followed a similar trend, but the increase in STS activity was not significant (n = 4). Owo-way ANOVA with Bonferroni post-test, $^{**}p < 0.01$. Experiments performed with laboratory technician Anne-Marie Hewitt.*

### 7.3.2 TNFα, IL-6 and HSD17β Expression

In human CRC STS activity is increased, HSD17β2 mRNA is decreased and HSD17β7 and 12 mRNA are up-regulated (Chapter 4). TNFα and IL-6 increased STS activity in CRC cells lines (Figure 7.1). The effect on HSD17β expression was also explored to assess if TNFα and IL-6's impact was extended to other oestrogen metabolism enzymes. Colo205 and Caco-2 cells treated with TNFα and IL-6 for 48 hours showed a reduced HSD17β2 mRNA expression trend. However, only significant changes were noted in Caco-2 cells treated with combination treatment ($^{*}p < 0.05$, Figure 7.2). As HCT116 had undetectable HSD17β2 mRNA (Figure 5.1)

they were not examined. HSD17β7 and 12 mRNA remained unchanged with TNFα and IL-6 treatment (data not shown, n = 2-3).

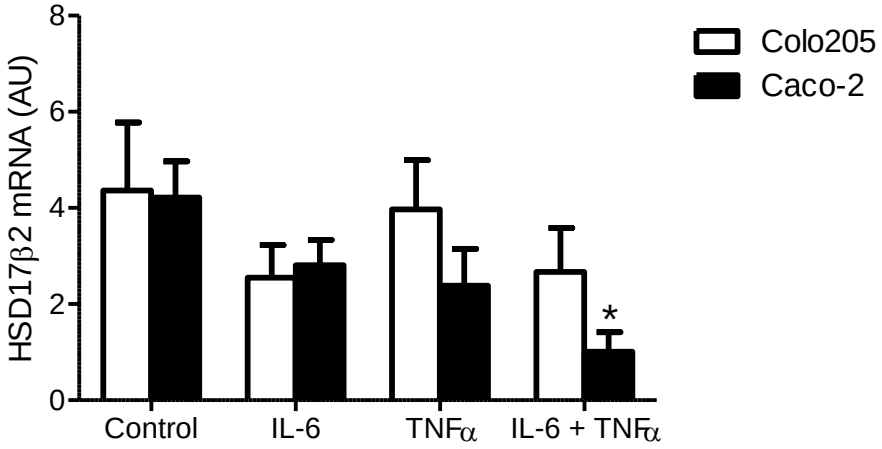



*Figure 7.2 HSD17β2 mRNA is not regulated by TNFα and IL-6. HSD17β2 mRNA was quantified using real time PCR in CRC cells treated with TNFα (40 ng/ml) and IL-6 (10 ng/ml) for 48 hours and displayed in arbitrary units (AU). Caco-2 cells had significant reduction in HSD17β2 mRNA after combination treatment, but no change was seen in Colo205 cells. One-way ANOVA with Bonferroni post-test, n = 3, $^{*}p < 0.05$.*

### 7.3.3 STS Activity is Increased Globally in Tg-TNF Mice

Tg-TNF mice globally overexpress human TNFα. To assess if TNFα regulates STS *in vivo,* protein and RNA were harvested from various tissues of 9 week old Tg-TNF mice and age-matched WT controls and STS activity measured. STS activity appeared higher in almost all tissues collected with significant increases in liver (Figure 7.3). There was not enough numbers to separate mice into males and females so gender differences could not be confirmed.

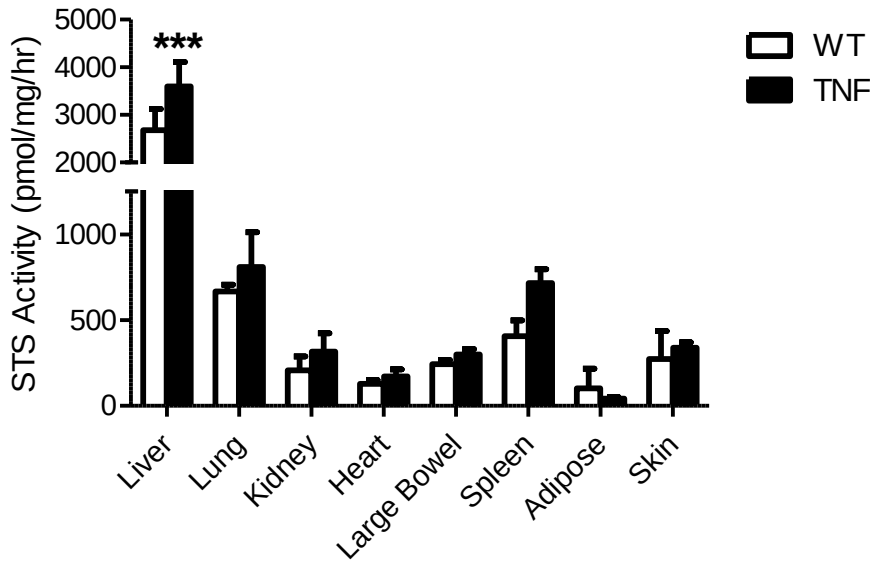



*Figure 7.3 TNFα increases STS activity in multiple tissue types. Tg-TNF mice have global increased STS activity. STS activity was measured in multiple tissues and found to be elevated in TNF-Tg mouse tissues compared to wild-type controls (WT); significant increases seen in the liver (n = 6-7). Experiments performed with masters student Mai Nguyen. Two-way ANOVA with Bonferroni post-test, $^{***}p < 0.001$.*

### 7.3.4 Tg-TNF Mice Oestrogen Enzyme mRNA Expression

To explore if increased STS activity (non-significant) found in the large bowel of Tg-TNF mice was related to STS mRNA expression real time PCR was performed. There was no change in STS mRNA and overall expression was low (Figure 7.4). IL-6 mRNA expression was also low in both Tg-TNF and WT mice with no significant changes (Figure 7.4 (A and C)). HSD17β2 mRNA was present but there were no differences between Tg-TNF and WT mice. HSD17β2 mRNA appeared higher in male WT mice compared to females however due to large variations in expression this was not significant (Figure 7.4 (B)). These Tg-TNF mice are understood to have global overexpression of human TNFα (Kontoyiannis et al., 1999, Keffer et al., 1991). However, human TNFα mRNA expression was low in both WT and Tg-TNF

mice (data not shown), with only a small but notable increase in female Tg-TNF colon (Figure 7.4 (D)).

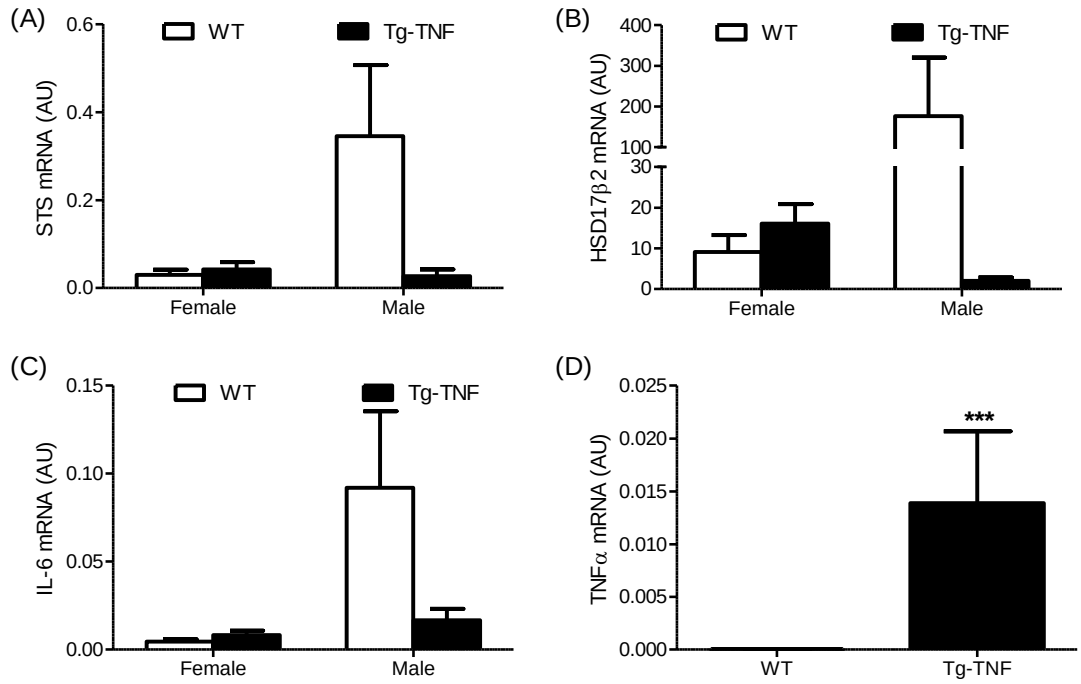



*Figure 7.4 STS and HSD17β2 mRNA is not regulated by TNFα in vivo. (A) STS (B) HSD17β2 (C) IL-6 mRNA expression is unchanged in the large bowel of male and female Tg-TNF mice. (D) Human TNFα was increased in female mice only (n = 5-6 for females and males). Two-way ANOVA with Bonferroni post-test ***p < 0.01.*

## 7.4 DISCUSSION

### 7.4.1 TNFα Regulates Colon STS Activity *in vitro*

TNFα increased STS activity in Colo205 and Caco-2 cells. HCT116 cells had an upwards trend in STS activity but it was not significant (Figure 7.1) which may be due to their comparatively lower basal STS activity (Figure 5.9). These data are in keeping with other cell lines; TNFα has been shown to increase STS activity in MCF7 breast cancer cells (Newman et al., 2000) and STS expression in LNCaP and PC-2 prostate cancer cell lines (Suh et al., 2011). The increase in STS activity

in CRC cell lines was not due to higher STS mRNA expression and so gene transcription indicating TNFα and/or IL-6 may increase STS activity through post-translational modification e.g. STS has four N-linked glycosylation sites with two (Asn47 and Asn259) affecting activity (Stengel et al., 2008). Alternatively, TNFα may increase STS substrate availability, such as altering $E_1S$ transport across the cell membrane.

HSD17β2 mRNA was reduced in human CRC and HSD17β7 and 12 increased (section 4.3.1). HSD17β2 has previously been shown to be regulated by retinoic acid (Su et al., 2007) and progesterone (Yang et al., 2001) and HSD17β7 upregulated by oestradiol (Shehu et al., 2011). TNFα and IL-6 do not appear to regulate HSD17β enzymes as expression remained essentially unaltered in CRC cell lines. Rises in STS activity could have a knock-on effect and increase HSD17β activity due to increased substrate, but in the results presented here, this may be limited by co-factor availability. As $E_1$ binds to ERα with 10% affinity of $E_2$, 2% for ERβ (Zhu et al., 2006) and just 0.1% for GPER (Thomas et al., 2005) only small changes in $E_1/E_2$ ratio could lead to large downstream effects. HSD17β2, 7 and 12 could be considered as therapeutic targets however due to the multitude of HSD17β enzymes and functional overlap this would be challenging. Nevertheless, the benefit of variable tissue expression could allow for tissue targeted inhibitors reducing side-effects.

### 7.4.2 TNFα Regulates STS Activity *in vivo*

To explore if TNFα induced STS activity was a phenomenon confined to cancer models, activity was also measured in a non-cancerous model. Tg-TNF mice globally overexpress human TNFα and have been developed for the study of inflammatory arthritis. Overall Tg-TNF tissues showed higher STS activity compared to WT but only liver showed significant increases (Figure 7.3). Therefore, TNFα also increases STS activity in noncancerous tissues and consequently may be part of a fundamental homeostatic response to inflammation rather than confined to a malignant phenomenon.

Tg-TNF mice are designed to have global overexpression of human TNFα. They are used primarily as an inflammatory arthritis model, (Keffer et al., 1991) however in addition to joint symptoms, overexpression of TNFα can lead to colitis. CRC has not been described. (Kontoyiannis et al., 1999). Conversely, when TNFα mRNA was measured, it was not raised in male mouse colon and only marginally increased in female mice (Figure 7.4). There was evidence of active arthritis in these animals despite their young age (9 weeks) and human TNFα increases were identified in other tissues such as muscle and joint (Keffer et al., 1991, Hardy et al., 2016). It is possible colon TNFα expression occurs later in their growth. However, despite low TNFα mRNA expression in male colon or increased STS mRNA expression in males and females, colonic STS activity appeared higher in both genders. As these experiments were in homogenised lysed tissue, alterations in cell membrane transport leading to increased $E_1S$ availability could not explain the increase in STS

activity. This suggests systemic inflammation rather than local TNFα exposure could post-translationally modify STS to increase activity in the colon.

### 7.4.3 Inflammatory Bowel Disease and STS

A proportion of IBD patients have systemic symptoms including arthritis however IBD associated arthritis does not lead to joint destruction and deformity and so is distinct from other inflammatory arthritides, such as rheumatoid (Gravallese and Kantrowitz, 1988). IBD increases CRC risk (Barral et al., 2015) however patients with rheumatoid arthritis actually have a reduced CRC risk. This is thought to be due to anti-inflammatory medications (Simon et al., 2015) as long-term use of nonsteroidal anti-inflammatories (NSAIDS) can reduce CRC risk by 40-50% (Smalley and DuBois, 1997). NSAIDS act through inhibition of cyclooxygenase -2 (COX-2) rather than TNFα. However, TNFα has been shown to increase COX-2 expression (Zhu et al., 2013, Medeiros et al., 2010, Shishodia et al., 2004) and so NSAIDS may indirectly affect STS. There is a growing body of evidence that shows TNFα regulates STS through NFκB pathways. STS expression in human MG-63 preosteoblasts was induced by NFκB (Dias and Selcer, 2016). Also, in prostate cancer PC-3 cells STS was induced by IGF-II via a PI3-kinase/AKt-NFκB pathway (Sung et al., 2013). Additionally, in hepatic tissue STS activated oestrogens also attenuated NFκB mediated inflammation showing a negative feedback loop (Jiang et al., 2016). Together these data suggests STS is regulated as part of the inflammatory response in multiple tissue types. In cancers such as CRC this TNFα-NFκB-STS pathway may become dysregulated leading to increased active

oestrogen production however further research would need to be done to confirm this.

### 7.4.4 STS and Colorectal Cancer Prognosis

Higher STS expression has been linked with poorer prognosis in CRC however functional STS activity was not examined (Sato et al., 2009). The relationship between STS activity and prognosis would be important to determine as due to STS post translational modifications mRNA and protein are not necessarily proportional to activity. In human CRC tissue, CRC cell lines treated with TNFα and Tg-TNF mouse colon STS mRNA remained unchanged despite increased STS activity. In CRC STS activity appears to be increased at an early disease stage (Chapter 4) but it is currently unclear whether higher STS activity has a positive or negative impact on CRC prognosis. Additionally, the effect of STS on IBD symptoms and CRC are unknown. Despite clinical and histopathologic differences between UC associated CRC and sporadic CRC, survival outcomes are similar. Females with UC-CRC have an improved outcome compared to males (Leowardi et al., 2016). This may be due to the consequences of differences in oestrogen enzyme expression between males and females (Chapter 4). Increased STS activity and higher local oestrogen concentrations in women may have a greater protective effect. Alternatively, STS may increase local androgens in males which may be detrimental to survival (Amos-Landgraf et al., 2014).

In mouse models inflamed stroma has been shown to promote progression of adenomas to adenocarcimonas (Okada et al., 2000). However, it is unclear if higher

STS activity and subsequent oestrogen increase is beneficial or detrimental. Acute inflammation and increased STS effects may vary per individual. However, chronic and sustained inflammation with increased STS activity may contribute to adenomas and CRC. ERβ loss in CRC (discussed further in Chapter 6) could switch oestrogen effects from apoptotic to pro-mitogenic with tumour cells exploiting initial protective measures to aid proliferation and metastasis.

## 7.5 CONCLUSION

TNFα increases STS expression and/ or activity in multiple cancer types including breast, prostate and CRC. However, TNFα associated STS activity is not a phenomenon confined to malignancy and occurs in multiple tissues types by STS post translational modification. Consequently, TNFα regulated STS activity may be a normal physiological homeostatic response of which the mechanism has yet to be fully elucidated. TNFα and increased STS activity may be detrimental in chronic inflammatory states such as poorly controlled IBD, obesity and cancer and in these circumstances anti-inflammatories and STS inhibition may be of clinical benefit.

# CHAPTER 8. FINAL DISCUSSIONS AND FUTURE STUDIES

The work presented in this thesis describes a pathway for localised colonic active oestrogen generation that ultimately increases proliferation in colorectal tumours, highlighting multiple potential drug targets. Previous published research has described parts of this oestrogen pathway. English and colleagues identified decreased oxidative HSD17β in CRC with reduced $E_2$ to $E_1$ conversion (English et al., 1999). Also, Sato and colleagues found STS expression was frequently raised in CRC. They also found higher STS mRNA and lower SULT1E1 mRNA correlated with a poorer outcome and the reverse with improved patient survival (Sato et al., 2009). Data presented here supports these published findings and attempts to combine both HSD17β and STS data finding CRC metabolism is frequently altered towards $E_2$ generation. Previous research focus has been on traditional oestrogen receptors ERα and ERβ however here it is shown that active oestrogens exert their effects in the colon through GPER increasing proliferation.

## 8.1 LC-MS/MS OESTROGEN METHOD DEVELOPMENT

Oestrogen sulphates are frequently ignored in quantitative analysis with focus on active unconjugated forms. However, $E_1S$ circulates at higher concentrations than either $E_1$, $E_2$ or in combination and has a significantly longer half-life. $E_1S$ acts as a reservoir for active oestrogen generation (Reed et al., 2005). Tissues able to uptake $E_1S$ and have STS activity can locally alter active oestrogen concentrations. The effect of $E_1S$ and STS activity has been recognised in breast cancer where STS expression is 50-200 times that of aromatase (Pasqualini et al., 1996) and STS inhibitors are in Phase II clinical trials (Stanway et al., 2006, Palmieri et al., 2016a, Palmieri et al., 2016b). However, despite this $E_1S$ is not routinely measured either

systemically or in tissue. This novel LC-MS/MS method acknowledges the importance of including $E_1S$ concentrations in oestrogen responsive tissues such as the colon.

Previous LC-MS/MS methods have analysed unconjugated oestrogens (Owen et al., 2014), $E_1S$ (Corona et al., 2010), or used two extractions and two LC-MS/MS methods to enable both sulphated and non-sulphated oestrogens to be quantified (Galuska et al., 2013). Alternatively, IA have been used to measure oestrogen sulphates separately or lengthy GCMS analysis (Stanway et al., 2007, Ranadive et al., 1998). This is a novel high-throughput LC-MS/MS method to quantify oestrogen and their sulphates simultaneously in cell culture validated to industry standard. In comparison to other methods this uses a single SPE without any derivitisation to quantify $E_1$, $E_2$, $E_1S$ and $E_2S$ in a five minute run. With a LLOQ of 14 nmol/l for $E_1$ and $E_2$ and 18 nmol/l for $E_1S$ and $E_2S$ it was not as sensitive as other oestrogen methods described above. With a more sensitive MS the more desirable pmol/l concentrations could be achieved and so be applied to serum and colon tissue. This method has been applied successfully to CRC cell lines in culture and showed differences in oestrogen metabolism which can potentially explain the differences in their proliferative responses.

## 8.2 COLORECTAL CANCER FAVOURS OESTRADIOL PRODUCTION

To characterise oestrogen metabolism in CRC and matched human colon tissue, expression of oestrogen metabolism enzymes were assessed. Findings were supportive of previous published research with reduced HSD17β2 expression and

increased STS activity. Here for a novel holistic view oestrogen oxidoreductive metabolism and desulphation were combined in one dataset.

STS activity is frequently overlooked with most studies focusing on mRNA and protein expression. STS can undergo extensive post translational modifications impacting on desulphation capability and thus measuring STS activity assesses functional changes. As there was no alteration in STS mRNA expression these data suggest that post translational STS modifications contribute significantly to STS activity in CRC and mRNA and protein data alone are insufficient. Sato and colleagues measured expression with STS/SULT1E1 expression ratio correlating with prognosis (Sato et al., 2009). The increase in STS activity shown in this thesis does follow a similar pattern to the expression findings by Sato and colleagues. However, here STS activity was also assessed by disease stage showing increases in STS activity occurred in early disease in both males and females and this was likely regulated by localised inflammation. SULT1E1 was impossible to assess here through a lack of cDNA primers and probes, suitable commercial WB antibodies and ex-vivo activity assays. Sulphated oestrogens have negligible ER receptor binding capability and are considered biologically inactive (Zhu et al., 2006). $E_2$ is also much more potent than $E_1$ (Zhu et al., 2006, Thomas et al., 2005). Therefore, only small STS/SULT1E1 and $E_1/E_2$ ratio changes could produce dramatic downstream effects. SULT expression is generally thought to be down-regulated in cancer (Mueller et al., 2015). However, even without assessing SULT1E1 activity here this increase in STS activity is an important finding suggesting a new biological target for CRC.

HSD17β1 is the principal enzyme responsible for $E_1$ to $E_2$ reduction however as with other published research was not expressed in colon tissue or CRC cell lines. Alternative enzymes therefore had to be responsible for this conversion. HSD17β7 and 12 were examined and both mRNAs were found to be expressed in CRC cell lines, benign and cancerous CRC human tissue with expression increased in CRC. HSD17β7 protein was not able to be examined by Western blot. HSD17β12 protein was detected and like HSD17β2 there was a mismatch between mRNA and protein with significant increases in mRNA not mirrored by protein. This discrepancy between HSD17β mRNA and protein has been previously described and acknowledged by other groups (Day et al., 2006, English et al., 1999). HSD17β activity has been found to closely correlate with mRNA rather than protein stipulating that co-factor availability may be the cause (Day et al., 2006, English et al., 1999). Overall HSD17β activity was shown in CRC cells using LC-MS/MS. However, this was not sensitive enough to show lower levels of activity and no inhibitors were available to assess oxidation and reduction reactions individually. Also, it was currently not possible to assess specific HSD17β activity in fresh tissue or protein extracts therefore although enzyme expressions are altered in CRC, end results remain to be defined.

Sex differences in mRNA were noted for HSD17β enzymes. Compared to males, females had lower HSD17β12 in healthy colon and higher HSD17β7 mRNA expression in both healthy and cancerous colon. Also, in advanced disease males had higher HSD17β7, females appeared to have lower HSD17β7 but this was not significant. This may be why there are differences in incidence and prognosis

between men and women and could lead to gender based treatment. However, overall these results show a potential drive towards $E_2$ generation in the majority of CRCs regardless of disease stage or sex: an increase in oestrogen desulphation via STS, reduced HSD17β oxidative expression and increased HSD17β reductive expression. HSD17β inhibitors appear to be attractive treatment targets and are currently being explored with HSD17β1 being targeted in breast cancer and endometrial disease and HSD17β3 in prostate cancer (Saloniemi et al., 2010, Day et al., 2008b). However, they have not yet reached clinical trials.

## 8.3 INCREASED STS ACTIVITY ENHANCES PROLIFERATION

Oestrogen-dependent cancers include endometrial, breast and ovarian and limiting oestrogen availability can reduce proliferation. Currently in breast cancer this is achieved through inhibition of oestrogen receptors or aromatase e.g. tamoxifen, anastrozole (National Institute for Health and Care Excellence, 2009). STS inhibitors reduce oestrogen generation from circulating $E_1S$ and have progressed from Phase I (Stanway et al., 2006) to Phase II clinical trials for breast cancer (Palmieri et al., 2016a, Palmieri et al., 2016b).

*In vitro* oestrogen treatments ($E_1$, $E_2$ and $E_1S$) increased proliferation in a dose dependent manner in HCT116 and HT29 cells which was in contrast to Caco-2 cells. These proliferation differences were in part explained by oestrogen metabolism as assessed by LC-MS/MS. HCT116 and HT29 cells responded very similarly to oestrogen treatment however their metabolism profiles differed. Even when oestrogen increases proliferation in CRC cells there may be multiple mechanisms

and metabolites responsible. This emphasises the difficulty in trying to extrapolate *in vitro* data to human disease. CRC is heterogeneous and thus *in vitro* findings may still be relevant but only apply to a subset of patients or cell populations. As with hormonal breast cancer targeting multiple oestrogen pathways is likely to be more successful at improving prognosis. However, care should be taken as steroidiogenesis can still occur through alternative pathways leading to recurrence. This is a particular problem in recurrent prostate cancer (Ferraldeschi et al., 2015).

CRC cell lines did have similarities with human tissue with no HSD17β1 expression, low HSD17β2 and mismatch between HSD17β mRNA and protein. However, other than Caco-2 cells they had low STS activity and so no CRC cell line tested was really representative of CRC. To overcome this problem and develop a model similar to CRC stable STS overexpressing HCT116 cells were developed. These cells had low HSD17β2 expression and higher STS activity as seen in human CRC. This is a strategy utilised in breast cancer research where aromatase (Zhou et al., 1990, Macaulay et al., 1994) or STS (James et al., 2001) was overexpressed in MCF7 breast cancer cell lines in order to more closely mimic activity seen in human breast tumours.

$HCT116_{STS}$ had increased proliferation in complete and SFBS phenol red free media. It is not possible to completely remove all oestrogen traces in media however this does suggests it was not solely increased oestrogen generation from STS that amplified proliferation in $HCT116_{STS}$ compared to $HCT116_{VO}$. $E_2S$ treatment augmented proliferation further in $HCT116_{STS}$ cells and this effect was abolished by

STS inhibitor STX64. This is similar to breast cancer where oestrogen generation through the sulphatase pathway in STS overexpressing MCF7 cells increased proliferation and was inhibited by STX64 (Foster et al., 2008). In colon cancer it was still unclear how oestrogen exerted its proliferative effects as these cells had no detectable ERβ or ERα expression.

## 8.4 OESTROGEN ACTS THROUGH GPER IN THE COLON

The colon lacks ERα expression and anti-proliferative ERβ is frequently reduced in cancerous colorectal tissue (Campbell-Thompson et al., 2001, Niv, 2015). These ER findings were supported by CRC cell lines studied here. Therefore, how oestrogen action occurs is not understood. The relatively newly recognised ER GPER may be a viable route and GPER was identified in both healthy and cancerous colon tissue and CRC cell lines.

GPER was active in CRC cells *in vitro* as agonist G1 increased proliferation in all cell lines (HCT116, HT29 and Caco-2 cells). Caco-2 cells did not respond to $E_1$ or $E_2$ treatment and they rapidly metabolised $E_2$ to $E_1$ which has low affinity for GPER. This suggests higher HSD17β2 activity may limit oestrogen proliferative response in CRC. Increased proliferation of HCT116$_{STS}$ cells was inhibited by GPER antagonist G15 in both complete, SFBS and oestrogen treated media. This strongly suggests GPER is fundamental to oestrogen action within the CRC.

Supporting *in vitro* data G15 significantly reduced proliferation in HCT116$_{STS}$ xenografts *in vivo*. In this study intact female mice were used. In humans CRC is

more common in men and post-menopausal women thus it would be good to follow this study in ovariectomised female mice and male mice with and without oestrogen treatment.

If novel oestrogen receptor targeted treatment is to be considered for CRC then how oestrogen receptor inhibitors act and cross react with ERα and ERβ is important to consider. Tamoxifen is a prodrug and when metabolised into its active metabolites competitively competes with oestrogen for ER binding (Desta et al., 2004). It is a common and successful oestrogen positive breast cancer treatment. However, tamoxifen also acts as a GPER agonist and is thought to have a role in tamoxifen resistant breast cancer (Catalano et al., 2014). Additionally, an older study noted women on tamoxifen treatment for breast cancer had an increased risk of CRC (Newcomb et al., 1999).

The importance of aromatase in the colon is controversial with many studies finding low aromatase expression or activity and no change in CRC (English et al., 1999, Foster, 2013, Sato et al., 2009). However, the aromatase gene (CYP19A1) has been linked to CRC risk (Slattery et al., 2011) and *in vitro* studies have shown aromatase activity in CRC cell lines (Fiorelli et al., 1999). In 2009 Sato et al concluded that locally produced oestrogens in CRC were mainly derived from the STS pathway (Sato et al., 2009). In 2012 they explored aromatase in CRC in more detail. They noted the colon had functional aromatase with expression and activity increased in CRC. However, aromatase status did not correlate with survival as they found for STS/SULT1E1 expression (Sato et al., 2012). Thus, STS may be the

dominant pathway for oestrogen generation in the colon and so inhibiting STS should have the biggest impact on oestrogen availability in CRC. If anti-oestrogen treatment in CRC is found to be beneficial then full assessment of oestrogen enzymes and receptors status may be needed to stratify different treatment groups.

## 8.5 TNFA INCREASES STS ACTIVITY

There is an inflammatory component in the microenvironment of most neoplastic tissues including CRC. Cancer-related inflammation is the seventh hallmark of cancer (Colotta et al., 2009). Inflammatory mediators induce genetic instability and the abundance of genetic heterogeneity within tumours suggests genetic instability is an ongoing process throughout tumour development (Wang and Lin, 2008).

Previously TNFα has been shown to increase STS expression and/or activity in breast MCF7 (Reed et al., 2005, Purohit et al., 1996, Reed and Purohit, 1997, Purohit et al., 1997) and prostate cancer LNCaP (Suh et al., 2011) cell lines. TNFα regulation of STS has now been expanded to include CRC cell lines *in vitro* and multiple benign tissues and organs of Tg-TNF mice *in vivo*. In these experiments TNFα increased STS activity without effecting STS mRNA suggesting TNFα may post-translationally modify STS or alter $E_1S$ availability across the cell membrane. Tg-TNF mice did not have increased TNFα expression in the colon itself indicating systemic inflammation can alter STS activity in the colon. STS is at least in part regulated by inflammatory modulator TNFα with TNFα increasing STS activity in benign and malignant tissues.

Inflammation induced STS activity may not be a unique CRC trait and in the acute setting may be of clinical benefit, such as modifying immune function through oestrogens or androgens. Interestingly, research has shown evidence that anti-oestrogen treatment may benefit the majority of cancers independent of ER expression due to the effects on the immune system (Svoronos et al., 2016). Chronic inflammation induced STS activity with active oestrogen generation may be detrimental. This homeostatic response could be commandeered in CRC contributing to genetic instability by accruing mutations. Long-term anti-inflammatories offer some protection against CRC reducing risk by 40-50% (Smalley and DuBois, 1997) This may be through TNFα and COX-2 indirectly down-regulating STS.

It would also be interesting to examine STS activity and inhibition in chronic inflammatory diseases such as IBD and arthritis to ascertain how STS affects symptoms and outcomes for these patients. By also assessing oestrogens and androgens this may even give insight into the gender differences in inflammatory disease.

## 8.6 SUMMARY

Together these *in vitro*, *ex vivo* and *in vivo* experiments support an active oestrogenic pathway in CRC that drives proliferation. Ultimately a combination of increased oestrogen availability through STS desulphation activity, alteration in $E_1/E_2$ ratio through decreased oxidation and increased reduction would shift towards the more potent $E_2$ which then stimulates proliferation through GPER (Figure 8.1).

Novel findings demonstrated in this study shed light on complex oestrogen metabolism in CRC. Previous focus on single enzymes does not inform of the full metabolism profile.

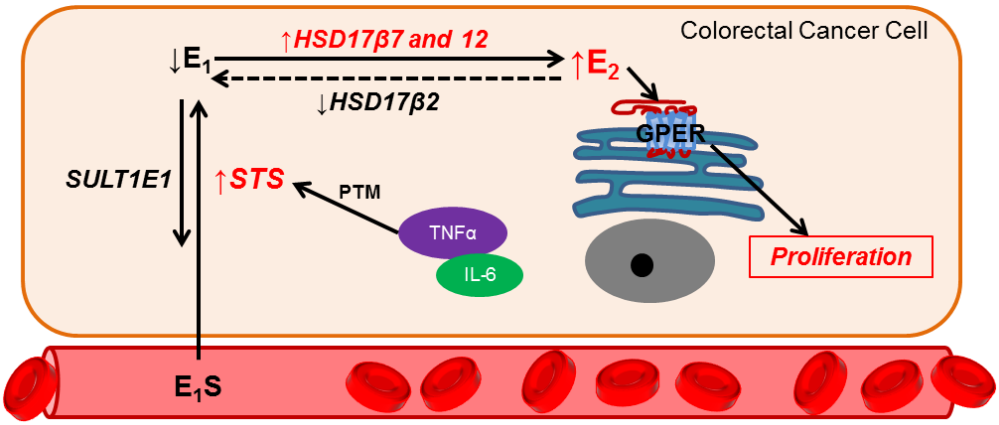



*Figure 8.1. Illustration of oestrogen metabolism in a colorectal cancer cell. Circulating oestrogen sulphate ($E_1S$) is taken up by the cell and desulphated by steroid sulphatase (STS) to oestrone ($E_1$). STS is increased in CRC through post-translational modification (PTM) by TNFα. $E_1/E_2$ ratio is shifted in favour of $E_2$ generation as oxidative HSD17β2 is decreased and reductive HSD17β7 and 12 are increased. $E_2$ then acts on GPER to stimulate proliferation.*

The exact timings of these post-translational and genetic changes have not been fully elucidated. It can be hypothesised that STS activity occurs early in CRC which may be due to the presence of TNFα in the matrix surrounding tumours. HSD17β2 reduction tends to occur in later stages and may reflect an oestrogenic switch in the tumour where the increase in local $E_1$ from STS is used to advance proliferation. The potential stages of these data have been added to the chromosomal instability pathway and are depicted in red in Figure 8.2.

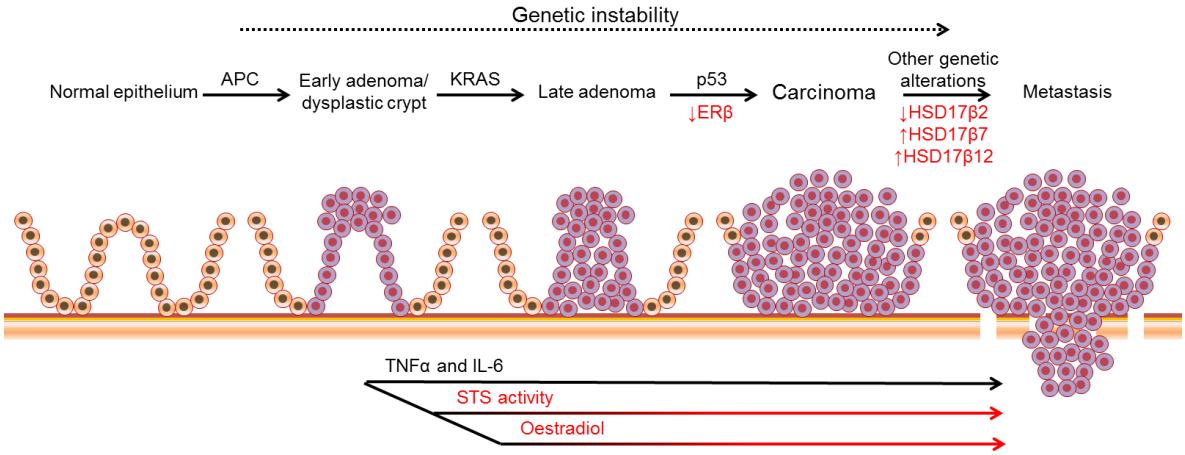



*Figure 8.2. Increase in oestradiol generation in colorectal cancer. Oestrogen metabolism changes in CRC overlaid on the colon cancer chromosomal instability pathway shown in Figure 1.4. Early adenoma causes local inflammation with resultant TNFα and IL-6 increasing STS activity. Later there is loss of ERβ and oxidoreductive HSD17β changes shifting the $E_1/E_2$ ratio to increase potent $E_2$ advancing progression from late adenoma to carcinoma and metastasis.*

## 8.7 FUTURE DIRECTIONS

To affirm oestrogen metabolism alterations in CRC tumours SULT1E1 and HSD17β activity changes would need to be determined in CRC cells and human tissue and circulating and tumoural oestrogen concentrations calculated. This could be achieved using LC-MS/MS with a newer more sensitive MS. There is evidence implicating sex hormones and CRC in both men and women, which correlate circulating oestrogen levels with increased risk (Lin et al., 2013, Clendenen et al., 2009). Few studies have recorded actual circulating concentrations of oestrogens in CRC patients due to the difficulty in measurement. A small study compared serum $E_2$ of 51 Indian men with CRC against 50 age-matched controls. They demonstrated an increase in serum $E_2$ in CRC patients. However, a significant limitation of this study is the use of immunoassay to measure $E_2$ within serum (Basu et al., 2015) and it would be beneficial to confirm these findings in an LC-MS/MS study.

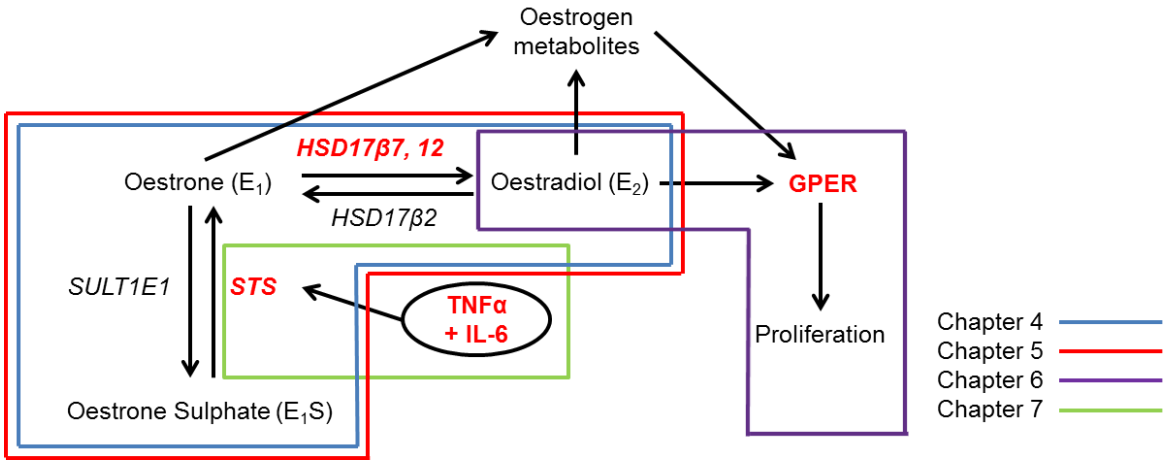



*Figure 8.3 Treatments targeting the oestrogen pathway in colorectal cancer. Potential treatment targets identified in each chapter are highlighted in red. Further research could detect additional therapeutics options such as oestrogen metabolites able to activate GPER and downstream pathways of GPER.*

This thesis has strategically examined oestrogen metabolism in CRC identifying multiple potential therapeutic targets (Figure 8.3). Generally it is more feasible to design inhibitors rather than increase expression or activity of an enzyme or receptor. Therefore, increasing SULT1E1 activity or specific oxidative HSD17β action to reduce oestrogen potency would be challenging. Instead the STS pathway for active oestrogen generation could be targeted, such as by preventing $E_1S$ uptake into the cell by organic anion-transporting polypeptides (Gilligan et al., 2017). $E_1S$ could be prevented from being desulphated by STS inhibitors, some of which are currently in breast cancer trials (Stanway et al., 2006, Palmieri et al., 2016a, Palmieri et al., 2016b). Generation of the most potent oestrogen $E_2$, could be reduced by selective reductive HSD17β inhibitors. Finally GPER inhibitors could prevent oestrogen proliferative action in the colon. In addition to the STS pathway the impact of aromatase inhibitors have yet to be assessed in CRC. As with other cancers such

as breast and prostate, a combination of different treatments, depending on the oestrogen pathway changes seen in each patient, is likely to be most effective.

This thesis has also highlighted other areas of research that may lead to new CRC treatments. STS post-translational modification e.g. glycosylation could be targeted to reduce activity. Anti-inflammatories have been shown to reduce CRC risk. It would be interesting to examine if they have any indirect effects on oestrogen metabolism enzymes through COX-2 and TNFα e.g. STS, HSD17β7 and 12, GPER. GPER has been shown to be a strong contender for oestrogen action which could be further validated by demonstrating stimulation through activation of down-stream effects such as connective tissue growth factor (Pandey et al., 2009). Also, other oestrogen metabolites may be able to activate GPER and these pathways have yet to be examined in CRC.

In addition further study is needed on how other sex steroids interact with the colonic environment. The colon has been documented to express progesterone (Oshima et al., 1999) and androgen receptors (Gu et al., 2009). In breast cancer the effect of sex hormones have been well studied, however using progesterone (Baird and Carroll) and androgens as treatment targets remains controversial (Garay and Park, 2012). This controversy extends to CRC risk and therapeutic application with no consensus as to the role of either androgens or progesterone in the colon. It is important to note that the protective effects of HRT on CRC risk were only with combined oestrogen plus progestin therapy and not oestrogen alone (Ritenbaugh

et al., 2008). Also, androgen effects on the colon may also help explain the marked gender differences in CRC (Roshan et al., 2016).

The drug targets identified in this thesis may be extrapolated to other cancers and diseases. Changes in oestrogen metabolism have been well characterised in breast cancer with anti-oestrogen treatment effective in many patients. Similar oestrogen enzyme alterations have also been documented in ovarian and endometrial cancer and may form future treatment targets (Ren et al., 2015, Rizner et al., 2017). However, what is unique to the colon is that it is an extension of the external environment and orally administered drugs are directly exposed to the gut. Drugs could be engineered to not be systemically absorbed and so could potentially be used uniquely to target local CRC and adenomas.

# APPENDICES

## APPENDIX I

Patient colorectal tissue sample characteristics.
Female:

| HBRC No | Patient's Age and Sex | Pathological diagnosis | TNM Staging | Dukes' Stage |
|---|---|---|---|---|
| 13654 (N)<br>13655 (N)<br>13656 (C)<br>13657 (C) | 81 F | Adenocarcinoma distal sigmoid colon | pT3 N1 Mx | C1 |
| 13658 (C)<br>13659 (C)<br>13660 (N)<br>13661 (N) | 73 F | Adenocarcinoma sigmoid colon | YpT3 N0 Mx | B |
| 13662 (C)<br>13663 (N)<br>13664 (N)<br>13665 (C) | 57F | MSI-H adenocarcinoma with loss of MLHI and PMS2 expression and BRAF mutation within codon 600 | pT2 N1 | C |
| 13666 (N)<br>13667 (C) | 84 F | Adenocarcinoma caecum | pT4 N0 Mx | B |
| 13668 (N)<br>13669 (C) | 72 F | Caecum MSI high adenocarcinoma | pT3 N2 | C |
| 13670 (N)<br>13671 (C) | 71 F | Invasive sigmoid colon adenocarcinoma | pT4 (4b-TNM 2005) N1 Mx | C1 |
| 13672 (N)<br>13673 (C) | 68 F | Adenocarcinoma, sigmoid/upper rectum | pT3 N1 M1 (liver and lung metastases) | D |
| S029320 (N)<br>S029321 (C) | 85 F | Moderately differentiated mucinous adenocarcinoma | pT3 N0 | B |
| S029322 (N)<br>S029323 (C) | 86 F | Invasive caecal mucinous adenocarcinoma | pT3 N0 Mx | B |
| S029324 (C)<br>S029325 (N) | 70 F | Adenocarcinoma | pT4 N0 Mx | B |
| S029326 (N)<br>S029327 (C) | 76 F | 4 adenocarcinomas | pT3 (3), pT2 (1) | B |
| S029328 (N)<br>S029329 (C) | 61 F | Invasive adenocarcinoma of caecum | pT2 N0 Mx | A |
| S029330 (N)<br>S029331 (C) | 86 F | Mucinous adenocarcinoma of colon | pT2 N0 Mx | A |
| S029332 (N)<br>S029333 (C) | 66 F | Invasive caecal adenocarcinoma | pT2 N0 Mx | A |
| S029334 (N)<br>S029335 (C) | 82 F | Adenocarcinoma of right colon | pT4 N1 Mx | C1 |
| S029336 (N)<br>S029337 (C) | 87 F | MSI-H adenocarcinoma | pT3 N1 | C |
| S029338 (N)<br>S029339 (C) | 67 F | Invasive sigmoid colon adenocarcinoma | pT2 N1 Mx | C1 |
| S029340 (N)<br>S029341 (C) | 82 F | Invasive caecal adenocarcinoma | pT2 N0 Mx | A |
| S029342 (N)<br>S029343 (C) | 63 F | Adenocarcinoma | pT3 N0 | B |

| HBRC No | Patient's Age and Sex | Pathological diagnosis | TNM Staging | Dukes' Stage |
|---|---|---|---|---|
| S029344 (N)<br>S029345 (C) | 57 F | Invasive caecal adeocarcinoma | pT3 N0 Mx | B |
| S029346 (N)<br>S029347 (C) | 72 F | Adenocarcinoma | pT3 N0 | B |
| S045959 (N)<br>S045963 (C) | 58 F | Adenocarcinoma | pT3 N0 pMx | B |
| S046076 (N)<br>S046077 (C) | 55 F | Adenocarcinoma | pT2 N0 | A |
| S045965 (N)<br>S045966 (C) | 63 F | Adenocarcinoma | pT4 N0 pMx | B |
| S045967 (N)<br>S045968 (C) | 85 F | Adenocarcinoma | pT4 N0 pMx | B |
| S045969 (N)<br>S045970 (C) | 81 F | Adenocarcinoma | pT4 N1 Mx | C |
| S045975 (N)<br>S045976 (C) | 72 F | Adenocarcinoma | pT3 N0 | B |
| S045981 (N)<br>S045982 (C) | 69 F | Adenocarcinoma | pT2 N1 Mx | C |
| S045983 (N)<br>S045984 (C) | 56 F | Adenocarcinoma | pT2 N0 Mx | A |
| S045985 (N)<br>S045986 (C) | 60 F | Adenocarcinoma | pT3 N1 | C |
| S045987 (N)<br>S045988 (T) | 60 F | Adenoma | | - |
| S045989 (N)<br>S045990 (C) | 63 F | Adenocarcinoma | pT4 N1 M1 | D |

Male:

| HBRC No | Patient's Age and Sex | Pathological diagnosis | TNM Staging | Dukes' Stage |
|---|---|---|---|---|
| 14780 (N)<br>14781 (N)<br>14782 (C)<br>14783 (C) | 76 M | Advance caecal adenocarcinoma | pT4 N2 | C |
| 14784 (N)<br>14785 (C) | 75 M | Adenocarcinoma upper rectum | YpT3 N1 Mx | C |
| 14786 (N)<br>14787 (C) | 55 M | Left colon adenocarcinoma | pT4 N0 | B |
| 14788 (N)<br>14789 (N)<br>14791 (C) | 75 M | Invasive sigmoid colon adenocarcinoma | pT3 N0 Mx | B |
| 14792 (N)<br>14793 (C)<br>14794 (C) | 53 M | Invasive adenocarcinoma of splenic flexure and sigmoid colon. Tumours invade serosa, extramural lymphovascular invasion | pT4 N2 Mx | C1 |
| 14795 (C)<br>14796 (N) | 84 M | Adenocarcinoma | pT4 N2 | C |
| S029282 (C)<br>S029283 (N) | 79 M | Invasive sigmoid colon adenocarcinoma | pT4 N2 Mx | C1 |
| S029284 (N)<br>S029285 (C) | 84 M | Adenocarcinoma | pT2 N1 Mx | C1 |
| S029286 (N)<br>S029287 (C) | 81 M | Adenocarcinoma | pT3 N1 Mx | C1 |
| S029288 (N)<br>S029289 (C) | 90 M | Adenocarcinoma | pT3 N1 | C |
| S029290 (C)<br>S029291 (N) | 82 M | Adenocarcinoma | pT3 N1 | C |
| S029292 (C)<br>S029293 (N) | 81 M | Adenocarcinoma | pT3 N0 | B |
| S029294 (C)<br>S029295 (N) | 83 M | Adenocarcinoma left colon | pT3 N0 Mx | B |
| S029296 (N)<br>S029297 (C) | 77 M | MSI-H caecal adenocarcinoma | pT2 N0 | A |
| S029298 (N)<br>S029299 (C) | 53 M | Adenocarcinoma | pT2 N0 Mx | A |
| S029300 (N)<br>S029301 (C) | 63 M | Moderately differentiated adenocarcinoma | pT2 N1 Mx | C1 |
| S029302 (N)<br>S029303 (C) | 50 M | Adenocarcinoma of sigmoid colon | ypT2 N0 Mx | A |
| S029304 (N)<br>S029305 (C) | 72 M | Invasive caecal adenocarcinoma | pT4 N1 Mx | C1 |
| S029306 (N)<br>S029307 (C) | 75 M | Moderately differentiated adenocarcinoma | pT3 N1 | C |
| S029308 (N)<br>S029309 (C) | 62 M | Adenocarcinoma | pT4 N2 Mx | C2 |
| S029310 (N)<br>S029311 (C) | 79 M | Adenocarcinoma in sigmoid colon | pT1 N0 | A |

| HBRC No | Patient's Age and Sex | Pathological diagnosis | TNM Staging | Dukes' Stage |
|---|---|---|---|---|
| S029312 (N)<br>S029313 (C) | 68 M | Adenocarcinoma | pT3 N1 | C |
| S029314 (N)<br>S029315 (C) | 60 M | Invasive sigmoid colon adenocarcinoma | pT3 N0 Mx | B |
| S029316 (N)<br>S029317 (C) | 85 M | Adenocarcinoma | pT3 N0 | B |
| S029318 (N)<br>S029319 (C) | 63 M | Invasive sigmoid colon adenocarcinoma | pT4 N0 M0 R0 | B |
| S045961 (N)<br>S045962 (C) | 83 M | Adenocarcinoma | pT3 N0 Mx | B |
| S045963 (N)<br>S045964 (C) | 67 M | Adenocarcinoma | pT3 N0 | B |
| S045971 (N)<br>S045972 (C) | 79 M | Adenocarcinoma | pT3 N1 Mx | C |
| S045973 (N)<br>S045974 (C) | 69 M | Adenocarcinoma | pT2 N0 Mx | A |
| S045977 (N)<br>S045978 (C) | 72 M | Adenocarcinoma | pT3 N1 M0 | C |
| S045979 (N)<br>S045980 (C) | 79 M | Adenocarcinoma | pT3 N0 Mx | B |
| S045991 (N)<br>S045992 (C) | 72 M | Adenocarcinoma | pT3 N2 | C |

## APPENDIX II

Figure II 1 shows both GeNorm and Normfinder selected RPLPO as the best individual standard gene for real-time PCR.

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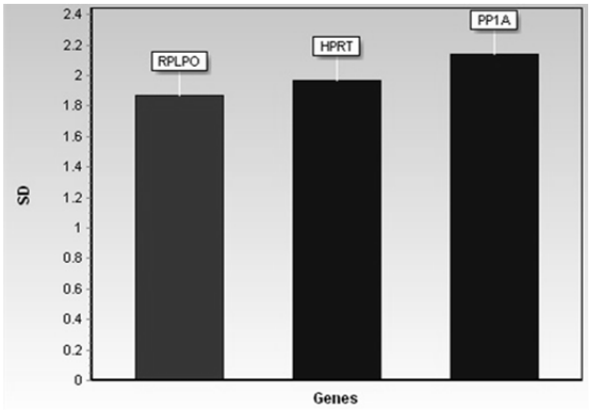
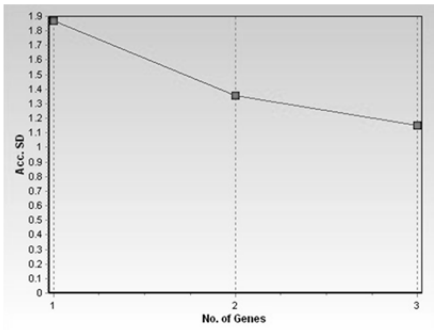
*Figure II 1. (A) Screenshot from Normfinder showing the more genes used the more accurate the data, but if only one is used RPLPO is the most reliable. (B) Screenshot from GeNorm showing that RPLPO and HPRT are the most reliable internal standard genes for real-time PCR.*

(A)



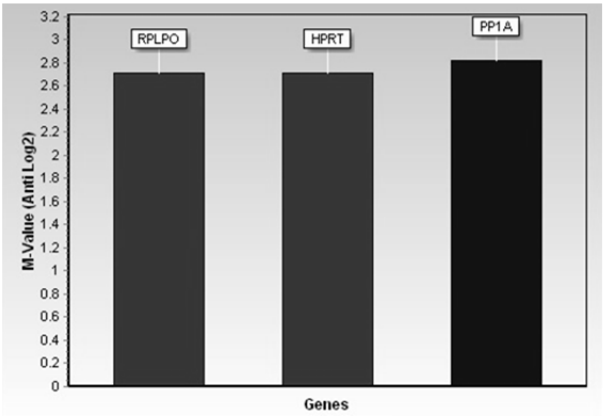




| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Gene Name | SD | Acc. SD | SD | 1.8692 |
| 2 | RPLPO | 1.8692 | 1.8692 | | |
| 3 | HPRT | 1.9663 | 1.3565 | Best Gene | RPLPO |
| 4 | PP1A | 2.1392 | 1.1516 | | |

**Output summary**

| | |
|---|---|
| **Best gene:** | **RPLPO** |
| **SD:** | **1.8692** |

(B)



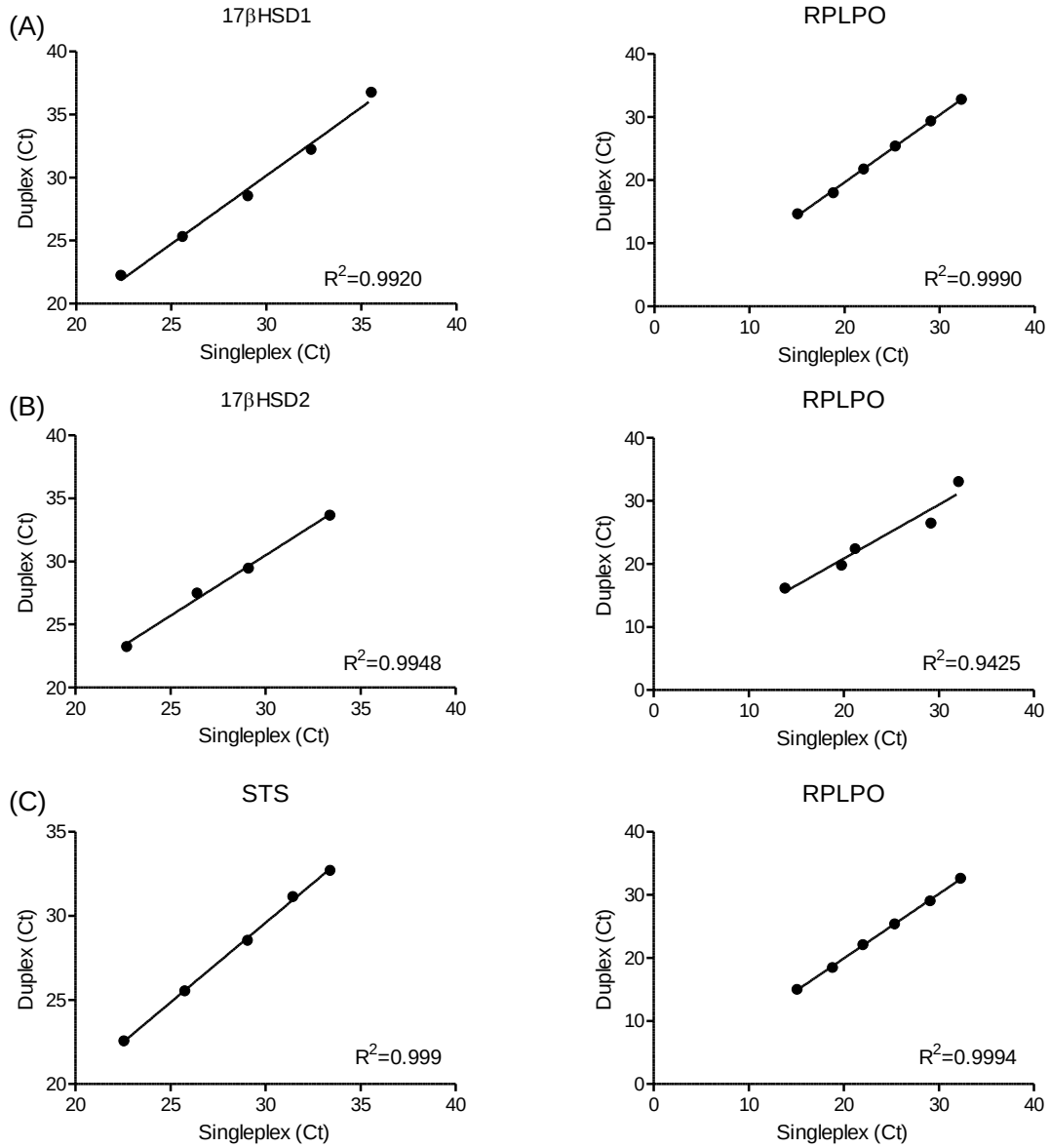

**Output summary**

| | |
|---|---|
| **Best genes:** | **RPLPO and HPRT** |
| **M-value:** | **2.7130** |

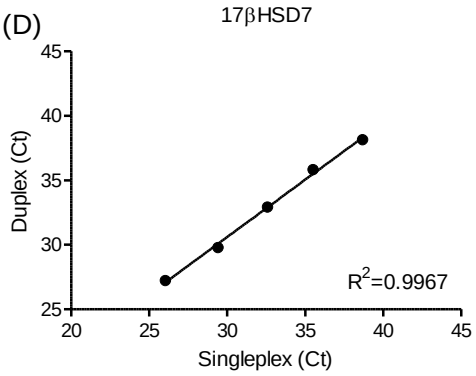
| | A | B |
|---|---|---|
| 1 | Gene Name | M-Value |
| 2 | PP1A | 2.8195114553... |
| 3 | HPRT | 2.7129777449... |
| 4 | RPLPO | 2.7129777449... |

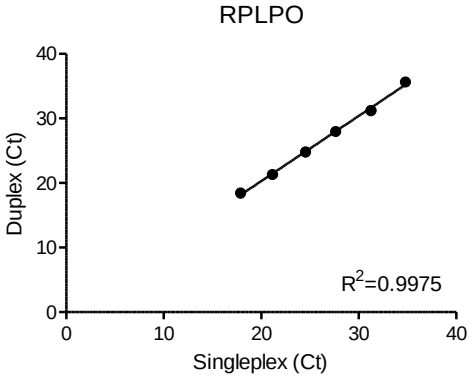
Figure II 2 shows testing for genes in duplex and singleplex. Genes not displayed and reactions performed on mice tissue were singleplex. Note SULT1E1 (I) could not be validated and so was not used

*Figure II 2. TaqMan™ showing duplex and singleplex reactions for those genes used in duplex as standard. All other genes were performed in singleplex.*

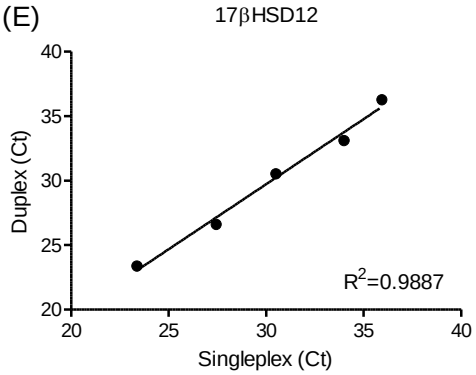


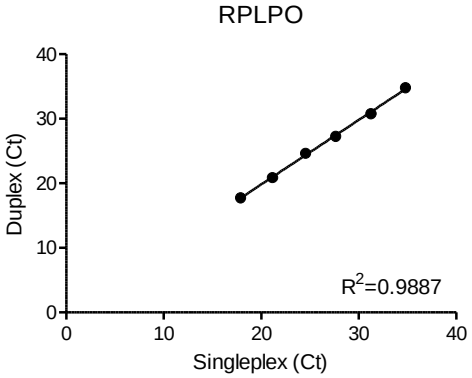
(D)


17βHSD7
Duplex (Ct)
Singleplex (Ct)
R2=0.9967

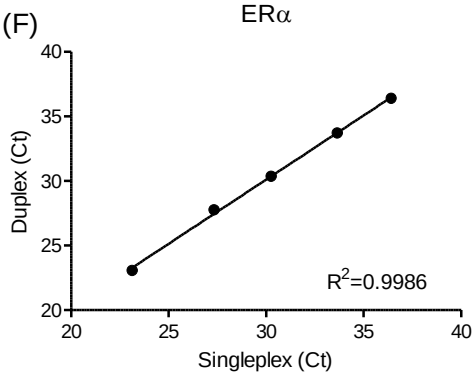

RPLPO
Duplex (Ct)
Singleplex (Ct)
R2=0.9975

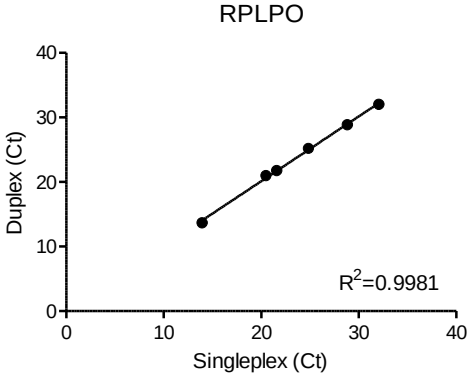
(E)


17βHSD12
Duplex (Ct)
Singleplex (Ct)
R2=0.9887

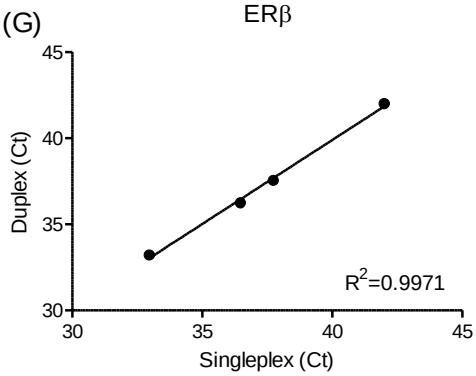

RPLPO
Duplex (Ct)
Singleplex (Ct)
R2=0.9887

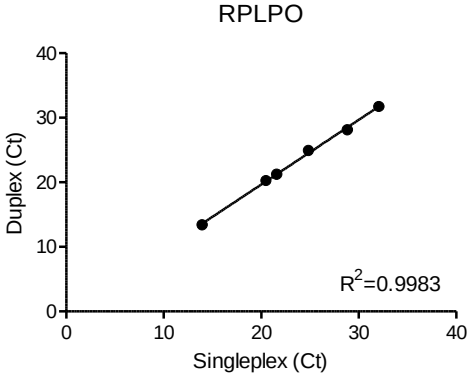
(F)


ERα
Duplex (Ct)
Singleplex (Ct)
R2=0.9986

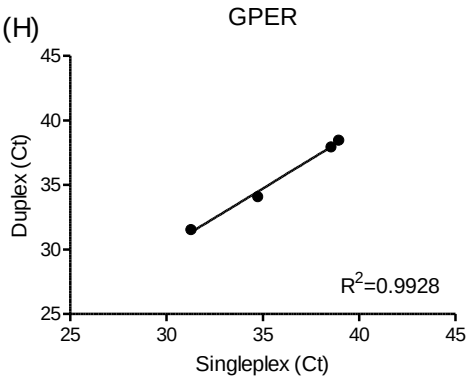

RPLPO
Duplex (Ct)
Singleplex (Ct)
R2=0.9981

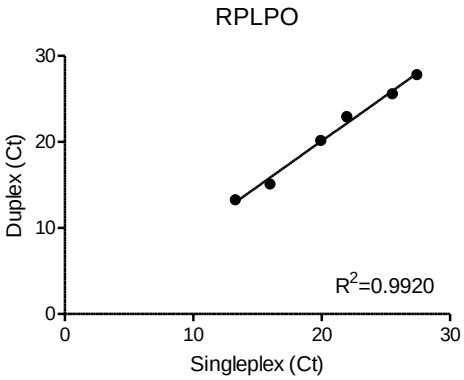
(G)


ERβ
Duplex (Ct)
Singleplex (Ct)
R2=0.9971

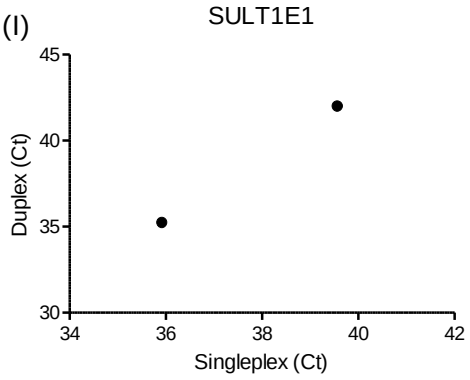

RPLPO
Duplex (Ct)
Singleplex (Ct)
R2=0.9983

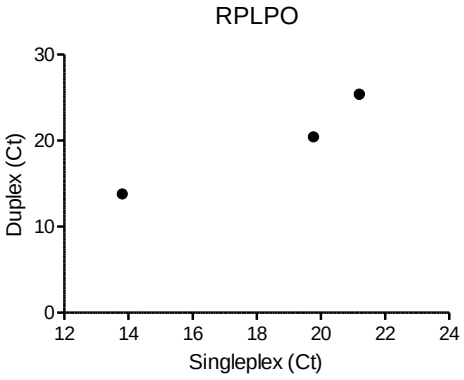
(H)


GPER
Duplex (Ct)
Singleplex (Ct)
R2=0.9928
45
40
35
30
25
25
30
35
40
45


RPLPO
Duplex (Ct)
Singleplex (Ct)
R2=0.9920
30
20
10
0
0
10
20
30

(I)

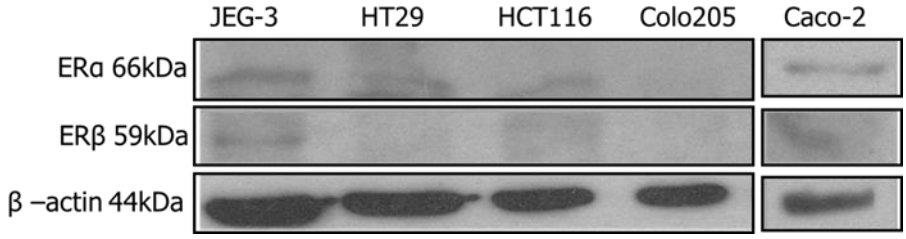

SULT1E1
Duplex (Ct)
Singleplex (Ct)
45
40
35
30
34
36
38
40
42


RPLPO
Duplex (Ct)
Singleplex (Ct)
30
20
10
0
12
14
16
18
20
22
24

## APPENDIX III

CRC cell line expression performed by previous group members.

*Figure III 1. Western blot performed by technician Anne-Marie Hewitt showing ERα and ERβ expression in CRC cell lines.*




Unpublished *in vivo* data of HCT116 cells overexpressing STS (HCT116[STS]) or VO (HCT116[VO]) in nude mice xenografts.

*Figure III 2. HCT116 [STS] xenografts in MF-1 mice have increased proliferation in vivo. (A) HCT116[STS] xenografts had increased growth rate compared to HCT116[VO] xenografts which was inhibited by STX64 (20mg/kg/thrice weekly). **p<0.01, ***p<0.001. (B) Wet tumour weights at 21 days post HCT116 cell inoculation. HCT116[STS] resulted in increased tumour burden, which was inhibited by STX64. *p<0.05, ***p<0.001. (C) Tumour images after removal. Performed by Dr P.A. Foster.*

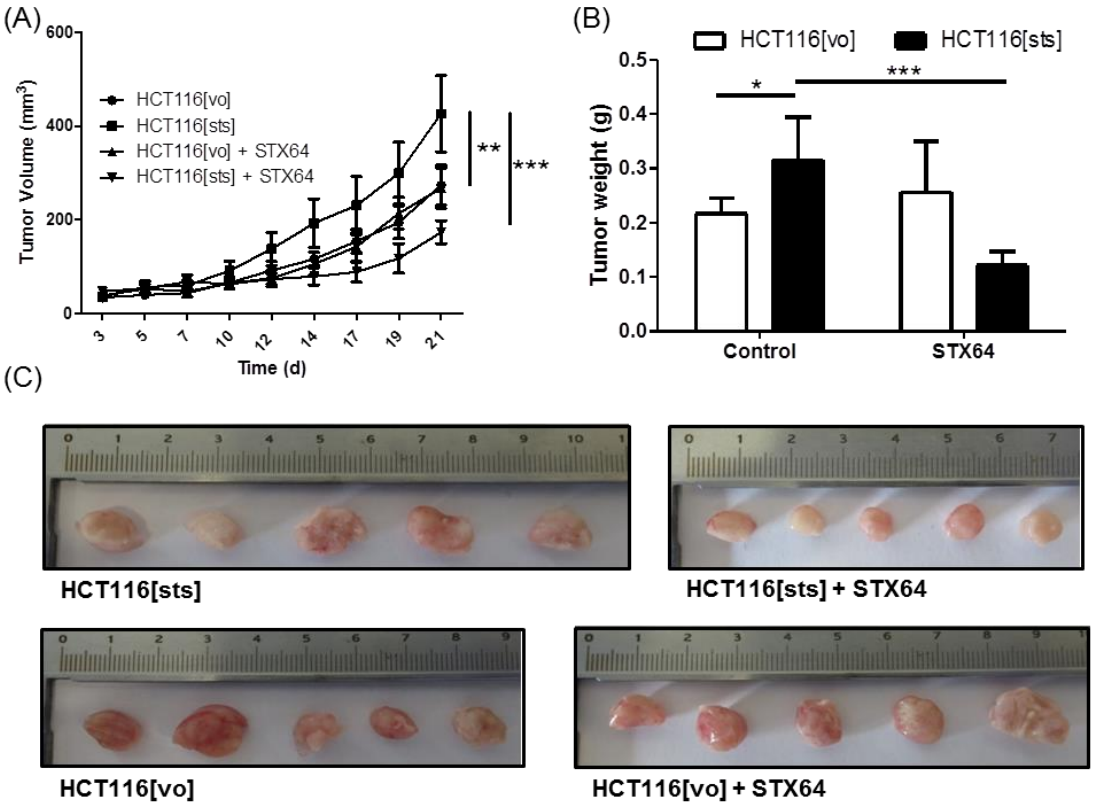
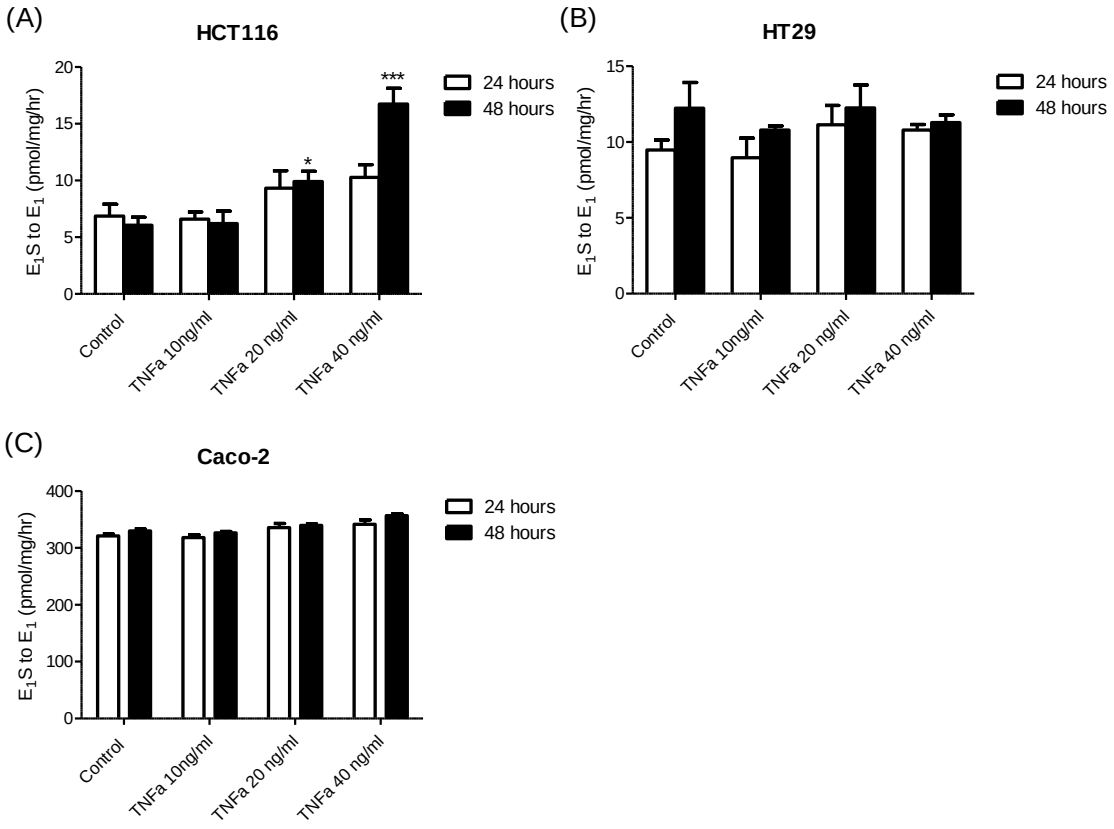


*Figure III 3. Cell lines treated with 10, 20 or 40 ng/ml of TNFα for 24 or 48 hours and STS activity measured. Only with higher concentrations and longer time points were any significant changes in STS activity seen. Two-way ANOVA with Bonferroni post-test, n=3* *p < 0.05, **p < 0.01, ***p < 0.001



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