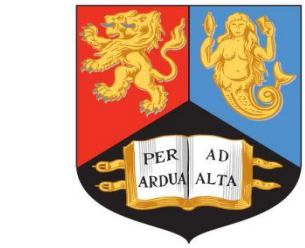

UNIVERSITY OF BIRMINGHAM

THE EFFECT OF INFLAMMATORY CYTOKINES ON STEROIDOGENESIS IN COLON CANCER CELLS

BY

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Abstract

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and has a high incidence and mortality rate. It is known that oestrogens impact CRC incidence and prognosis. Sulfated oestrogens, primarily oestrone sulfate (E1S), are taken into cells via organic anion transporting polypeptides (OATPs). Steroid sulfatase (STS) then desulfates this into oestrone (E1). E1 can be reduced by 17β-hydroxysteroid dehydrogenases (HSD17Bs) to form biologically active oestradiol (E2). Our group previously showed that STS activity and HSD17Bs expression are altered in CRC to favour E2 synthesis. However, what regulates STS activity, HSD17Bs and OATPs expression in CRC is poorly understood. My hypothesis is that inflammatory cytokines drive these changes in E2 synthesis. In this thesis, the Cancer Genome Atlas (TCGA) human colorectal adenocarcinoma RNA-Seq dataset (n = 433, normal=51 and CRC=382) was analysed to generate Kaplan–Meier survival curves and correlation graphs comparing normal and CRC tissue expression of TNFα, HSD17Bs, OATPs, and STS. How inflammatory cytokines impacted oestrogen uptake, enzyme activity and cell proliferation were further assessed through radiolabelled enzyme activity assays, LC-MS/MS, and proliferation assays. In CRC tissue, results demonstrated positive correlations between TNFα and STS (*P=0.036), HSD17B4 (**P=0.0045), HSD17B12 (*P=0.016), OATP2B1 (***P<0.0001), OATP3A1 (***P<0.0001), OATP3A1 (***P<0.0001), and OATP1C1 (***P<0.0001). In CRC cell lines, treatment with TNFα significantly increased STS activity as measured via the conversion of ^{3}H-E1S to ^{3}H-E1. An increase in STS activity was associated with an increase in CRC cell proliferation. HCT116 and Colo205 cells displayed the greatest proliferative responses compared to LoVo and SW620 cells. For HSD17Bs, TNFα treatment decreased HSD17B2 and HSD17B4 expression and increased

HSD17B7 and HSD17B12 expression, suggesting greater E2 synthesis. TNFα also significantly upregulated OATP2B1 expression in HCT116 and Colo205 cells. E1S uptake significantly increased in HCT116 and SW620 cells. Finally, CRC oestrogen metabolism, measured by LC-MS/MS, showed that TNFα reduced E2 loss in CRC cell lines and reduced E1 synthesis from E2. Taken together, these data show that TNFα significantly increases STS activity, alters HSD17B expression, and upregulates oestrogen uptake in CRC cells. This suggests that TNFα is an important regulator of oestrogen metabolism in CRC.

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PUBLICATIONS RELATING TO THESIS

Review article

Banibakhsh, A., Sidhu, D., Khan, S., Haime, H. and Foster, P.A., 2023. Sex Steroid Metabolism and Action in Colon Health and Disease. *The Journal of Steroid Biochemistry and Molecular Biology*, p.106371.

Poster presentations

Banibakhsh, A., Hardy, R. and Foster, P., 2019, November. Inflammatory cytokines dysregulate oestrogen metabolism in colorectal cancer. In *Endocrine Abstracts* (Vol. 65). Bioscientifica.

Banibakhsh, A., Hardy, R. and Foster, P., 2022, June. The Effect of Inflammatory Cytokines on Steroidogenesis in Colorectal Cancer. In *EACR 2022 Congress Abstracts*. 20 – 23 June 2022. Seville, Spain.

Summary

I presented a poster twice at the 2019 conference with the Society for Endocrinology and in 2022 with the European Association for Cancer Research. Both posters focused on how inflammatory cytokines alter oestrogen metabolism in colorectal cancer. Also, in 2023, I contributed to writing a Review article on Sex Steroid Metabolism and Action in Colon Health and Disease.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC- ATP-Binding Cassette

ACT- Adjuvant Chemotherapy

***APC*-** Adenomatous polyposis coli

BCA- Bicinchoninic Acid

BSA- Bovine serum albumin

BrdU- Bromodexyuridine

CD- Crohn's Disease

cDNA- Complementary Deoxyribonucleic Acid

CEA- Carcinoembryonic Antigen

CIMP- CpG island methylator phenotype

COADREAD- Colon and Rectal Adenocarcinoma

COX- Cycloxygenase

CRC- Colorectal Cancer

CYP19A1- Aromatase

DHEA- Dehydroepiandrosterone

DHT- Dihydrotestosterone

DNA Deoxyribonucleic acid

E1- Oestrone

E1S- Oestrone Sulfate

E2- 17β-oestradiol

E3- Estriol

EGF- Epidermal Growth Factor

EGFR- Epidermal growth factor receptor

ELISA- Enzyme-Linked Immunosorbent Assay

ER- Oestrogen Receptor

ER*α*- Oestrogen Receptor Alpha

ERβ- Oestrogen Receptor Beta

ERE- Oestrogen response elements

ERK- Extracellular signal–regulated kinases or classical MAP kinases

***ESR1*-** Oestrogen receptor 1 gene

***ESR2*-** Oestrogen receptor 2 gene

FAP- Familial Adenomatous Polyposis

FBS- Foetal Bovine Serum

FGly Formylglycine

FIT- Faecal Immunochemical Testing

FOBT- Faecal Occult Blood Testing

GI- gastrointestinal

GPER- G Protein-coupled Oestrogen Receptor

HAS- Human Albumin Serum

HNPCC- Hereditary nonpolyposis colorectal cancer

HRT- Hormone Replacement Therapy

HSD- Hydroxysteroid Dehydrogenase

HSD17B- 17 Beta Hydroxysteroid Dehydrogenase

HSD3B-3-β-Hydroxysteroid-Dehydrogenase

IBD- Inflammatory Bowel Disease

IL4- Interleukin 4

IL6- Interleukin 6

IFN-γ- Interferon-γ

kDa- Kilodalton

LC- Liquid-chromatography

LDL- Low Density Lipoproteins

LLE- Liquid-Liquid Extraction

MAPK- Mitogen-activated protein kinase

mRNA- Messenger Ribonucleic Acid

MS- Mass Spectrometry

MTBE- Methyl Tertiary Butyl Ether

NCI- National Cancer Institute

NFκB -Nuclear Factor-κB

NGS- Next-Generation Sequencing

NRAS- Oncogene

NSAIDs- Nonsteroidal Anti-Inflammatory Drugs

OATP- Organic anion transporter polypeptide

OCT- organic cation transporters

OS- Overall Survival

P53- Tumour Suppressor Gene

PCR- Polymerise Chain Reaction

PBS- Phosphate Buffered Saline

PPT- Protein Precipitation

RFS- Relapse-Free Survival

RPLPO- Large Ribosomal Protein

SEER- The Surveillance, Epidemiology, and End Results

SLC- Solute Carrier

SMAD4- Tumour Suppressor Gene

SNPs-Single Nucleotide Polymorphisms

STAT3- Signal transducer and activator of transcription 3

STS- Steroid sulphatase

SPE- Solid Phase Extraction

SULT1E1- Oestrogen Sulfotransferase

SUMF- Sulphatase Modifying Factor

TCGA- The Cancer Genome Atlas

TME- Tumour Microenvironment

TNFα- Tumour Necrosis Factor

TNM- Tumour Node Metastasis

UC- Ulcerative colitis

VEGF- Vascular Endothelial Growth Factor

VEGFR- Vascular Endothelial Growth Factor Receptor

WHI- Women's Health Initiative

mRNA- Messenger RiboNucleic Acid

qRT-PCR- Quantitative Real Time Polymerase Chain Reaction

sFBS- Stripped Foetal Bovine Serum

VAT- Visceral Adipose Tissue

Wnt- Wingless-Related Integration Site

CHAPTER 1. GENERAL INTRODUCTION

1.1 Colon anatomy and physiology:

The gastrointestinal (GI) tract is a system of organs that breaks down food to extract and absorb energy and nutrients, then excretes the waste as faeces. When food is swallowed, it passes through the oesophagus and into the stomach, where it is partially broken down before being delivered to the small intestine, where digestion continues and the majority of nutrients are absorbed. The large intestine is the last section of the GI tract and is separated into the caecum and appendix, colon, rectum, and anal canal. The colon is also divided into the ascending, transverse, descending, and sigmoid portions. The sigmoid colon enters the pelvic cavity through the pelvic brim and merges with the rectum there; this includes the anal canal, colon, rectum, and appendix (Figure 1.1) (Mahadevan, 2020).

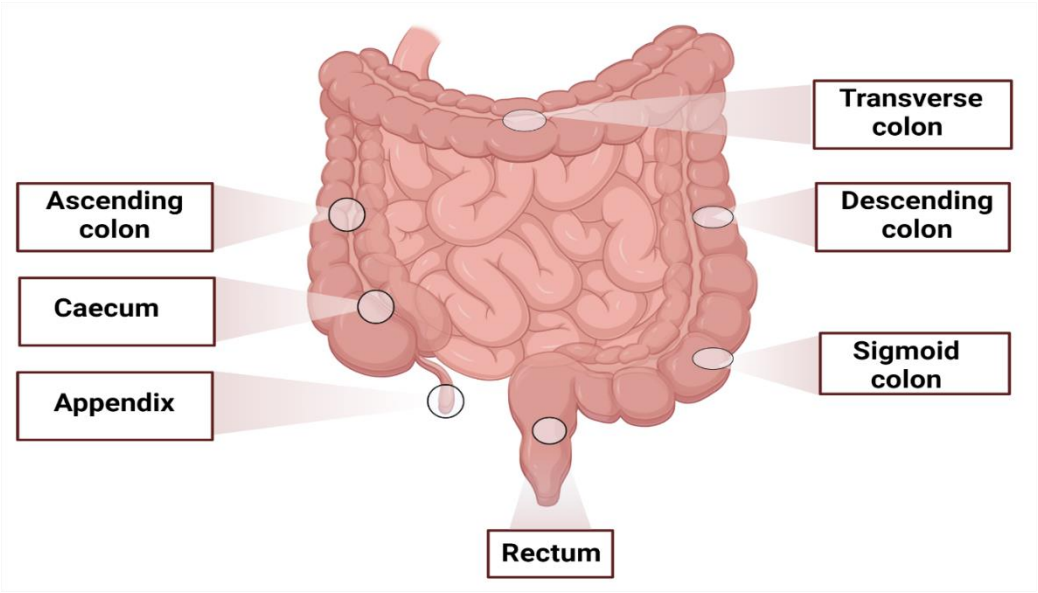

Figure 1.1: Large intestine's external sections. (Created with BioRender.com).

Mucosa, muscularis mucosae, submucosa, muscle layer (muscularis propria), and serosa are the five primary layers that make up the large intestine wall (Figure 1.2). The colon is the first segment of the large intestine, and it comprises of ascending, transverse, sigmoid, rectum, and anus. It is mainly located in the bottom part of the gastrointestinal tract and ileocecal junction to the anus. The colon resembles a cylinder tube lined with

mucosa and a lumen channel with 2-3 inches diameter. The proximal and distal colons can be distinguished based on absorption at each location and the mucosal lining (Ellis & Mahadevan, 2014).

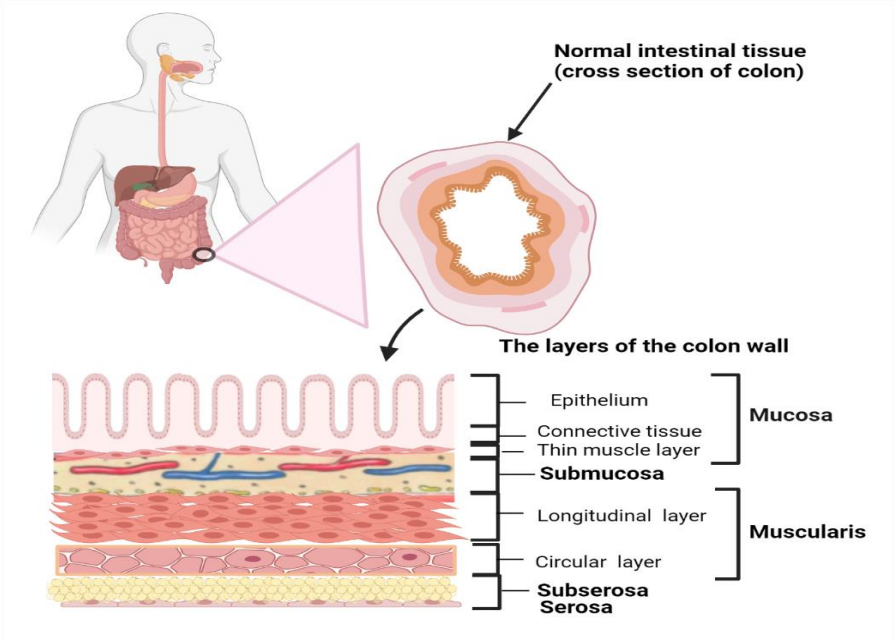

Figure 1.2: Layers of the colon wall consisting of the columnar mucosa, followed by the submucosa. Then there are the muscle layers (muscularis propria). The serosa is the outermost layer. (Created with BioRender.com).

The inner lining mucosa comprises a smooth columnar epithelial cell layer supported by the lamina propria, a thin connective tissue binding layer (Figure 1.2). Numerous lymph nodules in the lamina propria play an important role in disease prevention. The muscularis mucosa comprises two thin layers of smooth muscle surrounding the lamina propria that divides the mucosa from the submucosa layer. This muscular layer generates several tiny folds in the gastrointestinal canal, considerably increasing the absorptive surface area (Van de graaff,1986). The submucosa, which contains nerves, blood vessels, and connective tissue, lies beneath the epithelial layer. Also, the muscularis is composed of two smooth muscle layers, a circular band of muscle, and a longitudinal muscle layer

(muscularis propria) (Kapit & Elson, 2002). Rather than being continuous, the outer longitudinal layer is composed of three strips called teniae coli. As a result, the colon has a puckered appearance, with bulges called haustra along its length (Carlson, 2018). Food nutrients must be absorbed through the mucosa before the digestive process is complete, and through capillary networks in the small intestine, they enter the blood vessels of the submucosa. Finally, the colon is covered by the thin outermost layers of connective tissue and squamous epithelial cells (subserosa and serosa) (Van de graaff,1986).

The three main roles of the large intestine are to produce and absorb vitamins, to create and move faeces towards the rectum for excretion, and to absorb water and electrolytes. In the small intestine, 90% of water, most nutrients and most indigestible materials are absorbed before reaching the colon, a mechanism that transforms liquid chyme waste into semi-solid stools or faeces (Azzouz and Sharma, 2018). Faeces in the colon move ultra-slowly forward due to a segmental pattern of colonic motility programmed by the enteric nervous system and a motility pattern known as haustration, with this occurring when the circular muscle contracts in rings to divide the colon into sections (Wanamaker and Grimm, 2004).

The colon is prone to various disease states, some of which will be discussed later in this thesis. However, some of the most common conditions affecting the gastrointestine are outlined here. For example, colon and rectal cancers are a relatively common malignancy of the GI tracts, with common symptoms including abdominal pain, bleeding, changes in bowel habits, and weight loss (Astin et al., 2011). Diverticular disease is characterised by the formation of small pockets or pouches called diverticula in the colon wall. This can result in symptoms such as abdominal pain, bloating, and bowel changes (Feuerstein and Falchuk, 2016). Both types of inflammatory bowel disease (IBD), such as Crohn's disease

(CD) and ulcerative colitis (UC), are thought to be caused by the combination of genetic, immunological, and environmental factors, similar to the majority of autoimmune and chronic inflammatory disorders (Danese and Fiocchi, 2004). An immune response to gluten can result in the autoimmune condition known as coeliac disease in people who are genetically prone to it. The disease primarily affects the small intestine (Lebwohl et al., 2018). The research in this thesis mainly focuses on colorectal cancer.

This introduction discusses colorectal cancer and how steroidogenesis and inflammatory cytokines impact its pathogenesis. It will cover the current literature and outline how this thesis will contribute to understanding oestrogens and the colon. Furthermore, this chapter will explain the research's purpose, goals, and hypotheses, along with the aims and objectives.

1.2 Colorectal cancer (CRC):

1.2.1 Incidence

In 2020, there were an estimated 19.3 million new cancer cases and approximately 10 million cancer deaths worldwide (Ferlay et al., 2021). CRC is the third most common cancer in the world in terms of incidence and the second highest in mortality (Ferlay et al., 2019). When combined, the top 10 cancers account for more than 60% of newly diagnosed cancer cases and more than 70% of cancer deaths (Sung et al., 2021). Breast cancer in women is the most frequently diagnosed cancer (11.7% of all cases), followed by lung (11.4%), CRC (10.0%) and prostate (7.3%). Lung cancer is the most common cause of cancer death (18.0% of all cancer deaths), followed by CRC (9.4%), liver (8.3%), and breast cancer (6.9%) (Sung et al., 2021).

The incidence of CRC varies about nine times worldwide, with the highest in Europe, Australia / New Zealand and North America (Sung et al., 2021). With approximately 42,900 new cases of CRC each year in the UK, CRC is the fourth most common cancer in the UK, accounting for 11% of all new cancer cases (2016-2018) (Cancer Research UK, 2022). CRC-related mortality is very high in high-income countries, but CRC-related incidence and mortality are also increasing in developing countries (Hossain et al.,2022). However, due to a considerable increase in cases discovered in the elderly population, the global incidence of CRC is expected to more than double by 2035, with the most significant increase occurring in developing countries (Papamichael et al., 2015).

1.2.2 Risk factors for developing CRC

Many risk factors are linked to the development of CRC. They can be divided into two main groups: non-modifiable and modifiable risk factors (Edwards et al., 2010). Approximately 70-75% of cases of CRC are sporadic and associated with modifiable risk factors (e.g., lifestyle, diet, and obesity), while the remaining 25–30 percent are linked to non-modifiable risk factors (e.g., age and family history) (Sharma et al.,2022).

1.2.2.1 CRC and modifiable risk factors

Some modifiable factors, including excessive alcohol use, obesity, lack of exercise, smoking and diet, have all been linked to an elevated risk of CRC (Tabung et al.,2018). In obese individuals, inflammatory conditions in adipose tissue may contribute to CRC risk and development. The visceral adipose tissue (VAT) plays a major role in the systemic low-grade inflammation associated with obesity by secreting proinflammatory cytokines. According to current research, adipocytes within the tumour

microenvironment (TME), which is also intimately linked to VAT, may go through phenotypic alterations that aid in the development of tumours (Chaplin et al., 2022). The two most frequently employed obesity measurements are waist circumference (WC), which primarily reflects abdominal fatness, and BMI, which evaluates overall body fatness. According to some data, WC is a more significant risk factor for CRC than BMI (Moore et al., 2004). About a 4% higher risk of CRC was linked to every 10 cm increase in WC (Keum and Giovannucci, 2019). The two behavioural factors that are most important for CRC development are obesity and inactivity, which together likely account for most individual differences. Furthermore, physical activity has been linked to a 25% decreased risk of CRC development. The chance of acquiring CRC is up to 50% higher for the most sedentary people (Rawla et al., 2019).

Diet modifies inflammation, hence dietary patterns with a higher propensity for inflammation may increase the risk of CRC (Tabung et al.,2018). Furthermore, consuming red meat, particularly processed meat, has been associated with an increased risk of CRC (Aykan, 2015).

A meta-analysis investigated the link between cigarette smoking and colorectal, colon, and rectal cancer. The findings demonstrated that, in comparison to non-smokers, smokers had a greater risk of CRC incidence and mortality, respectively. Moreover, smoking rates, duration, pack-years, and age of initiation all elevated the chance of developing CRC (Liang et al., 2009).

Finally, persistent ethanol intake increases the risk of developing some cancers, including those of the large intestine, liver, and the female breast. Acetaldehyde, the first metabolite of ethanol, was determined by the International Agency for Research to be carcinogenic to humans (Seitz and Stickel, 2010).

1.2.2.2 Non- modifiable risk factors for CRC

Non-modifiable risk factors such as personal histories of polyps or adenomas, genetic factors, or a family history of CRC or hereditary risks like familial adenomatous polyposis (FAP) and Lynch syndrome (hereditary nonpolyposis colorectal cancer - HNPCC) (Sharma et al., 2022). Several non-modifiable factors, including (IBD), FAP and HNPCC, can raise the risk of CRC development (Edwards et al.,2010). Individuals with hereditary cancer syndromes are at a higher risk of CRC than the general population at any age because they have germline mutations in high-penetrance genes that mostly follow an autosomal dominant pattern (Rahner and Steinke, 2008). Also, if a first-degree relative has CRC, the risk of CRC is about twice that of the average person (Edwards et al., 2010).

Globally, population deaths and cases of CRC increase rapidly after age 50, with 90 percent of deaths occurring after this age (Keum and Giovannucci, 2019). In many other high-income nations, including Canada, Germany, and the United Kingdom, the death rate of people under 50 is also rising. Although the cause of the rise in CRC incidence and fatalities among young people is uncertain, it is likely to be related to the increase in risk factors for the disease associated with lifestyle (Sedlak et al., 2023). Along with the conditions outlined above, there is a significant gender bias in the development of CRC, with women having a lower risk than age-matched men (Arnold et al., 2017) and men

develop CRC 4–8 years earlier than women and at a rate that is 1.5 times greater than women, which points to the potential protective function of oestrogen or androgens in CRC development (Kim, N., 2022).

1.2.3 The aetiology of CRC

CRC is a disease that occurs only in the colon or rectum and is caused by the abnormal proliferation of glandular epithelial cells in the colon. Sporadic, genetic, and colitis-associated CRC are the three main kinds (Hossain et al.,2022). Among the five subtypes of CRC (adenocarcinoma, carcinoid tumour, gastrointestinal stromal tumour, lymphoma, and sarcoma), adenocarcinoma is the most common (95%) (Huo et al., 2017). CRC usually begins as a polyp, a noncancerous growth that develops on the inner lining of the rectum or colon (Winawer and Zauber, 2002). CRC develops from a polyp that starts as an abnormal crypt and then progresses into an early adenoma before becoming colon cancer (Figure 1.3). This process usually takes 10-15 years to complete but can go more quickly in some circumstances. It is driven by the accumulation of mutations and epigenetic changes (for example, in patients with Lynch syndrome) (Kuipers et al., 2015).

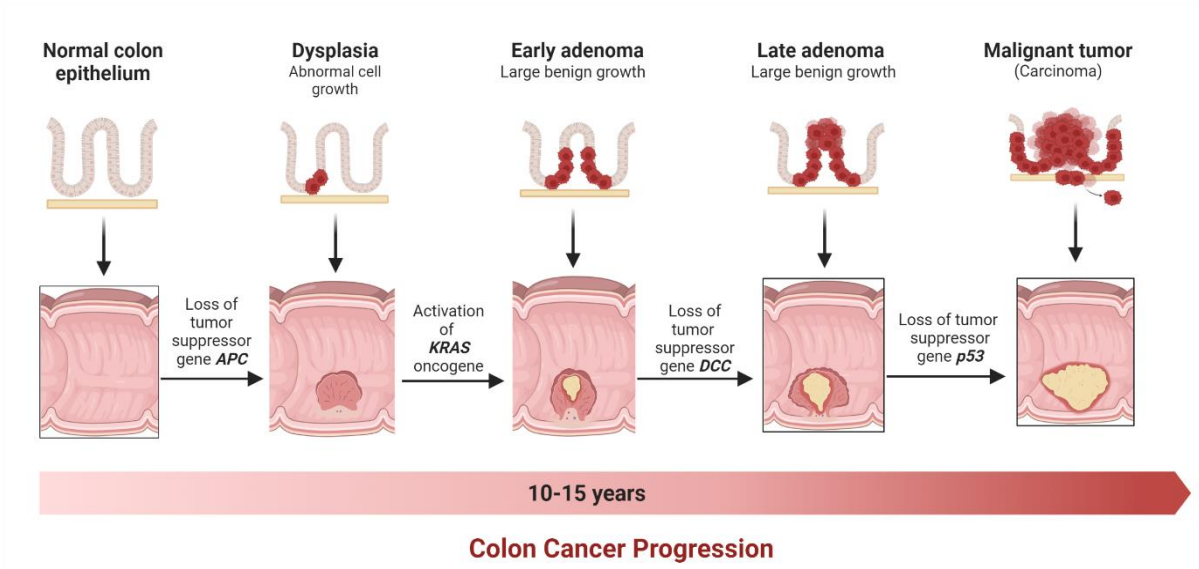

Figure 1.3: Colon cancer develops by a chromosomal instability pathway that includes the development of genetic mutations from the normal epithelium to adenoma, carcinoma, and metastasis. (Created with BioRender.com).

For oncogenes and tumour suppressor genes, the loss of genomic and epigenomic stability speeds up the accumulation of mutations and epigenetic changes. This causes colon cells to become malignant through rounds of clonal expansion, which select cells with the most aggressive and malignant behaviour (Kuipers et al., 2015). Most sporadic CRCs are assumed to develop from benign adenomas, whose epithelium can produce finger-like projections (villous adenomas), glands (tubular adenomas), or a combination of the two (Davies et al.,2005). Microsatellite and chromosomal instability, the CpG island methylator phenotype, and mutations in oncogene and tumour suppressor genes are some of the molecular factors contributing to CRC progression. Additionally, differences in microRNA expression profiles encourage the development of abnormal crypt foci and polyps, modifications to the tissue microenvironment, the establishment of carcinoma, angiogenesis, and, ultimately, metastasis (Coronel-Hernández et al., 2022).

To monitor the progression of CRC, Fearon and Vogelstein first proposed the adenoma-cancer sequence model. In this model, specific mutations are directly associated with different stages of tumour development (Fearon and Vogelstein, 1990). It assumes that the accumulation of genetic and epigenetic mutations causes epithelial dysplasia and hyperplasia of the colon, ultimately leading to CRC (Vogelstein and Kinzler, 1993). The sequence usually begins with a mutation in Adenomatous polyposis coli (*APC*) (formation of a precancerous adenoma) and ends with a mutation in *P53*. Then, the disease progresses through the stages of carcinoma (Phelps et al., 2009). According to this model, tumour initiation is caused by mutations at the level of the *APC* gene involved in the formation of adenomas and the development of "dysplastic crypts" (see Figure 1.2). Following this stage, the appearance of additional mutations at the levels of *KRAS, p53, and SMAD4* encourages tumour promotion and progression. It is characterised by increased growth of adenomas and the spread of individuals, especially malignant clones, followed by tumour invasion and metastasis (Testa and Castelli, 2018).

Inflammatory bowel disease (IBD) can also lead to CRC, which is the most frequent cancer among these patients. IBD, as well as the hereditary disorders of familial adenomatous polyposis and hereditary nonpolyposis CRC syndrome, are the three most major high-risk disorders for CRC (Forbes, 2008). IBD affects the GI tract, and IBD-related CRC (IBD-CRC) is one of its serious complications. Only 1%-2% of patients with IBD develop IBD-CRC, but it is responsible for 15% of IBD-related deaths (Leong and Koo, 2009). The Wnt pathway and CIMP pathway are two well-known pathways by which the development of IBD-CRC is likely triggered because of chromosomal and microsatellite instability, resulting in mucosal dysplasia (Claessen et al.,2010). As a result

of the involvement of these pathways, persistent inflammation is believed to play a prominent role in the development of cancer. Chronic inflammation is believed to be responsible for the increased risk due to changes in the microenvironment. Also, inflammation-induced chronic proliferation enhances the risk of dysplasia in epithelial cells (Romano et al., 2016). Pathogenesis of IBD-CRC has been linked to multiple cytokines and pathways (Claessen et al.,2010). IBD is fundamentally caused by tumour necrosis factor (TNFα), which has been the target of pharmacological therapies. This cytokine causes inflammation by increasing interleukin (IL) 1b and IL6 production, activating adhesion molecule expression, fibroblast proliferation, activation of pro-coagulant factors, and cytotoxicity of the acute-phase response (Sakurai et al., 2014).In CRC, where the inflammatory process and immunogenic responses drive tumour formation, cytokines are crucial components of tumour immunology. Due to their stimulation of the primary oncogenic factors, nuclear factor B and inducer of transcription 3 (STAT3) in intestinal cells promote proliferation and resistance to apoptosis in CRC. (West et al., 2015; Hossain et al.,2022).

Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) are the most common inherited colon diseases. FAP is an autosomal dominantly inherited disease that affects roughly 1 in 7000 people, where patients typically develop multiple benign colonic polyps (Kinzler & Vogelstein,1996). HNPCC is a dominantly inherited autosomal disease caused by heterozygous germline sequence mutations in DNA mismatch repair genes, primarily MLH1 and MSH2 (Hitchins et al., 2005). Only 5-10% of CRCs are caused by inherited mutations in well-known cancer-related genes. In comparison, the majority of CRCs are sporadic (75%) and occur in people without a

family history or genetic predisposition (Yamagishi et al.,2016). Most CRCs arise from adenomas, which are precursors of preclinical development. Progression from early-stage adenomas to invasive cancers can take years (Kuntz et al., 2011; Schreuders et al., 2015).

1.2.4 CRC diagnosis

CRC can be detected through screening tests or after the onset of symptoms. CRC screening involves a variety of procedures, each with unique properties. The most often used tests are colonoscopy, sigmoidoscopy, and faecal occult blood testing (FOBT). Adenomas are typically found and removed during colonoscopy and sigmoidoscopy, so these tests are largely used for preventive screening. A colonoscopy is a requirement for all CRC screening tests (either for primary screening, for those who test positive for FOBT, sigmoidoscopy, or both) (Kalager et al., 2018). These tests play a vital role in the early detection of CRC, and faeces tests are mostly used for early detection screening due to their high sensitivity to invasive cancers. FIT faecal immunochemical testing and FIT faecal DNA testing are commonly used for screening CRC in stool samples, both of which are non-invasive methods of detecting blood or abnormal DNA in the stool (Toth III et al., 2024). National recommendations have been made for FIT to be used as a triage test in primary care for patients with symptoms due to its high sensitivity and negative predictive value at the lower threshold of ≥10μg Hb/g stool (Bailey et al.,2021).

Adults aged 60 to 74 years are provided a faecal occult blood test as part of the UK CRC screening programme in England every two years, and this has been found to be effective in reducing CRC mortality (Hewitson et al., 2007). The most common symptoms of CRC are blood in the stool, altered bowel transit with prolonged constipation or altered bowel transit with diarrhoea, significant weight loss, and loss of appetite. Symptoms also depend on the size and location of the disease (Yamada et al., 2011). Colonoscopy, chest and abdomen CT scans, as well as tumour biopsies, are used to confirm the diagnosis (David et al., 2010). The histological analysis of the biopsy samples can classify CRC as intermediate or poorly differentiated based on the shape of the cancer cells (Jass, 2007). Also, a laboratory test that assesses the blood's concentration of the tumour marker carcinoembryonic antigen (CEA) can identify CRC (Williams et al., 2016). As a result of the findings, CEA may not be appropriate as a particular biomarker for CRC because it can be expressed at higher levels in both CRC and stomach cancer (Ma et al., 2022).

Since the mid-1980s, there has been a steady drop in CRC incidence rates, which has been linked to both altered risk factors and the implementation of screening (Edwards et al., 2010; Siegel et al., 2016). According to studies, dietary and lifestyle adjustments could help prevent 20% of cases of CRC (Kirkegaard et al., 2010). Numerous cancers have modifiable risk factors, and lowering such risks often lowers the incidence of cancer over the long term. Therefore, it is necessary to analyse the impact of altering CRC risk factor prevalence over a long period to see the impact (Edwards et al.,2010). However, the recent significant declines in CRC are most likely caused by the increased use of colonoscopies for screening, which can detect and remove precancerous lesions (Siegel et al., 2012). Ninety percent of patients diagnosed with the early-stage localised disease

survive for five years, emphasizing the significance of early detection. When the disease has metastasized outside of the colon but is still localised, this rate falls to 71 percent, and when the diagnosis of CRC is coupled with distant metastases, it falls to just 13 percent (Williams et al., 2016).

1.2.5 CRC classification and staging

The TNM tumour categorization system is the most popular method of cancer staging (Kehoe and Khatri, 2006). For the routine prognosis and treatment of CRC, the American Joint Committee on Cancer (AJCC) TNM staging system provides the most accurate guidance (Galon et al., 2014). This typical tumour staging provides information on the size of the primary tumour (T). For instance, T1-T4 are used to determine the size and extent of the tumour, with progressive enlargement and invasiveness from T1 to T4, whereas T0 indicates the absence of the tumour. An invasion of the submucosa is characterised by T1 in CRC, while an invasion of the visceral peritoneum or adjacent structures is characterised by T4. Invasion of muscularis propria occurs in T2, and invasion of subserosa occurs in T3 (Rosen and Sapra, 2022). The spread of the tumour in the regional lymph nodes (N), while N0 implies that there is no regional nodal spread, N1 to N3 show varying degrees of nodal spread, with a gradually distal spread from N1 to N3. N1 in CRC denotes the presence of 1-3 regional nodes. N2 can have 4-6 regional nodes. However, N3 means there are at least seven regional nodes involved (Rosen and Sapra, 2022). Finally, whether there is evidence of distant metastases (M) of the primary tumour is also classified. If there are no signs of distant metastasis, a tumour is categorised as M0, and if there are, it is categorised as M1. In the classification of CRC, M1a indicates spread to one location, M1b to two or more, and M1c to the peritoneal surface (Rosen

and Sapra, 2022). TNM staging for CRC is based on survival analysis of Surveillance, Epidemiology, and End Results (SEER) data. According to SEER analysis results, rectal cancer is also classified into stages I to IV (Gunderson et al., 2010).

As mentioned above, CRC development is categorised into four stages from stage 0 to stage IV (see Table 1.1). This is the same as other tumours and malignancies: initiation, promotion, progression, and metastasis. The liver, lung, and bone are the most typical metastatic sites for CRC. It is difficult to estimate how long each stage will take, but decades will probably be needed to form CRC (Markowitz and Bertagnolli,2009). In cancer grading, cells and tissue are described microscopically. The cells and tissue structures of low-grade tumours appear relatively normal. It is considered that these tumours are well-differentiated. Higher-grade tumours have more abnormal cells, and their tissue is abnormally shaped. In general, higher-grade tumours have a worse prognosis and are more aggressive. As a result, they are considered poorly differentiated (Rosen and Sapra, 2022)

Stage	Criteria
Stage 0	Indicates carcinoma in situ. Tis, N0, M0
Stage I	Localized cancer. T1-T2, N0, M0
Stage II • IIA (T3N0) • IIB (T4aN0) • IIC (T4bN0)	Locally advanced cancer, early stages. T2-T4, N0, M0.

Stage III • IIIA (T1-2N1 or T1N2a) • IIIB (T3-4aN1, T2-3N2a or T1-2N2b) • IIIC (T4aN2a, T3-4aN2b or T4bN1-1).	Locally advanced cancer, late stages. T1-T4, N1-N3, M0.
Stage IV	Metastatic cancer. T1-T4, N1-N3, M1.

Table 1.1: The different stages of CRC based on TNM classification. The schematic representation of the progression of CRC from stage 0 to IV (Rosen and Sapra, 2022).

A growth that does not invade (metastasize) to different regions of the body is referred to as a non-cancerous (benign) soft tissue tumour. A (benign) polyp or adenoma develops as a result of hyperproliferation (stage 0). Adenocarcinomas that infiltrate the muscularis propria can develop from ten percent of adenomatous polyps (stage I). The tumour expands in size and continues to infiltrate serosal (stage II) and visceral peritoneal tissue (stage III). Additionally, lymphatic or blood vascular metastases could develop (stage IV) (NIH, 2022). Patients who are diagnosed with high-stage CRC typically have a worse chance of survival than those who are diagnosed with low-stage cancers (Pei et al., 2021). The survival of patients with stage IIB/IIC CRC was lower than that of those with stage IIIA according to data from the Surveillance, Epidemiology and End Results (SEER) programme based on the AJCC 7th (Edge et al., 2010) and 8th TNM staging systems (Amin et al., 2017).

1.2.6 CRC treatment options

A colon polyp typically takes 10 to 15 years to develop into a cancerous tumour. As a result, CRC can be identified by routinely screening and removing polyps at an early stage (Hossain et al., 2022). A few of the currently accepted forms of treatment are surgery, chemotherapy, cryosurgery, targeted therapy, radiation therapy, and radiofrequency ablation. Based on the tumour's stage (0-IV) and spreading, a treatment plan is chosen (Williams et al., 2016). For patients with non-metastatic CRC, surgery is the primary curative procedure. The quality of the surgery, the quality of pre-operative staging, and the choice of treatment, however, are all highly correlated with patient outcomes (Kuipers et al., 2015).

1.2.6.1 Chemotherapy drugs and their use in CRC

Surgery and, in some cases, adjuvant chemotherapy are the main forms of treatment for CRC (McGee and Benson, 2014). Radiotherapy has not been found to improve outcomes when combined with chemotherapy, which is the primary adjuvant therapy for CRC (Martenson et al., 2004). For the treatment of CRC, cytotoxic drugs such as oxaliplatin, irinotecan, and 5-fluorouracil (5-FU) are frequently utilized as chemotherapy agents. Some CRC patients, however, do not react to this regime and/or experience severe toxicities (Yau, 2019). Many chemotherapeutic medication regimens are regarded as first-line in the neoadjuvant/adjuvant curative setting. The primary component of cytotoxic chemotherapy is fluoropyrimidine (FP), which can be given intravenously as 5-FU or orally as Capecitabine in a variety of potential combinations (Cassidy et al., 2011). 5-FU is usually administered within a combination regimen such as FOLFOX (5-FU, leucovorin, and oxaliplatin) or FOLFIRI (5-FU, leucovorin, and irinotecan). There are

many ways in which 5-FU works on cancer cells, including stimulating apoptosis and autophagy. Still, its main mechanism is to prevent the replication of DNA by inhibiting the cellular thymidylate synthase (Longley et al., 2003).

Stages 0-II CRC are often treated with surgery, and adjuvant therapies are used for stage II colon cancer (McGee and Benson, 2014). Patients with stage II CRC who do not belong to a high-risk category are not typically advised to get adjuvant chemotherapy (ACT). Patients with T4 tumours should be offered ACT. In contrast, those with other high-risk factors, such as sampling of fewer than 12 lymph nodes in the surgical specimen, poorly or undifferentiated tumour grade, intestinal obstruction, or tumour, may also be given ACT (Baxter et al., 2022). Furthermore, adjuvant oral fluoropyrimidine monotherapy, which has comparable oncological results to intravenous fluoropyrimidine-based treatment and less chemotherapy-related side effects, can be a useful and practical alternative in high-risk stage II colon cancer (Cho et al., 2022). Stage III CRC is typically treated with surgery and ACT, and stage IV and recurrent CRC are typically treated with surgery, chemotherapy, and targeted therapy.

Unfortunately, there is currently no known cure for CRC (Hossain et al., 2022). ACT should begin within eight weeks of surgery. Delaying the start of ACT for longer than eight weeks after surgery significantly reduced overall survival (OS) but not relapse-free survival (RFS). This gap could be from factors not directly related to CRC (post-operative problems, social status), or it could be the result of a more precise assessment of death (Des Guetz et al., 2010).

1.2.6.2 Targeted therapies used against CRC

Similar to other cancers, genetic and epigenetic modifications are thought to contribute to the development of CRC. As discussed above, *TP53* (a tumour suppressor gene) and *KRAS* (an oncogene) mutations are two of the most frequently documented genetic changes in CRC (Tortola et al., 1999). There are several genomic changes associated with resistance to targeted therapy with epidermal growth factor receptor (EGFR) monoclonal antibodies, including KRAS, NRAS, and BRAF mutations. This provides a molecular basis for selecting a suitable agent to treat metastatic CRC (Sepulveda et al., 2017). EGFR overexpression contributes to tumour progression in a number of cancer forms, including CRC (Cheng et al., 2021). The serine/threonine-specific protein kinase (AKT), JNK, and mitogen-activated protein kinase (MAPK)/ERK signalling pathways that control DNA synthesis, cell proliferation, death, and motility are all regulated by the transmembrane tyrosine kinase receptor known as EGFR (Yau, 2019). In CRC patients, blocking the EGFR with monoclonal antibodies such as cetuximab or panitumumab in combination with a chemotherapy formula containing 5-FU, leucovorin, and oxaliplatin (FOLFOX) (Tveit et al., 2012) or a chemotherapy formula combination of 5-FU, leucovorin, and irinotecan (FOLFIRI) results in better treatment response (Peeters and Jay Price, 2010).

1.2.7 Prevention strategies for CRC

In public health, one of the main challenges is preventing carcinogenesis (primary prevention) and detecting it early (secondary prevention). The primary prevention of CRC is achieved through genetic counselling, chemoprevention, and dietary modification (Marin et al., 2012). Also, increasing the knowledge of the modifiable risk variables may

help us develop new primary preventive methods that can further lower risk (Giovannucci and Willett, 1994).

Chemoprevention is one approach to lower CRC risk that has been thoroughly explored over the past several decades (Sporn, 1976). Several medications, including aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and hormone replacement therapy, have been found to affect the risk of CRC (Sninsky et al., 2022). Although aspirin and NA-NSAIDs have demonstrated the greatest promise, recommendations for their usage as chemopreventive drugs have only been made for those with predisposition syndromes associated with an elevated risk of CRC, such as Lynch syndrome or FAP (Katona and Weiss, 2020). NSAIDs, which are, in general, cycloxygenase (COX) inhibitors, are among the oldest and most frequently used medications in clinical practice (Marin et al., 2012). Mesalamine, a drug used to treat IBD, has been discovered to exhibit chemoprotective properties against CRC in IBD patients; the growth and survival of CRC cells have also been demonstrated to be inhibited by mesalamine (Stolfi et al., 2012). The results of a study published in 2021 involving more than 94,000 participants found that those who used aspirin on a daily basis had a decreased risk of CRC compared to those who did not (Guo et al., 2021). As a result, the American Gastroenterological Association's Clinical Practice Update recommended that certain individuals, based on age and cardiovascular risk profile, take aspirin for CRC prevention (Liang et al., 2021).

1.2.8 Resistance and Recurrence of CRC

Early detection (Stage 0 – II) of CRC makes it perfectly treatable through surgery and subsequent chemotherapy. The failure rate of treatment in higher stages of cancer is

increased by the higher risk of recurrence and cancer drug resistance. Only 40% of CRC cases may now be detected in the early stages by screening, and CRC may return after surgery and post-operative care. Chemotherapeutic drugs work to kill cancer cells, but they also damage healthy cells in the surrounding environment. Nearly all CRC patients receiving modern chemotherapies developed resistance, which reduced the effectiveness of anticancer drugs and ultimately resulted in chemotherapy resistance (Hossain et al.,2022).

According to research by Martínez and colleagues, people who consumed cooked red meat had a higher chance of developing an adenoma again than those who did not consume red meat (OR,1.85; 95% CI, 1.10-3.13) (Martinez et al., 2007). The risk of CRC and adenomas is lowered by physical activity (Wolin et al., 2011), and the probability of an adenomatous polyp recurrence was considerably lower in males in the physically active quartile than in the least physically active quartile (Sardo Molmenti et al., 2014). Determining gender-specific techniques for screening, treatment, and prevention procedures will assist in lowering mortality and enhancing the quality of life. This is because sex/gender-related biological and socio-cultural differences in CRC risk exist (Kim,2022). The greater mortality of CRC in males appears to be caused by exogenous and/or endogenous causes pre-diagnosis that result in higher incidence rates since there are small sex differences in the data from pathways to diagnosis to survival (White et al., 2018). The biological basis for the sexual difference in CRC includes sexual dimorphism in gene and protein expression as well as in endocrine cellular signalling. Long-term and short-term genomic regulation of colon cancer development by oestrogen and X-linked genes opens up new possibilities for precision medicine and molecular targeted therapies.

(Abancens et al., 2020). Also, right-sided (proximal) colon cancer is more common in women than in males and is linked to a more aggressive form of neoplasia than left-sided (distal) colon cancer (Kim, 2022).

1.3 Steroid hormones and the colon:

1.3.1 Hormones of the gastrointestinal tract

The earliest gut hormones discovered were gastrin (1902), secretin (1905), and cholecystokinin (1928). Then, over 50 hormones and bioactive peptides have been identified that regulate the gastrointestinal tract's normal physiology. These include absorption, digestion, secretion, and motility. Therefore, the gastrointestinal tract is considered a major hormonal organ. They also have a role in the pathogenesis of gut mucosal atrophy and malignancies by modulating GI mucosal proliferation (Jaladanki & Wang, 2011; Banibakhsh et al., 2023).

Numerous studies have shown that additional hormones, such as steroids, can be synthesised and metabolised by the GI tract. There are high levels of CYP11A1, CYP17, and CYP11B1 and cortisol production in colorectal cancer cell lines (Caco2, HT29) as well as primary human intestinal tissue. There is also a constitutional expression of 3βHSD1 and 2 in Caco-2 cells (Sidler et al., 2011; Bouguen et al., 2015). Furthermore, human intestinal tissue, contains steroidogenic enzymes and active steroids (Cima et al., 2004), and it is the primary source of de novo synthesised glucocorticoids, which contributes to intestinal homeostasis and has been linked to the pathophysiology of IBD (Bouguen et al., 2015). Intestinal epithelial cells can also metabolise sex steroids,

particularly oestrogen, which could influence the development of CRC (Bouguen et al.,2015).

1.3.2 Steroid Metabolism

In the cortex of the adrenal gland, steroidogenic cells synthesise oestrogens and androgens from cholesterol through a series of pathways that begin in the mitochondria. The majority of cholesterol found in the body comes from low density lipoproteins (LDL), which transport dietary cholesterol into cells. Also, cholesterol can be synthesised from acetate de novo.

Three different layers of the adrenal cortex are involved in the synthesis of steroid hormones: the zona glomerulosa, which produces aldosterone; the zona fasciculata, which produces cortisone and cortisol; and the zona reticularis, which produces the androgens DHEA and androstendione (Miller & Auchus, 2011; Miller, W.L., 2013). In human adrenal glands, gonads, and other peripheral tissue, enzymes involved in the biosynthesis of active steroid hormones from cholesterol are separated into two classes. The hydroxysteroid dehydrogenase (HSD17Bs) enzymes which catalyse redox reactions are typically reversible, whereas cytochrome P450 (CYP) enzymes which catalyse oxidative reactions are mostly irreversible (Hanukoglu, 1992). The steroidogenesis of steroid hormones involves a multistep and multienzyme process by which precursor cholesterol is converted into pregnenolone and then metabolised into other biologically active steroids (Figure 1.4.). In order to produce steroids, cholesterol must be broken down enzymatically, where the cytochrome P450 cholesterol sidechain cleavage enzyme (*CYP11A1* or P450scc) catalyses the first and rate-limiting step, which leads to

pregnenolone, the precursor of all steroid hormones (Payne & Hales, 2004). Pregnenolone is thus further transformed into the final hormone product through a series of steps along various pathways, depending on the enzymes present in that tissue. As a result, the capacity of each cell to synthesise distinct steroid hormones is determined by the unique expression of these enzymes (Hanukoglu, 1992).

Pregnenolone in the zona glomerulosa is converted into progesterone by HSD3B2, which is then converted into 11-deoxycorticosterone (DOC) by CYP21A2. In this stage, DOC is used as a substrate for CYP11B2 (aldosterone synthase). This enzyme has 11-hydroxylase, 18-hydroxylase, and 18-oxidase activities, which catalyse the three final steps leading to the formation of aldosterone (Hattangady et al., 2012). CYP17A1 and cytochrome P450 reductase (CPR) are both present in the zone fasciculata layer of the adrenal cortex, which direct the hydroxylated products of pregnenolone and progesterone towards glucocorticoid synthesis, resulting in cortisol (Rege et al., 2014).

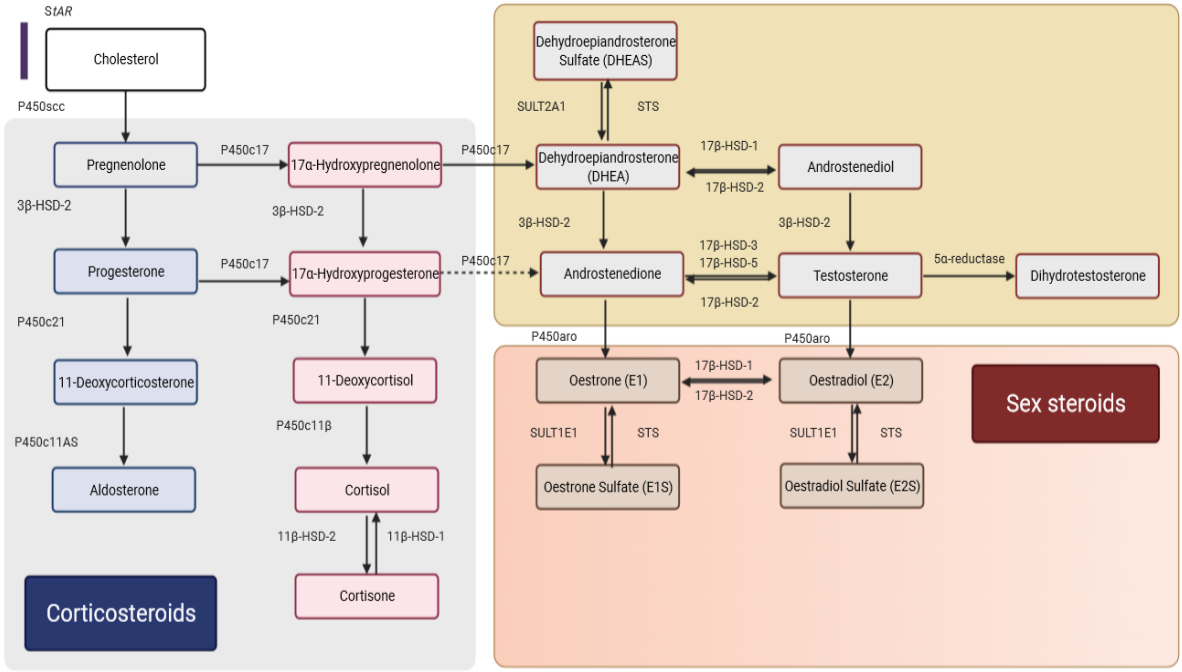

Figure 1.4: Main Steroidogenesis Pathways in Humans. All steroids, including progestogens, mineralocorticoids, androgens, glucocorticoids, and oestrogens, have cholesterol as a common precursor. P450scc is the enzyme that converts cholesterol to pregnenolone. (Created with BioRender.com).

A single enzyme, cytochrome P450c17, catalyses 17β-hydroxylation of pregnenolone and progesterone, as well as C17-C20 bond cleavage of 17β-hydroxypregnenolone and 17β-hydroxyprogesterone, in order to produce dehydroepiandrosterone (DHEA) and androstenedione (Zhang et al., 1995). As a sex steroid precursor, DHEA is abundantly produced in the adrenal cortex' zona reticularis (Li et al., 2020). In peripheral tissues, DHEA is converted into androstenedione via 3-β-hydroxysteroid-dehydrogenase (HSD3B), which is mainly involved in the synthesis of testosterone (T) and estrone (E1). Dihydrotestosterone (DHT) results from further dehydroxylation of testosterone by 5-alpha reductases. Testosterone can also be aromatized to produce 17β -estradiol (E2), the oestrogen with the greatest ER affinity (Swerdloff et al., 2017).

The level to which the different steroidogenic and metabolising enzymes are expressed in each cell of peripheral target tissues affects the rate at which the adrenal precursor hormones DHEA-S and DHEA are converted into androgens and/or oestrogens (Labrie et al., 1997). Female adrenal precursors DHEA-S, DHEA, and 4-dione play a greater role in peripheral oestrogen formation than male adrenal precursors do. While oestrogen production by the ovaries entirely stops at menopause in women, androgen secretion by the testes remains at a high level throughout life in men, leaving the adrenals as the only source of sex hormones in women. Before menopause, women produce 75% of their oestrogens intracrinely from peripheral tissues and close to 100% after menopause (Adams, 1985; Labrie et al., 2003).

1.4 Oestrogen Synthesis, Metabolism and Transport in the normal colon

Typically, oestrogens are associated with female reproductive development. However, they are known to affect many other tissues, including the colon. Here, oestrogen metabolism and action will be discussed, as well as how this potentially impacts normal colon function.

There are two pathways by which oestrogen is synthesised, through either aromatase or steroid sulfatase (STS) action. These enzymes convert inactive steroid hormone precursors to the biologically most active oestrogen, E2. The peripheral conversion of androstenedione (Adione) to E1, regulated by aromatase, is a simple way to synthesise biologically active oestrogens. Aromatase activity is minimal in the colon (Foster, 2013), and aromatase immunoreactivity in normal colonic epithelial cells is weak and localised (Sato et al., 2012).

Desulfation of sulfated oestrogens via STS, which hydrolyses oestrogen sulfates into their unconjugated, biologically active forms **(**Strott, 2002), is also a primary pathway for

oestrogen metabolism**.** E_1S in circulation is taken up by cells via organic anion transporting polypeptides (OATPs) prior to sulfate removal by STS. After desulfation (see Figure 1.5), E_1 is further reduced by reductive members of the 17β-hydroxysteroid dehydrogenase (HSD17Bs), such as 17β-hydroxysteroid dehydrogenase type-1 (HSD17B1), to form E_2, which has the highest binding affinity for ERs (Zhu et al., 2006). The conversion of E_2 to E_1 can also be catalysed by other oxidative HSD17Bs, such as HSD17B2.

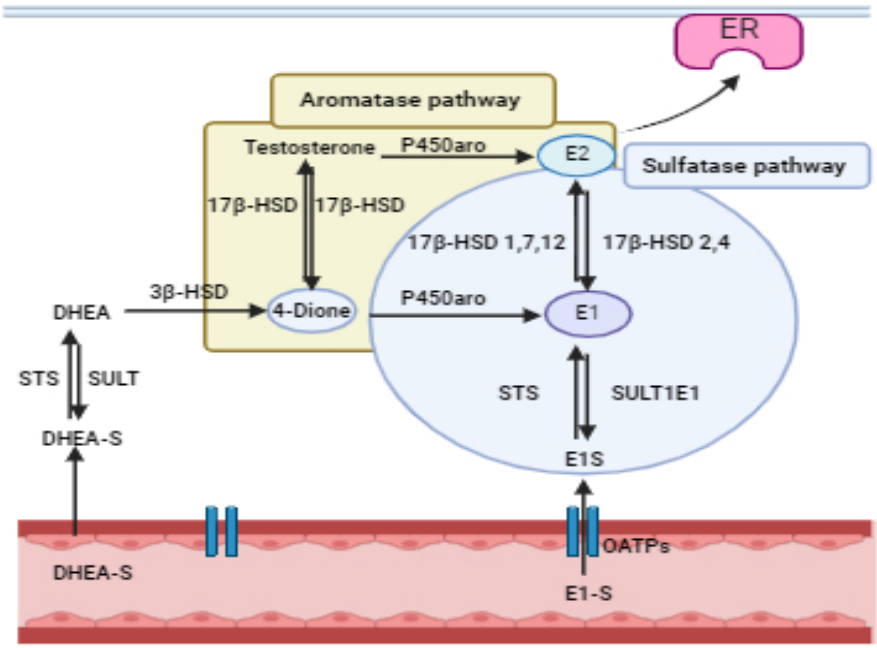

Figure 1.5: The sulfatase pathway and aromatase pathway**.** E_1S is taken up by the cells by OATPs and activated by the removal of sulfate by STS. E_1 is subsequently reduced by reductive members of the superfamily of HSD17Bs, such as HSD17B1, to form E_2, which has the highest binding affinity for ERs. Also, the conversion of E_2 to E_1 can be catalysed by oxidative HSD17Bs. For inactivation, E_1 can be sulfonated by oestrogen sulfotransferase SULT1E1 to E_1S. For the aromatase pathway, testosterone is converted to E_2 by P450 aromatase (CYP19A1). DHEA-S is mainly derived from the circulation and is hydrolysed to form DHEA, demonstrating STS involvement in activating androgens.

In tissue and serum, oestrogens are mostly found in their inactive sulfated form (Pasqualini et al., 1996), and the vital precursor for E_2 in the sulfate pathway is inactive E_1S, which has a 5-10 fold higher level in the blood than unconjugated oestrogens like E_1, E_2, and oestriol (E_3). E_1S also has a longer half-life and is thought to act as an oestrogen synthesis reservoir (Chetrite et al., 2000; Reed et al., 2005).

1.4.1 STS

STS belongs to the arylsulfatase (ARS) family of enzymes that catalyse the hydrolysis of sulfate ester bonds in a wide range of substrates (Parenti et al., 1997). Seventeen human sulfatase proteins and their genes have been identified (Diez-Roux & Ballabio, 2005), with sequence homologies ranging from 20% to 60% (Schmidt et al.,1995). STS is a membrane-bound microsomal enzyme that is mostly found in the rough endoplasmic reticulum (Ghosh, 2007). It is active in nearly all human tissues, with the highest activity in the placenta and liver (Reed et al., 2005). The human STS gene maps to Xp22.3-Xpter on the short distal arm of the X chromosome (Yen et al., 1987). It spans over 200 kbp, and the regulatory region is situated in the first 100 kbp of the human STS gene, while the STS-coding region is found downstream (Nardi et al., 2009).

1.4.1.1 Regulation of STS

STS goes through a variety of post-translational modifications that enhance its activity. The crucial one is cysteine (C)75, which is required for sulfatase-modifying factors 1 and 2 (SUMF1 and SUMF2) to generate C-alpha formylglycine (FGly) at the active site of STS. In addition, STS has four potential N-glycosylation sites, but only two are utilised (Asn47 and Asn259), and only alterations at these locations reduce activity (Foster &

Mueller, 2018). However, very little is known about the mechanisms that regulate STS expression, post-translation, and activity. Many malignant tissues have higher STS mRNA and activity levels compared to normal tissues, suggesting that STS plays a significant role in hormone-dependent tumour development. Since STS is ubiquitously expressed, the regulation of STS expression does seem to be tissue-specific. It is sensitive to a variety of feedback mechanisms, as evidenced by the positive link between STS and ER homologs mRNA (Zaichuk et al., 2007). The proinflammatory cytokines IL-6 and TNFα have been shown to affect STS enzyme kinetics in various cancers in addition to the possibility that oestrogens influence STS activity (Purohit et al., 1996; Reed & Purohit, 1997). These studies perhaps indicate that STS activity is regulated by post-translational changes, presumably through STS glycosylation. However, cytokines may not just be influencing STS activity through increased expression or post-translational changes. These cytokines may change cell membrane permeability to sulfated steroids, which would increase substrate availability, resulting in a perceived rise in STS activity (Mueller et al., 2015).

1.4.2 SULT1E1

Oestrogens are inactivated by sulfation, the main pathway for oestrogen metabolism in target tissues (Xu et al., 2012). The enzyme SULT1E1, also known as oestrogen sulfotransferase, sulfates estrone and oestradiol with high efficiency. The sulfation of active 17β-oestradiol (E2) generates inactive estradiol sulfate, which can be reactivated following the desulfation steps of oestrogen sulfatase (Choi et al., 2005). As E1S has a plasma concentration 10–20 times higher than those of the unconjugated oestrogens estrone and oestradiol and a longer plasma half-life, it serves as a reservoir for active

oestrogens (Ruder et al., 1972). A negatively charged sulfonate group (SO3-) is transferred from the universal donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a suitable substrate during sulfonation (Kauffman, 2004). In order to synthesise PAPS, ATP sulfurylase and APS kinase bind together within a bifunctional enzyme of approximately 56 kDa (Lyle et al., 1994). PAPSS has two isoforms in humans, and each isoform displays a distinct subcellular localisation, but their amino acid sequences are 77% similar (Xu et al., 2000). PAPSS1 is predominantly located within the nucleus, whereas PAPSS2 is mainly located in the cytoplasm (Besset et al., 2000; Schröder et al., 2012). The SULT1E1 enzyme's ability to sulfurylate oestrogens at physiological concentrations plays an important role in regulating their binding to oestrogen receptors in target tissues. It consists of 294 amino acids. There are two E2 bound to each subunit of the native enzyme, which has a size of 35 kDa (Zhang et al., 1998).

1.4.3 17β-hydroxysteroid dehydrogenases (HSD17Bs)

The HSD17Bs family of enzymes was initially discovered in the placenta in 1959 (Langer & Engel, 1959). These enzymes play a key role in the synthesis of sex steroid hormones, primarily through oestrogen and androgen metabolism (Bouguen et al.,2015; Oduwole et al., 2003). Fourteen HSD17B members have been identified, and they are numbered according to the order in which they were discovered. They prefer to function as NADPH-dependent ketosteroid reductases or as NAD+-dependent hydroxysteroid oxidases. In these transformations, two large gene superfamilies are involved: the aldo–keto reductases (AKRs) and the short-chain dehydrogenase reductases (SDRs) (Jörnvall et al., 1995; Bauman et al., 2004).

For sex steroid metabolism, HSD17B1, 3, 5, 7, and 12 catalyse reduction, while 2, 4, and 14 catalyse oxidation. Reduced oestrogens and androgens (E_2 and testosterone, respectively) have a higher affinity for binding to their respective receptors than their oxidized equivalents (E_1 and androstenedione, respectively). Furthermore, the oxidizing reaction is considered to inactivate the effects of sex hormones. Oxidizing enzymes are more broadly expressed than their reductive counterparts, and they are occasionally decreased or eliminated in cancer. As a result, the production of E_2 and/or testosterone is generally favoured in hormone-responsive cancers (Adamski et al., 1995; Vihko et al., 2002).

1.4.3.1 Role of HSD17B enzymes in oestrogen synthesis in the colon

The presence of 11β-HSD and HSD17Bs enzymes in the colon and gastrointestinal tract have previously been linked to human steroid metabolism (Sano et al., 2001; Whorwood et al., 1994). In human colonic mucosa, which primarily expresses isoforms 2 and 4, various isoforms of the HSD17Bs enzyme with varied enzymatic activity have been found and thoroughly investigated (English et al., 2001). Inhibitors of HSD17Bs, in particular HSD17B1, which regulate the concentration of active oestrogens, could be used to treat cancer (Marchais-Oberwinkler, 2011), and several researchers have investigated it (Day et al.,2009; Day et al.,2013).

1.4.3.1.1 HSD17Bs type1

The first HSD17B enzyme discovered was HSD17B1 (Langer & Engel, 1958). The *HSD17B1* gene encodes a 328 amino acid protein with a molecular mass of 34.95 kDa that is localized to 17q11-q21 (Miettinen et al., 1996). It catalyses the conversion of dehydroepiandrosterone (DHEA) to androstenediol (Adiol) and works as a reductase converting E_1 to E_2 (Miettinen et al., 1996). Regarding E_2 synthesis, HSD17B1 is the most active enzyme (Zhang et al., 2015). HSD17B1 is mostly expressed in the ovaries and

placenta (Labrie et al., 1997), but it has also been found to be expressed in breast epithelial cells (Vihko et al., 2002) and female human breast cancer (Song et al., 2006). A postmenopausal women's breast cancer tissue accumulates E2, possibly due to higher aromatase activity, STS activity, and HSD17B reductive activity. A recent study by Miyoshi et al. showed that breast cancer tissues of postmenopausal women are higher in E2/E1 ratio and HSD17B1 expression than those of premenopausal women. As a result, HSD17B1 may be responsible for the accumulation of E2 in breast cancer tissues (Vermeulen et al., 1986; Miyoshi et al., 2001; Oduwole et al., 2004). Increased oestrogen-dependent proliferation and cancer development could result from a high incidence of HSD17B1 in malignant breast tissue (Vihko et al., 2002).

1.4.3.1.2 HSD17B type 2 and 4

In the *HSD17B2* gene, 387 amino acids are encoded by six exons, and the protein has a molecular mass of 43 kDa. It is located at 16q24.1-q24.2. The endoplasmic reticulum is most likely the site of HSD17B2 (Wu et al., 1993). HSD17B2 is expressed in the lungs, placenta, liver, kidney, prostate, pancreas, colon, small intestine, endometrial (Labrie et al., 1997) and breast epithelial cells (Vihko et al., 2002). In malignant breast cells, HSD17B2 expression is also present (Sasano et al., 1996). Furthermore, HSD17B2 may protect normal breast cells from the mitogenic effects of E2 (Vihko et al., 2002). In the *HSD17B4* gene, 737 amino acids and the protein has a molecular mass of 80 kDa. It is located at 5q2 (De Launoit & Adamski, 1999). HSD17B4 is expressed in the liver, heart, testis, lung, skeletal muscle, prostate, pancreas, kidney, thymus, ovary, gut, placenta, and various human breast cancer cell lines (Labrie et al., 2000). Both HSD17B2 and HSD17B4 catalyse the converting of E2 into E1 by oxidative activity (Miettinen, 1996;

Peltoketo et al., 1998; English et al., 2000), and they catalyse the converting of androstenediol (5-diol) into DHEA by oxidative activity (Adamski et al., 1995). Moreover, HSD17B2 also catalyses testosterone to androstenedione (Vihko et al., 2002).

1.4.3.1.3 HSD17B type 7 and 12

Human HSD17B7 is involved in the biological action of sex steroid hormones and plays a key role in their regulation. The *HSD17B7* gene, which is located on chromosome 10p11.2, has nine exons and eight introns and encodes a 37 kDa protein (Krazeisen et al., 1999). Human HSD17B7 is expressed in a variety of human tissues, including the adrenal gland, small intestine, lung, liver, and thymus, as well as the pituitary gland, prostate, lymph node, kidney, trachea, and female human breast cancer (Song et al., 2006). HSD17B7 also functions as a reductase, converting 5α-dihydrotestosterone (DHT) to the oestrogenic metabolite 5α-androstane-3β,17β-diol (3βA-diol), facilitating the reduction of the keto group in either the 17- or 3-position of the substrate. Törn et al. (2003) found that it exhibits minimal 3βHSD-like activity towards progesterone and 20-hydroxyprogesterone, resulting in inactivation of these progesterones. Furthermore, HSD17B7 is an intracrine regulator of steroid hormone metabolism in peripheral tissues that creates an oestrogenic environment by synthesising E_2, converting androgens into oestrogenic metabolites, and inactivating progesterone.

HSD17B12 is a protein found in the endoplasmic reticulum. The *HSD17B12* gene, located on chromosome 11p11, encodes 17HSD12. This gene, which includes 11 exons, is widely expressed. The translated protein has a molecular weight of 34.3 kDa and 312 amino acids (Luu-The et al., 2006; Visus et al., 2011). Its major purpose is to help the

cytosolic fatty acid synthase complex produce long-chain fatty acyl CoA by assisting in fatty acid synthesis and chain extension (Lukacik et al., 2006), although it has also been shown to have a role in oestrogen metabolism (Luu-The et al., 2006; Rantakari et al., 2010). Organs involved in lipid metabolism, such as the kidney, liver, heart, and skeletal muscle, have high expression of HSD17B12. Its presence has also been verified in endocrine organs such as the pancreas, pituitary, testis, adrenal, and placenta, as well as in the gastrointestinal tract (Sakurai et al., 2006). The expression of HSD17B12 in breast carcinoma was substantially higher than in normal tissue (Song et al., 2006).

1.4.4Oestrogen receptors

Once pre-androgens and oestrogens have been metabolised to their active forms by the enzymes listed above, they express their function in the body through the nuclear receptors ERα *(ERS1*) and ERβ (*ERS2*) or at the cell receptor GPER (GPER-30) (Cui et al., 2013).

1.4.4.1 ERα and ERβ

The first nuclear ER to be identified was ERα, encoded by the gene *ERS1* on chromosome 6, and the corresponding gene was cloned in 1985 (Jensen et al., 1966). The *ERS2* gene, which is found on chromosome 14, codes for ERβ, which was discovered many years later, in 1996, in rat prostate and ovary tissues (Kuiper et al., 1996).

ERα is primarily expressed in the uterus and ovaries, as well as in the breast, liver, bone, white adipose tissue, and kidney. In contrast, ERβ is expressed in the central nervous system (CNS), lung, cardiovascular system, ovary, male reproductive organs, prostate,

colon, kidney, and immune system. ERs are nuclear receptor proteins that are mostly present in the nucleus, while they can also be found in the cytoplasm and mitochondria (Jia et al., 2015). Moreover, cytoplasmic organelles, including the mitochondria and endoplasmic reticulum, have been found to contain ERs. However, it has been hypothesised that mitochondrial ERs are involved in apoptotic/anti-apoptotic signaling (Yager & Chen, 2007). In the normal human colon, ERβ1 and ERβ2 mRNA have been discovered (Moore et al.,1998).

1.4.4.2 G-protein-coupled oestrogen receptor1 (GPER)

The integral membrane ER, known as GPER, is capable of rapidly initiating oestrogen-responsive signaling without the intervention of ERα and ERβ (Thomas et al., 2005). In the late 1990s, several groups succeeded in cloning this receptor, known at the time as GPR30 (Carmeci et al., 1997; Feng et al.,1997; Prossnitz & Barton, 2011). GPERs are physically distinct from ERα and ERβ and are expressed in both the plasma membrane and cytoplasm. GPER is present in significant amounts in the neural tissue, breast cancer, prostate, vascular epithelial, ovarian, placental, hepatic, heart, and lymphoid tissues. It shares structural similarities with the receptors for several chemokines, including interleukin, angiotensin, and others, and indicates that it might be a peptide receptor (Thomas et al., 2005).

1.4.4.3 ER signalling pathways

ERs, which include the two unique receptors ERα and ERβ, as well as GPER, mediate oestrogen signalling in human cells (Prossnitz & Barton, 2011). Also, there are ER-independent processes even though ER is required for the majority of oestrogen-mediated signalling pathways. Two distinct ER-dependent processes are categorized as genomic

and non-genomic consequences result from ER activation, depending on whether transcriptional regulation is the end effect (Cui et al., 2013). ER-dependent pathways can start either in the nucleus or at the plasma membrane in addition to being genomic or nongenomic. Compared to ER-dependent pathways, E2 starts ER-independent signalling pathways by controlling enzymatic processes or interacting with nuclear receptors for non-sex steroid hormones (Richardson et al., 2012). The majority of oestrogen's transcriptional effects are nucleus-initiated and are controlled by ERα and ERβ. The nuclear ligand-activated transcription factors in both receptors either activate or repress the transcription of target genes (Cui et al., 2013). When activated, nuclear ERs transcriptionally regulate the expression of specific target genes (Liu et al., 2020).

These nuclear receptors regulate target gene transcription by binding to oestrogen-response DNA regions, dimerising following ligand binding, and translocating to the nucleus (Ascenzi et al., 2006; Williams et al., 2016). In a process known as transcription factor cross-talk, the receptors can also interact with other transcription factor complexes, such as activating protein-1 (AP1) and nuclear factor-κB (NFκB) (Paech et al., 1997; Frasor et al., 2009; Williams et al., 2016). Furthermore, non-genomic effects through the phosphorylation and control of enzymes that affect cell physiology, such as kinases and phosphatases, as well as action through the membrane-bound GPER, have been identified (Levin, 2005). Additionally, ERs are linked to rapid cellular signalling, which is assumed to be predominantly mediated by membrane-associated versions of these receptors (Prossnitz & Barton, 2011).

ERα is known to mediate the proliferation of CRC cells, while ERα expression in normal colon tissue and CRC tissue is typically low. When ERβ expression decreases in cancer

cells, and the ratio of ERα / ERβ rises, ERα expression becomes dominant, inhibiting apoptosis and promoting cell proliferation (Barzi et al., 2013).

1.4.5 Organic anion transporting polypeptides (SLCO/OATP):

Cells need to constantly absorb nutrients and signalling molecules while also releasing metabolic end products for disposal. Most of these compounds, even if very lipophilic, cannot diffuse through plasma membranes and thus require transport proteins to cross cell membranes (Hagenbuch & Stieger, 2013). Among these are organic anion transporting polypeptides (OATPs), which belong to the *SLCO* family and carry a wide range of structurally varied amphipathic substrates, such as steroid hormones, which are necessary for the proliferation of hormone-dependent cancers (De Bruyn et al., 2011). The first OATP, rat OATP1A1, was identified in rat liver by expression cloning (Jacquemin et al., 1994), and the first human OATP, OATP1A2, was isolated through hybridization screening (Kullak-Ublick et al., 1995). Then, several other OATPs from humans and rodents have been found and characterized, and more than 300 members of the OATP family have now been identified in more than 40 species, including 11 human transporters and many rat and mice transporters (Hagenbuch & Stieger, 2013). OATPs play an important role in the uptake and distribution of both endogenous compounds and xeno-biotics across plasma membranes (Hediger et al., 2004; Hagenbuch & Stieger, 2013). For example, in relation to this thesis, E_1S cannot cross the cell membranes of peripheral tissues because of its negative charge. As a result, its transport into cells is dependent on OATPs (Obaidat et al., 2012). The OATPs are the main sulfated steroid influx transporters, and each one has a unique uptake kinetics and substrate selectivity for various conjugated steroids. It should be observed that several OATs (OAT1, OAT3,

OAT4, and OAT5) can transport sulfated steroids, especially E1S, in the human placenta (Cha et al., 2000) and kidney (Sweet et al., 2002).

For cellular uptake, hydrophilic sulfated steroids require active transmembrane transport. Since these endobiotics are often organic anions, a large number of transporter proteins from the solute carrier (SLC) and ATP-binding cassette (ABC) superfamilies regulate cellular influx and efflux. The majority of transporters appear to be bidirectional, whereas ABC transporters typically mediate efflux, whereas SLC transporters mediate influx (Roth et al.,2012). The SLCO and the SLC22A superfamilies, two of the 52 gene families that make up the SLC transporters, contain transporters that are involved in the transport of sulfated steroids. OATPs are members of the SLCO superfamily (Hagenbuch & Stieger, 2013), while the organic anion transporters (OATs) and the organic cation transporters (OCTs/OCTNs) are members of the SLC22A superfamily (Koepsell, 2013).

1.4.5.1 Classification of OATPs

Several human OATPs, including SLCO1B1, SLCO1B3, and SLCO1A2, are encoded by genes found on chromosome 12, although genes for other OATPs are dispersed throughout the rest of the genome. The 12 transmembrane domains of all OATP family members have intracellular amino and carboxy termini, and there is a large extracellular loop between the ninth and tenth transmembrane regions (Hagenbuch & Meier, 2004; Konig, 2011).

In 2004, the HUGO Gene Nomenclature Committee introduced and accepted an amino acid sequence-based classification and nomenclature system. Each newly identified OATP, according to this classification, has a unique name, whether it is a unique member

of the family or a known orthologue name. This classification depends on the percentage of identity: Proteins with more than 40% identity belong to the same family (denoted by a number), and proteins with more than 60% identity belong to the same subfamily (denoted by a letter) (Hagenbuch & Meier, 2004). In contrast to Oatp, which is utilized for nonhuman transporters, OATP is used for human transporters. (Hagenbuch & Meier, 2004). The related gene symbols are the same as the protein symbols in terms of family number, subfamily letter, and chronological number. They start with *SLCO* for human genes and *Slco* for rodent genes (e.g., *SLCO1B1* for OATP1B1, *Slco1a1* for mouse OATP1A1) (Hagenbuch & Stieger, 2013).

Based on similarities in sequence (amino acid identity) of the human and rodent, the OATP superfamily is classified into six subfamilies (OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6). There are currently 11 identified human OATPs, with the OATP1 family being the most characterized (Thakkar et al., 2015). Bile acids, conjugated steroids and thyroid hormones are just a few examples of the huge variety of structurally unrelated substances they can transport. They can also carry exogenous substrates, including immunosuppressants, anti-inflammatory, and anti-cancer medications (Rawłuszko-Wieczorek et al., 2015).

OATP1A2, OATP1B1, OATP1B3, and OATP1C1 are the four members that comprise the OATP1 family. OATP2A1 and OATP2B1 are two members of the OATP2 family, both of which exhibit relatively limited substrate specificity when compared to other OATPs. OATP4A1 and OATP4C1 are both members of the OATP4 family. OATP3A1, OATP5A1, and OATP6A1 are members of the OATP3, OATP5, and OATP6 families, respectively (Thakkar et al., 2015). OATP3A1 and OATP4A1 are two OATPs that have

wide expression and are involved in the uptake of E1-S in hormone-dependent breast cancer cells (Nozawa et al., 2004). In hormone-dependent cancers, steroid hormones and their conjugates are crucial for the growth of tumour cells (Nozawa et al., 2004; Nozawa et al., 2005; Hong & Chen, 2011). For example, a study by Maeda et al. (2010) showed that OATP1B3 efficiently transported E3S in breast cancer cells, but this transport was responsible for just 6% of the overall E3S transport.

Other species have been shown to have orthologs of human OATPs. For instance, mice have at least four known members of the Oatp1a family (Oatp1a1, Oatp1a4, Oatp1a5, and Oatp1a6) compared to humans, who only have one OATP1A family member (OATP1A2). Humans have two OATP1B transporters (OATP1B1 and OATP1B3). However, mice only have one rodent homologue of Oatp1b2 (Thakkar et al., 2015).

The *SLCO* superfamily's currently authorized human members. "The Organic Anion Transporter (OAT) Family" is where OATPs are classified in Milton Saier's transporter classification database (Saier et al., 1999). Currently, more than 300 members of the OATP superfamily have been detected and/or expected from more than 40 species, up from the 52 members of the OATP superfamily identified in 2004. With 27 members, the OATP1 family is the largest, followed by the OATP6 family (Hagenbuch & Stieger, 2013). OATPs' exact method of transport is not entirely known, and it may differ for each transporter depending on the substrate (Hagenbuch & Stieger, 2013). Closely related OATPs can have different substrate profiles, as is the case with OATP1B1 and OATP1B3, each with their specific substrates (Schulte & Ho, 2019).

gene symbol	**protein name**	**Predominant substrates**	**Tissue distribution/subcellular expression**
SLCO1A2	OATP1A2	Bile salts, organic anions and cations, E1S and DHEAS	Brain (endothelial cells), kidney (apical), intestine (apical), liver (cholangiocytes), eye (ciliary body)
SLCO1B1	OATP1B1	Bile salts, organic anions, E1S and DHEAS	Liver (hepatocytes)
SLCO1B3	OATP1B3	Bile salts, organic anions, E1S and DHEAS	Liver (hepatocytes)
SLCO1C1	OATP1C1	T4, T3, rT3, BSP and E1S	Brain (blood-brain barrier), testis (Leydig cells)
SLCO2A1	OATP2A1	Prostaglandins	Ubiquitous
SLCO2B1	OATP2B1	E-3-S, DHEAS, BSP, E1S and DHEAS	Liver (hepatocytes), placenta, intestine (apical), eye (ciliary body)

SLCO3A1	OATP3A1	E-3-S, prostaglandin and E1S	Testis, heart, brain, ovary
SLCO4A1	OATP4A1	aurocholate, T3, prostaglandin and E1S	Ubiquitous
SLCO4C1	OATP4C1	Digoxin, ouabain, thyroid hormones, methotrexate	Kidney (basolateral)
SLCO5A1	OATP5A1		
SLCO6A1	OATP6A1		Testis

Table 1.2: The human members of the superfamily of Organic anion transporting polypeptides (Obaidat et al., 2012; Hagenbuch & Stieger, 2013).

1.4.5.2 Tissue distribution

OATP expression has been studied at both the mRNA and protein levels. OATPs have generally been found in epithelial or endothelial cells in almost every organ, and they are commonly (but not usually) found in polarized cells' basolateral membrane (Konig, 2011). Some OATPs are thought to be organ-specific because of their restricted expression. In contrast, others are ubiquitously expressed (Table 1.2 lists all of the *SLCO* superfamily, predominant substrates and the tissue distribution). Some OATPs, including OATP1B1, OATP1B3, OATP1A2, and OATP2B1, are mostly expressed in organs crucial to drug disposition, such as the liver, kidney, and intestine, as well as in the blood-

brain barrier (Schulte & Ho, 2019), are examples of such a restricted expression. Another example is the rat brain specific OATP1C1 (Sugiyama et al., 2003). Human OATP2A1, OATP3A1, and OATP4A1 are examples of OATPs that are ubiquitously produced. Their mRNA was discovered in almost all tissues examined (Roth et al., 2012).

This thesis will focus on the OATPs that play a role in sulfated steroid uptake, as shown in Table 1.2, including the OATP1, OATP2, OATP3 and OATP4 families. Some OATPs are expressed throughout the body (e.g., OATP1A2, OATP2B1, OATP3A1, and OATP4A1), while others are limited to specific tissue locations, such as OATP1B1 and OATP1B3 (liver), and OATP1C1 (brain, testis) (Hagenbuch & Meier, 2004). The fourth member, OATP1C1, is known for its high affinity for thyroid hormones T3 and T4. It also transports conjugated steroid hormones (Pizzagalli et al., 2002; van der Deure et al., 2008).

1.4.5.2.1 OATP1

One of the best-characterized families is the OATP1 family, which contains three transporters, OATP1A2, OATP1B1, and OATP1B3, which transport a variety of typical OATP substrates, such as thyroid hormones, prostaglandins, bile acids, statins, antibiotics, anticancer drugs and steroid hormone conjugates (Hagenbuch & Gui, 2008).

OATP1A2 expression has been identified in a variety of healthy tissues and cell types, and high expression of OATP1A2 has been observed at physiological barriers, such as in the blood-brain barrier (Gao et al., 2000; Bronger et al., 2005; Kusuhara & Sugiyama, 2005), the brush border membrane of the distal nephron (Kullak-Ublick et al., 1995; Lee et al., 2005), the bile duct cells cholangiocytes, and enterocytes (Lee et al., 2005; Thakkar

et al., 2015), ciliary body epithelium (Gao et al., 2005) and testis and prostate (Tamai et al., 2000), and in the small intestine apical membranes. OATP1A2 expression in the intestine is generally not thought to be highly expressed in this tissue (Tamai et al., 2000; Glaeser et al., 2007; Meier et al., 2007; Gröer et al., 2013). Furthermore, OATP1A2 is thought to be crucial for the transport of neuroactive steroids across the blood-brain barrier (BBB). In fact, this transporter has been demonstrated to uptake sulfated steroids, including DHEA-S (Grube et al., 2018). In vitro transport activity towards SLCO1A2 substrate E1S is decreased in some single nucleotide polymorphisms (SNPs) of the *SLCO1A2* gene (Lee et al., 2005).

OATP1B1 and OATP1B3 are considered to be liver-specific transporters (Roth et al., 2012) that are almost exclusively located at the basolateral membrane of hepatocytes under normal situations, while OATP1B3 expression being greater around the central vein than around the portal vein (Tamai et al., 2000; Cui et al., 2003; Konig, 2011). A recent study that measured the protein expression of OATP1B1 and OATP1B3 indicated that both proteins were present in membrane fractions separated from human hepatocytes in an equal quantity (Ji et al., 2012). However, OATP1B3 mRNA expression was low in the kidney (Hilgendorf et al., 2007). There are inconsistent findings about expression in the intestine, with one study reporting OATP1B1 and OATP1B3 mRNA expression in the small intestine (Glaeser et al., 2007). In addition to another discovery, these transporters are not expressed in either protein or mRNA throughout the whole intestinal tract (Drozdzik et al., 2014).

The transport function of OATP1A2, OATP1B1, and OATP1B3 depends on electrogenic gradients, and the variation of external pH strongly affects these transporters (Kato et al.,

2010; Martinez-Becerra et al., 2011). It remains controversial whether OATP1B1 and OATP1B3-mediated transport is pH-dependent since Mahagita et al. (2007) reported that E1S transport was not affected by pH changes.

OATP1C1 is expressed in glial cells throughout the hypothalamus (Alkemade et al., 2011), at the blood-brain barrier (Roberts et al., 2008), in testicular Leydig cells (Pizzagalli et al., 2002), and in the ciliary epithelium (Gao et al., 2005). Brain and testis express the highest levels of OATP1C1, and due to its high affinity for reverse T3 and T4, it is the most abundant protein in these tissues (Friesema et al., 2006). The thyroid hormone transporter OATP1C1 may play an important role in the transport of these hormones to target tissues due to its high affinity for thyroid hormones (Buxhofer-Ausch et al., 2013).

1.4.5.2.2 OATP2

OATP2A1 and OATP2B1 are two members of the OATP2 family. Both exhibit very limited substrate specificity compared to other OATPs.

OATP2B1 is encoded by the *SLCO2B1* gene and was first cloned in 2000 (Tamai et al., 2000). OATP2B1 transports a wide range of substances, including thyroid hormones, steroid hormone conjugates, prostaglandins, and other medications (Thakkar et al., 2015). Also, OATP2B1 is a widely expressed uptake transporter that is highly expressed at the mRNA level in a variety of organs. OATP2B1 was found in the placenta (St-Pierre et al., 2002), small intestine (Kobayashi et al., 2003, keratinocytes (Schiffer et al., 2003), and hepatocytes (Kullak-Ublick et al., 2001), in human platelets (Niessen et al., 2009), in skeletal muscle (Knauer et al., 2010), in the mammary gland (Pizzagalli et al., 2003), at the blood-brain barrier (Bronger et al., 2005; Iusuf et al., 2012), in the ciliary body

epithelium (Gao et al., 2005), in the heart (Grube et al., 2006b), as well as in the ovary, testis, pancreas, lung, and spleen (Tamai et al., 2000).

In an acidic pH environment, OATP2B1 transports a wide range of endogenous products and drugs, whereas at a pH of 7.4, it transports mainly steroid hormones (Hagenbuch and Gui, 2008). OATP2B1 has a high affinity for steroid hormone conjugates: It enhances the uptake of E1S, DHEAS and pregnenolone sulfate (PS) in the presence of progesterone (Grube et al., 2006). Also, progesterone's stimulation of OATP2B1 function is largely specific to sulfated steroids, while it inhibits or does not affect drug substrates such as atorvastatin and glibenclamide (Koenen et al., 2012).

1.4.5.2.3 OATP3

The OATP3 family include OATP3A1.

OATP3A1 is highly expressed in non-cancerous tissues, where it transports a variety of hormones, prostaglandins, and medications (Obaidat et al., 2012); OATP3A1 is found in normal breast tissue at the plasma membrane of epithelial cells (Kindla et al., 2011), the testes, the choroid plexus, and the ciliary body epithelium (Gao et al., 2005). Two splice variants have been demonstrated to express in a cell type-specific manner in the brain and the testes (Huber et al., 2007).

1.4.5.2.4 OATP4

The OATP4 family includes OATP4A1 and OATP4C1.

OATP4A1 is widely expressed and participates in the transportation of a number of endogenous substrates, including prostaglandins, hormones, and bile acids (Thakkar et al., 2015). Many tissues, including the lung, heart, liver, skeletal muscle, placenta, pancreas, and kidney, express SLCO4A1 mRNA (Fujiwara et al., 2001; Tamai et al., 2000). OATP4A1 was found in syncytiotrophoblasts (Loubiere et al., 2010; Sato et al., 2003) and the ciliary body epithelium at the protein level (Gao et al., 2005).

1.4.5.3 Transport mechanisms of OATPs

The mechanism of OATP-mediated transport is still debated, but all agree that it is ATP- and sodium-independent (Roth et al., 2012; Mueller et al.,2015). Also, OATP expression is regulated by both transcriptional and post-transcriptional mechanisms (Hagenbuch & Stieger, 2013). Their expression is regulated by transcription factors, DNA-dependent gene silencing, and posttranscriptional modifications (Svoboda et al., 2011). This process may alter the expression of transporters in cancer and/or shift the transporter from the plasma membrane to the cytosolic compartment, changing the OATP functional properties (Obaidat et al., 2012).

OATPs are bidirectional transporters, and data indicates that they may function as electroneutral exchangers. Some OATPs, for example, exchange substrates for glutathione (Franco and Cendrowski, 2006), glutathione conjugates (Li et al., 2000) or intracellular bicarbonate (Leuthold et al., 2009). Though glutathione does not influence OATP1B1 and OATP1B3 uptake, transport mechanisms might differ between OATPs (Mahagita et al., 2007). Research indicates that both local pH conditions and cell membrane potential affect these two transporters in different ways (Martinez-Becerra et al., 2011).

E1S, DHEAS, and oestradiol -17β-glucuronide are just a few of the endogenous steroid hormones and hormone conjugates that are transported by the OATP1A2, OATP1B1, OATP1B3, and OATP2B1 (Kullak-Ublick et al., 1998, 2001; König et al., 2000; Miyagawa et al., 2009; Buxhofer-Ausch et al., 2013; Schulte & Ho, 2019).

1.5 Oestrogen Synthesis, Metabolism and Transport in CRC

1.5.1 Evidence from epidemiological studies

There are complex effects of oestrogen on GI physiology with varying degrees of influence on the bowel. In vivo and in vitro studies on animals have shown that gonadal steroids influence smooth muscle contractility in the GI (Fisher et al., 1978; Bruce & Beshudi, 1981). Also, there is evidence that sex hormone receptors are present along the GI tract, which could explain changes in GI function during menstruation (Wald et al., 1981; Simmons et al., 1988). Furthermore, according to Hoff and Chang (1979), ovariectomy is associated with colonic crypt hypoplasia.

Numerous studies have shown the role of sex hormones in the development and risk of many types of cancer, including breast cancer and CRC, since Lacassagne demonstrated in 1955 that oestrogen administration increases mammary cancer incidence in mice. It has also been found that hormones play a significant role in CRC. Among nuns, Fraumeni et al. in 1969 noted that there was an elevated mortality rate for this disease, as well as other hormonally related cancers, such as ovarian and breast cancers. Compared to the majority of the female population, nuns are nullipara, meaning they experience no

disruption of their menstrual cycles throughout life, leading to a longer lifetime exposure to oestrogen.

Numerous studies have also examined the effect of hormone replacement therapy (HRT) on cancer incidence. The Nurse Health Study examined > 100,000 healthy women to determine the relationship between HRT use and new breast cancers. Colditz et al. (1995) found that both forms of HRT increased the risk of breast cancer, with the relative risk of breast cancer being 1.3 for those using oestrogen alone and 1.4 for those using oestrogen and progestogens together. In 1991, the Women's Health Initiative (WHI) was launched and consisted of clinical trials and observational studies. Between 1993 and 1998, 161,808 generally healthy postmenopausal women participated in clinical trials involving hormone therapy, diet modification, calcium/vitamin D supplementation, and observational studies in the United States (Stefanick et al., 2003; Hays et al., 2003). WHI findings indicate that combining HRT and oral contraceptive pills (OCP) may prevent CRC by 40% and 20%, respectively, suggesting that oestrogen and progestin may be protective. HRT, however, was associated with higher tumour grades in women diagnosed with CRC (Fernandez et al., 2001; Rossouw et al., 2002). Chlebowski et al. found that CRCs occurring in patients taking HRT tend to be more advanced and have more positive lymph node involvement. Thus, while oestrogens may protect against developing CRC, they may also promote tumour cell proliferation once cancer develops.

Further data from the WHI suggests that postmenopausal women's endogenous oestrogen concentrations increase their risk of CRC even after adjusting for known risk factors like

insulin concentrations and waist size (Gunter et al., 2008). In contrast, this study suggests a borderline positive association between circulating E1 and CRC, particularly in the proximal part of the colon. Clendenen et al. (2009) also showed a 60% higher risk of CRC in women with the highest level of circulating oestrogen concentrations than for those in the lowest concentrations, for women who are overweight or obese, but not for those who are normal weight. While this heterogeneity was not statistically significant, prior studies found limited evidence that sex hormones and CRC are associated differently by body weight. In a previous study, Murphy et al. found an inverse association between circulating E1 and E2 and CRC risk. In a meta-analysis of endogenous circulating sex hormone concentrations in postmenopausal women (including E1, E2 DHEA, progesterone, and sex hormone-binding globulin), no relationship was found between CRC and circulating sex hormones (Mori et al., 2021).

According to Alford et al., one-third of large bowel cancers are endocrine-dependent. Ten of 33 (30%) tumours expressed high-affinity E2 receptors. The E2 was compared between 51 Indian men with CRC and 50 age-matched controls. They found that serum E2 levels were higher in patients with CRC. The use of immunoassays to determine E2 levels in serum is a major limitation of this study (Basu et al., 2015). In addition, due to the study design, a causal link between serum E2 and CRC development could not be demonstrated. Both factors may be confounded. CRC patients are more likely to be obese (Renehan et al., 2008) and to have elevated serum E2 (Schneider et al., 1979).

In premenopausal women, oestrogens are primarily produced by the ovaries. Women and men can also produce oestrogens locally in peripheral tissues, especially adipose tissues, by aromatizing androgens (Mattsson and Olsson, 2007). The incidence of CRC is lower in females than in males, suggesting that ovarian steroids may contribute (Abd ElLateef et al., 2020). According to studies conducted on nuns in 1969, high levels of endogenous oestrogens may lead to a greater incidence of CRC over a lifetime. In contrast, with exogenous HRT, the colon may be protected from a short, concentrated oestrogenic dose (Foster, 2013). This perhaps suggests that the gut responds differently to endogenous and exogenous oestrogen in women.

1.5.2 Role of oestrogen metabolising enzymes, oestrogen receptors and oestrogen transporters on CRC

Oestrogens are known to play a role in regulating cell proliferation and apoptosis in cancer cells of hormone-sensitive tumours in the endometrium, ovary (Ness, 2003), breast (Travis & Key, 2003), and other hormone-sensitive tissues, such as the colon (Secky et al.,2013). However, the research on the link between CRC and oestrogen remains controversial. In CRC with dysregulated oestrogen metabolism, E2 synthesis is favoured, but the molecular mechanisms are unknown (Gilligan et al., 2017). In oestrogen-dependent tissues, oestrogen is produced peripherally from inactive steroids via the aromatase or sulfatase pathways (Foster, 2013). Oestrogens are readily generated from androstenedione (Adione) via peripheral conversion to E1, a reaction controlled by the aromatase enzyme complex. Then, by the action of oestrogen sulfotransferase (SULTs) and phenol sulphotransferase, a large number of these hydrophobic oestrogens can be converted into hydrophilic oestrogen sulphates (Foster, 2013). STS, which hydrolyses

E1S *in situ*, has been proposed as a source of postmenopausal tumour oestrogen (Reed et al. 2005). E1S has a long half-life in plasma and is formed via sulfotransferases at higher levels than E1 (Ruder et al., 1972; Noel et al., 1981; Reed et al., 2005). As a result, circulating E1S could act as an E1 reservoir that enters tumour cells through organic anion transporters (Mueller et al., 2015). By STS, E1S is converted to E1, which can then be converted to E2, which has a high affinity for ERs by HSD17Bs. In tumour cells, STS and HSD17Bs levels are higher than in normal tissues, indicating that this may be the main route in which oestrogen is produced (Labrie, 2015; McNamara & Sasano,2015). Through the binding and activation of their nuclear ER, ERα and ERβ mediate the genomic effects of oestrogens and GPER for non-genomic effects (Revankar et al., 2005; Kirker et al., 2013).

The enzymes HSD17Bs, SULT1E1, and STS, which are expressed in the colon, are required for oestrogen metabolism. The enzymes STS and SULT1E1, which mediate E_1 sulfation, have been found in both normal and cancer human colon tissue (Foster, 2013; Munroe & Change, 1987). Furthermore, any alterations in these enzymes' and receptors' peripheral ratios are expected to influence CRC progression (Foster, 2013).

1.5.2.1 Importance of STS in CRC

Several pathologies have been associated with dysregulation of sulfation and desulfation processes (Reed et al., 2005; Mueller et al., 2015). Sulfatase and aromatase pathways produce oestrogens within oestrogen-dependent tumours, such as breast carcinoma, from circulating inactive steroids (Suzuki et al.,2007). Very little is known about STS

expression and activity in CRC. However, in other cancers, STS is known to be directly involved in oestrogen desulfation and its availability. For example, the mRNA expression of STS in breast cancer has been studied by several groups, according to Utsumi et al. STS mRNA levels are significantly higher in breast cancer tissue than in non-malignant tissue. Miyoshi et al. examined 181 tumour samples over 34 months. As a result, a significant increase in STS mRNA expression has been demonstrated in malignant breast tissue (Utsumi et al. 1999, Miyoshi et al. 2003). STS activity in breast carcinoma is 50 to 200 times higher than aromatase activity (Pasqualini et al., 1996), and STS and SULT1E1 expression are both prognostic factors (Suzuki et al., 2003), whereas aromatase is not (Silva et al., 1989).

Several types of tumours have expressed STS and SULT1E1 intratumorally. In human endometrial carcinomas, increased STS and decreased oestrogen sulfotransferase expression were observed (Utsunomiya et al., 2004). Another study conducted by Ishibashi et al. (2005) showed that E2 concentrations inversely correlate with SULT1E1 and positively correlate with STSs and HSD17B1, with the presence of STS activity also detected in 97% of ovarian cancer specimens (Chura et al., 2009). These studies demonstrate that patients with low STS activity had significantly longer progression-free survival times than those with high STS activity.

With regards to CRC, Sato et al. (2009) were the first to implicate STS and SULT1E1 protein expression as prognostic indicators in this malignancy, with high STS and low SULT1E1 expression indicating poor survival. STS and SULT1E1 are present in normal

(Munroe & Chang,1987; Her et al., 1996) and carcinoma tissue (Sato et al., 2009) and may play metabolizing roles in colonic disorders. There was a higher concentration of oestrogen in CRC tumours compared to normal colonic mucosa in CRC tumours. The STS promotes the synthesis of E1, while the oestrogen sulfotransferase inactivates E1. Patients with STS negative and oestrogen sulfotransferase positive had significantly longer survival, while those with positive STS but negative SULT1E1 were associated with worse clinical outcomes (Sato et al., 2009). In the colon cancer studied samples, STS and SULT1E1 expression was found in 60% and 40% of the samples, respectively. However, only SULT1E1 expression was found in non-neoplastic colonic tissues (Sato et al., 2009; Foster, 2021). STS activity in human CRC was significantly elevated, and STS overexpression accelerated the growth of CRC both in vitro and in vivo. Inhibiting STS with Irosustat can effectively block this (Gilligan et al., 2017).

1.5.2.2 Role of HSD17B enzymes in CRC

Since the early 1980s, a previous epidemiological investigation has provided useful information on the role of E_2 in CRC risk (Singh & Langman, 1995). Measurements in human tissue biopsies showed that the usual colonic mucosa has a high capacity for E_2 metabolism, and E1 inhibits colon proliferative activity but not E2 (English et al., 1999). The HSD17Bs have previously been examined in colon carcinomas, and HSD17B2 and HSD17B4 are the most prevalent, metabolizing E2 to E1 (English et al., 1999; English et al., 2001). Consequently, colon cancers were suggested to have an increased E2 to E1 ratio, although definitive metabolism experiments were not performed.

In the colon, *HSD17B1* gene expression may play an important role in producing E2 and modulating endogenous E2 in preventing cancer. The decrease in HSD17B1 protein and transcript levels in CRC is consistent with this hypothesis (Rawłuszko et al., 2011; Abancens et al., 2020). Healthy colons express HSD17B2 mainly in the epithelium of the colon lumen and, to some extent, in the epithelium of the crypt (English et al., 1999). HSD17B1 and ERα are weakly or not expressed, whereas HSD17B2 and ERβ are widely expressed at relatively high levels (Foster, 2013). The expression of type 2 and 4 isozymes of HSD17B (HSD17B 2 and HSD17B4) appears to be linked to the presence of HSD17B activity in the colon, with mRNA expression for the latter being substantially lower in tumours compared to normal mucosae (English et al., 2000). HSD17B2 is strongly expressed in the healthy colon's small intestine and surface epithelium, but its expression was reduced in CRC tissues and cell lines (English et al.,1999; Oduwole et al.,2003; Gilligan et al., 2017). Among female patients with low expression levels of HSD17B2, the 5-year overall survival rate was 54%, while none of the female patients with overexpression of HSD17B2 survived. A higher HSD17B2 mRNA level is an independent indicator of a poor prognosis in female patients with CRC. However, there was no association between HSD17B2 and prognosis in male CRC patients (Oduwole et al.,2003). Furthermore, in some cancer types, HSD17Bs already have prognostic value. In addition, a decrease in HSD17B2 and HSD17B4 expression is linked to the development of CRC (Vihko et al., 2002). A high expression of HSD17B1, HSD17B2, and HSD17B4 is associated with poor prognoses in prostate and breast tumours, respectively (Rasiah et al., 2009; Oduwole et al.,2004; Gunnarsson et al., 2008).

Both HSD17B7 and HSD17B12, which convert E1 to E2, are expressed in CRC. HSD17B7 is expressed more highly in CRC than in healthy colon tissue, and this is the same for HSD17B12 (Gilligan et al., 2017). Also, HSD17B7 was found to be highly expressed in human ductal carcinoma and breast cancer cells, providing evidence that E2 may upregulate this enzyme (Shehu et al., 2011). Furthermore, studying 41 breast cancer tissues, it was found that HSD17B12 was expressed in 83% of them, and its expression was higher in malignant tissue (Song et al., 2006).

1.5.2.3 ERα, ERβ and GPER in CRC

Cancer development and metastasis, extracellular matrix remodelling, and treatment resistance are all strongly correlated with ER activation (Liu et al., 2020). ERα and β were first discovered in normal colon epithelium and then in certain CRC cells in the 1990s (Singh & Langman, 1995; Enmark et al., 1997). While the colonic epithelium and CRC do not express the ERα protein, ERβ has been shown in numerous studies to be the most common form of ER (Foley et al.,2000; Konstantinopoulos et al., 2003; Xie et al., 2004; Castiglione et al., 2008). With the development of CRC, ERβ expression is progressively less dominant in the colon mucosa (Caiazza et al., 2015). Additionally, ERβ is reduced in CRC, which is associated with a poorer prognosis (Foster, 2013). Work by Stevanato Filho et al. (2018) showed that patients with CRC with elevated ERβ. Expression has a better prognosis. Also, ERβ has gained popularity as a potential target for the therapy of many cancers, including CRC, in recent years (Williams et al., 2016). Since ERα is expressed at a very low level of expression in healthy colonic mucosa, little is known about how it may contribute to CRC. Furthermore, patients with CRC who have elevated ERα expression also have significantly worse survival rates (Liang et al., 2018).

In general, ERα and ERβ play diverse roles in carcinogenesis and tumour growth, with ERα acting as an oncogene and ERβ acting as a tumour suppressor (Peng et al., 2003). ERα is widely expressed in cells and plays a crucial part in both hormone-dependent and hormone-independent malignancies. ERα expression plays a major role in the advancement of hormone-related cancers like breast, ovarian, and endometrial cancers through controlling the PI3K/AKT signaling pathway (Liu et al., 2020). It is believed that oestrogen-induced growth-stimulating effects in breast cancer are predominantly caused by the ERα isoform (McDonnell & Norris, 2002). ERα plays a critical role in the development of hormone-dependent breast cancer, mostly through activating transcriptional programmes that promote cell proliferation, resistance to cell death, or angiogenesis in response to E2 stimulation (Arnal et al., 2017). Breast tumours can be classified as ER-positive or ER-negative based on their ER status (Allred et al., 2004). ER+ breast cancer, which makes up roughly 70% of all breast cancers, depends on ERα for its development (Nicolini et al., 2018). Also, the prognosis may be improved with ERα positivity, and ERα-negative tumours are more aggressive and metastatic (Dunnwald et al., 2007; Louie and Sevigny, 2017). Also, the progression of prostate cancer depends on ERα (Royuela et al., 2001). Ovarian cancer is a hormone-sensitive cancer, and according to previous studies, oestrogen receptors (Erα and Erβ) expression was detected in 60–100% of ovarian cancer cases (Lindgren et al., 2004; De Stefano et al., 2011). ERβ mRNA expression is reduced in cancerous tissues, such as breast, prostate, and ovarian tissues. This suggests that ERβ expression loss may contribute to carcinogenesis (Iwao et al., 2000; Horvath et al., 2001; Skliris et al., 2003; Chan et al., 2008).

Since patients and cell lines studied for CRC lack ERα or ERβ, oestrogen effects in advanced CRC tumours may be transmitted via GPER. In colonic epithelia, the GPER receptor (also known as GPER1 or GPR30) is the dominant ER once the tumour develops (Gilligan et al., 2017). The GPER1 signaling pathway induces a wide variety of responses in different tissue types that can either promote or suppress tumour growth and progression. Based on the stage of the disease and ER and GPER expression levels, the expression of GPER may act as a tumour suppressor or promoter in CRC (Jung, 2019). It has been demonstrated in a study by Liu et al. (2017) that activation of GPER by its specific agonist, G-1, inhibits cell growth and promotes apoptosis in CRC cells (HCT116 and SW480).

There is a significant association between high GPER1 expression and poor relapse-free survival in women with stage 3 or stage 4 CRC, suggesting oestrogen-dependent signaling may play a role in CRC progression and survival (Bustos et al., 2017). Also, it has been found by Gilligan et al. (2017) that oestrogen increases the proliferation of CRC cells expressing GPER but not ER through a positive feedback loop in which GPER stimulates the expression of STS, which in turn activates conjugated oestrogen, accelerating the progression of cancer.

Although GPER1 has been implicated in the suppression of ovarian cancer (Ignatov et al., 2013), also a selective agonist of GPER-1, G-1, or 17β-oestradiol has been shown to stimulate GPER1, which is linked to the proliferative responses in cancer cells derived from ovary, testis, breast, and endometrium, as well as thyroid (Filardo et al., 2012). In

addition to endometrial cancer, GPER1 is expressed in thyroid, ovarian, testicular, prostate, and pancreatic cancers (Pepermans et al., 2021).

1.5.2.4 OATPS and cancer

As this thesis focuses on oestrogens and CRC, this section will specifically look at the OATPs involved in sulfated oestrogen uptake and their potential impact on cancer. In order to investigate CRC incidence and treatment, OATPs are important as steroid hormone precursors are uptaken into cancer cells by OATPs; they may also contribute to tumour growth controlled by hormones. These precursors are converted to active oestrogens and androgens by steroid sulfatase and 17-β-dehydrogenase (Buxhofer-Ausch et al., 2013; Rawłuszko-Wieczorek et al., 2015). OATPs are required for the transport of sulfated steroids into cells (Roth et al.,2012). Six different OATPs (OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1, OATP4A1) transport E1S effectively (Mueller et al., 2015). OATP4C1 and OATP1C1 are also known to transport E1S (Mikkaichi et al., 2004). These sulfated-hormone transporters are not well expressed in many normal endocrine tissues, but their expression increases in hormone-dependent malignancies (Mueller et al., 2015). In addition to their expression in different malignancies and tumours, OATPs also play a significant role in cancer development by enhancing the uptake of E1S and DHEAS into cancer tissues (Obaidat et al., 2012; Thakkar et al.), 2015, as well as anticancer drug disposition (Thakkar et al., 2015). The differential expression of OATPs between cancerous and normal tissues has been investigated as a potential therapeutic approach (Okabe et al., 2008). It may be possible to identify OATPs overexpressed in cancerous tissues as diagnostic markers and therapeutic targets because malignant tissues with increased OATPs transporting chemotherapeutic substrates could be more susceptible to the cytotoxic effects of these

agents (Buxhofer-Ausch et al.,2013). In hormone-dependent tumours, differentiation levels have been associated with the expression of OATPs, which may serve as a disease stage marker (Pressler et al., 2011).

1.5.2.4.1 Breast cancer

Breast cancer cells have higher levels of steroid transporter OATPs (i.e., OATP1A2, OATP2B1, OATP1B3, OATP4A1, OATP4C1) compared to normal tissues (Pizzagalli et al., 2003; Al Sarakbi et al., 2006; Bleasby et al., 2006; Meyer zu Schwabedissen et al., 2008; Wlcek et al., 2008; Kindla et al., 2011; Buxhofer-Ausch et al., 2013). Miki et al. were the first to report that OATP1A2 mRNA and protein were expressed in breast cancer in contrast to non-cancerous breast tissues and later in other cancers such as colon cancer and prostate cancer. According to Miki et al. (2006) and Meyer zu Schwabedissen et al. (2008), breast cancer tissues express 10-fold more OATP1A2 than surrounding normal tissues. The presence of OATP2B1 has been detected in the luminal epithelium of invasive ductal carcinoma tissues, while it is confined primarily to the myoepithelium of normal breast tissues (Pizzagalli et al., 2003). A study by Maeda et al. demonstrated that two subclones of MCF-7 breast cancer cells with different oestrone-3-sulfate (E3S) uptake activities were analysed for expression of OATP family members, suggesting that among several candidate transporters, OATP1B3 may be responsible for E3S uptake into these cells. Recent research indicates that hormone-dependent cell lines of breast cancer have higher levels of expression of OATP1B1 than normal epithelial cells (Mueller et al., 2015). Also, Wleck et al. demonstrated that breast cancer tissues and cell lines have higher OATP4C1 mRNA levels.

1.5.2.4.2 Other types of cancers

In **prostate** cancer**,** the OATP1B1, OATP1B3 and OATB2B1, compared to healthy prostates, show higher levels of expression in prostate cancer specimens (Hamada et al., 2008; Yang et al., 2011; Buxhofer-Ausch et al., 2013; Lancaster et al., 2013). The upregulation of some OATPs is also thought to promote prostate cancer cell survival and growth (Cho et al., 2014). Also, in **liver** cancer**,** the expression of OATP1B1 and OATP1B3 is mainly found in normal liver tissues, though their levels are often downregulated in hepatic tumours (Buxhofer-Ausch et al., 2013). In addition, Wleck et al. (2011) found elevated OATP3A1 levels in both primary and metastatic liver tumours. Additionally, **pancreatic** cancer samples have higher levels of OATP1B3 expression than normal pancreas (Buxhofer-Ausch et al., 2013; Lancaster et al., 2013). Also, Kounnis et al. (2011) and Kounnis et al. (2015) reported high levels of OATP1A2 and OATP1B1 expression in pancreatic cancers. It has been demonstrated by Pressler et al. that OATP2B1 mRNA expression is lower in liver tumours and pancreatic tumours more than in nonmalignant tissues (Pressler et al., 2011). Moreover, OATP2A1 expression is higher in cholangiocarcinoma and liver metastases from colon cancer. On the other hand, decreased OATP2A1 levels have been observed in cancers of the kidneys, stomach, lung and ovary. Ovarian cancer also has been linked to OATP1B1 so far (Thakkar et al., 2015).

1.5.2.4.3 OATPs in Colorectal Cancer

The solute carriers for organic anions (*SLCO*) gene superfamily includes many organic anion-transporting polypeptides (OATPs), expressed in the small intestine and colon and found in many tissues in the body, may contribute to the resistance to pharmacological treatment as they take up a variety of medications used to treat CRC (Marin et al., 2020). In healthy colon tissue, **OATP1A2**

expression is detected (Tamai et al., 2000); compared with surrounding healthy tissues, Ballestero et al. found that CRC tumours showed a decreased mRNA expression of OATP1A2. A reduction in the expression of **OATP2A1** in CRC is associated with a greater concentration of prostaglandin E2, which works to promote the proliferation of cells, prevent apoptosis, and promote neovascularization. The extracellular concentrations of PGE2 in CRC may interact with surface G protein-coupled receptors to cause different signaling cascades (Holla et al., 2008). **OATP2B1** is expressed in several solid tumours. These include colon cancer cell lines (CX-1 and Caco-2) (Tamai et al., 2000; Hayeshi et al., 2008). The expression of OATP2B1 in neoplastic colon specimens is also higher than in normal colon specimens (Kleberg et al., 2012).

Normal colon tissue lacks **OATP1B1** and **OATP1B3** mRNA, but this gene is significantly expressed in gastrointestinal tract cancers, including colon cancer (Ballestero et al., 2006; Buxhofer-Ausch et al., 2013; Thakkar et al., 2015). Ballestero et al. found that CRC showed significantly increased mRNA expression of **OATP1B1**, and it is linked to a higher tumour grade (Pressler et al., 2011). There have been several studies showing that **OATP1B3** is expressed in CRCs, which is distinct from its expression pattern in normal colon (Lee et al., 2008; Evangeli et al., 2017). A study by Evangeli et al. showed that in 30 CRC samples examined, 27 of them showed OATP1B3 antibody binding, while three others showed a mutant form of OATP1B3, which prevented antibody binding during immunohistochemical staining. Also, according to Nagai et al., the lung, colon, and pancreatic cancer cells and tissues express alternative OATP1B3 transcripts, and the cancer-specific isoform of OATP1B3 was detected in four of the seven colon cancer specimens examined, which differed considerably from that in healthy tissue (Nagai et al., 2012). The overexpression of OATP1B3 mRNA in a CRC cell line (HCT-116$^{p53+/+)}$ was linked to decreased drug-induced apoptosis and decreased p53

transcriptional activity. These anticancer agents become resistant by modulating the function of p53, a cancer suppressor gene. By altering p53-dependent pathways, HCT116 cells that induced the expression OATP1B3 showed reduced SN38-induced apoptosis, yielding a survival advantage during chemotherapy (Lee et al., 2008). A small subset of CRC patients and HCT116 and Caco-2 cells were found to have hypermethylated DNA in the **OATP3A1** promoter region. At the same time, a hypomethylation of **OATP4A1** was detected in CRC tissues and cell lines, resulting in a significant decrease in the mRNA expression of OATP3A1 and an increase in the expression of OATP4A1 (Rawuszko-Wieczorek et al., 2015). Furthermore, a study conducted by Ancona et al. (2006) observed significant upregulation of OATP4A1 in cancerous samples from colon cancer patients, with the expression level of OATP4A1 2.44-fold ±0.39 in cancerous samples and 0.46-fold ±0.72 in non-cancerous samples. OATP4A1 may be a useful marker for the poor prognosis of CRC. Furthermore, it promotes cell proliferation, migration, invasion, and carcinogenesis in CRC cells (Ban et al., 2017). Among CRC cell lines, OATP4A1 is the most abundantly expressed, followed by OATP2B1 (Gilligan et al., 2017). These data suggest that OATP overexpression and sulfated steroids they take up may also play a role in CRC proliferation.

1.6 Cancer and inflammation:

Over the last century, inflammation has been considered a positive prognosis and a defensive mechanism against illness. Since the last decade, it has gradually become clear that chronic inflammation contributes to carcinogenesis, and an inflammatory environment facilitates the progression, metastasis and progression of cancer (Grivennikov et al.,2010; Kuraishy et al., 2011). Indeed, chronic inflammation appears to

be linked to 20% of all malignancies (Perwez & Harris, 2007). Also, studies have shown that cytokines secreted by infiltrated lymphocytes promote tumour growth (Grivennikov et al.,2011). As Rudolf Virchow observed in 1863, leukocytes within tumours suggest a possible link between inflammation and cancer (Balkwill & Mantovani, 2001). The adaptive immune response's involvement in regulating the growth and frequency of human tumours has been controversial (Landskron et al.,2014). Carcinogenesis is the outcome of a complex interaction between cell-intrinsic and cell-extrinsic processes that encourage persistent proliferation, resistance to apoptosis, reprogramming and remodeling of the stromal environment, and genetic instability (West et al.,2015). Moreover, the inflammatory response and the carcinogenic process share a number of molecular targets and signaling pathways (Landskron et al.,2014). Furthermore, inflammation can increase the risk of cancer by supplying molecules such as cytokines, chemokines, proangiogenic factors, cell survival signals to avoid apoptosis, and extracellular matrix modifying enzymes from cells infiltrating the tumour microenvironment (TME) (Hanahan & Weinberg, 2011), and most solid tumours contain inflammatory infiltrates (Grivennikov & Karin, 2011). Essential elements of the TME include adaptive immune cells (T and B lymphocytes) as well as innate immune cells (macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) are present along with cancer cells and their surrounding stroma (cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, and mesenchymal cells) (de Visser et al., 2006; Grivennikov et al.,2010), which together determine the ultimate phenotypic of cancer cells, as well as recurrence, metastasis, and the treatment resistance entity of cancer (Zafari et al., 2022).

In normal physiology, cytokines are released by immune system cells, mostly activated T cells and tumour-associated macrophages (TAMs), as well as stromal cells, including endothelial cells and fibroblasts, and they regulate proliferation, differentiation, immune cell activation, cell migration, cell survival, and cell death (Zamarron & Chen,2011; Mager et al.,2016). The TAMs make up up to 50% of the tumour mass (Pathria et al., 2019). In addition, TAMs can promote tumour growth by stimulating angiogenesis, remodeling the extracellular matrix, as well as invasion and dissemination of tumour cells (Weissleder and Pittet, 2008; Qian and Pollard, 2010**).** Approximately 80% of human tumours containing TAMs have a bad prognosis (Bingle et al., 2002). Tumour cells can express antigens and become targets of an adaptive immune response mediated by T lymphocytes (Boon et al.,1994). Cytokines regulate the immunological and inflammatory environment to favour anti-tumour immunity (IFNγ, IL-12, TRAIL) or promote tumour growth (IL-23, IL-6, IL-17). TNFα, FasL, EGFR ligands, TGF-b, IL-6, and TRAIL all have direct effects on cancer cell proliferation and survival (Grivennikov et al., 2010). The expression of these genes is primarily regulated by the transcription factor nuclear factor kappa B (NF-κB), which is constitutively active in most cancers and is stimulated by carcinogens, tumour promoters, carcinogenic viral proteins, chemotherapeutic drugs, and γ-irradiation (Aggarwal et al.,2006). One of the most significant molecules linking chronic inflammation to cancer is NF-κB, a transcription factor required for inflammatory responses whose process is closely controlled by a number of mechanisms and bacterial endotoxins such as lipopolysaccharide. Pro-inflammatory cytokines like TNFα are also a key driver of NF-κB activation (Taniguchi and Karin, 2018).

1.6.1 Inflammatory involvement in CRC:

Chronic inflammation is a key hallmark of many cancers, including CRC (Kundu & Surh, 2008; Coussens et al., 2013; Elinav et al., 2013; Zhao et al.,2021). An increased risk of tumours is associated with chronic inflammation caused by infections, abnormal immune responses, or environmental factors, such as smoking, inhaled pollutants, or certain dietary factors (Grivennikov et al.,2010). Obesity and IBD are two inflammatory conditions that increase the risk of CRC, suggesting that chronic inflammation may initiate and promote CRC development (Waldner & Neurath,2009; Flores et al., 2015). IBD and CRC share many signaling pathways, including those involved in cell survival, proliferation, angiogenesis, and inflammation (Salla et al., 2023). Furthermore, oestrogen and its receptors play a role in the onset, progression, and severity of inflammatory bowel illnesses (IBDs) and CRC (Secky et al.,2013; Garcia-Villatoro and Allred, 2021; Choi and Kim, 2022).

IBDs, or inflammatory bowel disorders, are complicated polygenetic diseases that are defined by an excessive or unneeded inflammatory immune response to the microbial flora that lives in the gut lumen. IBD is characterized by chronic inflammatory responses and corresponding tissue loss caused by inappropriate immune cell activation from the innate and/or adaptive immune systems (McGuckin et al., 2009). Crohn's disease (CD) and ulcerative colitis (UC) are two common inflammatory bowel disorders (IBDs) that affect both the upper and lower gastrointestinal tract. An analysis of 5529 patients with IBD performed by a Canadian-matched cohort study found that the incidence rate ratio (IRR) for colon cancer was similar for patients with UC and CD with (IRR 2.75 and IRR

2.64, respectively). In addition, patients with UC had high IRR (IRR 1.90) (Bernstein et al., 2001). Also, IBD symptoms, illness severity, and extraintestinal manifestations are all significantly different across the genders (Choi and Kim, 2022). Sex hormones, such as oestrogen, progesterone, and androgen, are involved in the aetiology of sex differences in IBD (Xu et al., 2022). A higher risk of CRC exists in those with IBD. A major prognostic factor for individuals with IBD is CRC, which accounts for 10–20% of all mortality in those with IBD (Jewel Samadder et al., 2017). CRC is thought to be the result of a process that starts with no dysplasia and progresses to indeterminate dysplasia, low-grade dysplasia, high-grade dysplasia, and lastly, aggressive adenocarcinoma. However, CRC can develop without going through each of these steps (Triantafillidis et al., 2009). Additionally, the presence of obesity also directly contributes to CRC cancer development, independent of hormonal influences. Obesity, for example, promotes intestinal progenitor and stem cell proliferation by activating macrophages and dendritic cells (Kizil et al., 2015). As a result, a series of cytokines are released, including IL-22, IL-6, IL-17, and TNFα (Pourvali and Monji, 2021). Inflammation has been linked to CRC formation in recent years. The pathways leading to the initiation, growth, and metastasis of CRC involve multiple cytokines, immune cells, and immune mediators. The role of inflammation in pathogenesis has been further studied outside of previously explored mechanisms and pathways. Several traditional CRC risk factors, such as obesity, smoking, and diabetes, as well as some more novel ones, such as gut microbiome, gut mycobiome, and exosomes, have also been associated with inflammation (Khalyfa et al., 2021). Finally, numerous studies have shown that long-term use of steroidal anti-inflammatory drugs (NSAIDs), including aspirin, reduces cancer risk, the incidence and mortality, and improves survival (Chan et al.,2005; Yan et al.,2006; Bibbins-Domingo, 2016; Hua et al., 2017; Figueiredo et al., 2021).

1.6.2 Cytokines and their role in CRC.

Besides cancer cells, CRCs consist of non-malignant cells such as tumour-infiltrating lymphocytes and cancer-associated fibroblasts (CAFs). Infiltrates of cytokine-producing immune-inflammatory cells are common in CRC (De Simone et al.,2015), and CRC prognosis and pathogenesis are heavily influenced by cytokine networks (Mager et al.,2016). An imbalance between pro- and anti-inflammatory cytokines may contribute to CRC development (Borowczak et al., 2022). Depending on the tumour microenvironment, cytokines can modulate an antitumoural response, as well as induce cell transformation and malignancy, depending on the balance of pro- and anti-inflammatory cytokines, their relative concentrations, the activation state of surrounding cells, and the expression content of cytokine receptors (See Figure 1.6) (Zamarron & Chen, 2011). CRC progression and invasion are driven by infiltrating T cells and interferon-γ (IFN-γ) (Galon et al., 2006). Also, TNFα, IL-1β, and IL-6, which are produced by gut immune cells in response to localized inflammation, stimulate complex crosstalk amongst gut microenvironment cells, including paneth cells, goblet cells, macrophages, and enterocytes which promote chronic inflammation in IBDs. It promotes metastasis throughout the development of CRC, immunosuppression, and drug resistance (Bhat et al., 2022).

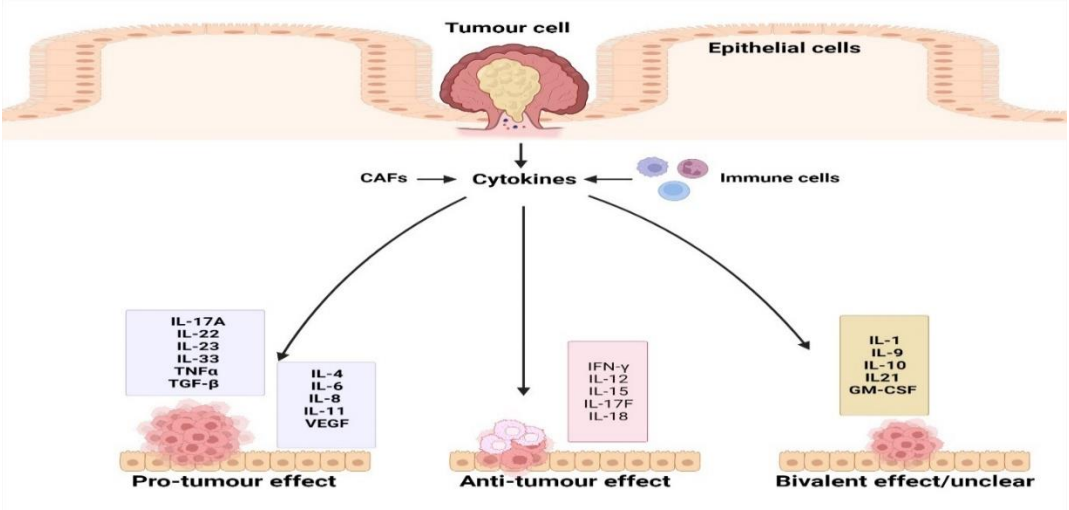

Figure 1.6: Cytokines participate in the pathogenesis of CRC. Cytokines can modulate an antitumoural (inhibit CRC development), pro-tumour, or bivalent response. (Created with BioRender.com).

Many pro-inflammatory cytokines have been implicated in the development and progression of CRC. For example, Langowski et al. (2006) discovered elevated expression of IL-23 in numerous kinds of human cancer, including CRC, suggesting that it plays a significant role in inflammation-associated tumour growth. Interleukin-17, a proinflammatory cytokine, plays an important role in CRC; both IL-17A and IL-17RA levels are elevated in CRC, which is linked to increased IL-17-driven inflammatory responses, such as an increase in VEGF and VEGFR expression, and all of these responses are involved in many cancer hallmarks.

In the design and development of medications for cancer prevention and treatment in CRC, IL-17 and its signaling pathways appear to be attractive novel targets (Xie, 2015). Patients with CRC had noticeably higher plasma levels of three cytokines/chemokines than those with adenomatous polyps, including IL-8, interferon gamma-induced protein

10 (IP-10), and TNFα. It may be possible to establish these proteins and cytokines as novel biomarkers for CRC or colon cancer progression (Choi et al., 2013). Thus, CRC progression is most likely influenced by local cytokine concentrations. This thesis will examine the role TNFα and other cytokines impact oestrogen metabolism in CRC and thus will now focus on those specific inflammatory mediators.

1.6.2.1 TNFα

Many types of leukocytes, as well as some non-hematopoietic cells, produce TNFα, a potent pro-inflammatory cytokine (Vassalli,1992). This pleiotropic pro-inflammatory cytokine is involved in a wide range of cellular processes and has a well-known role in the pathophysiology of several inflammatory disorders (Noti et al.,2010). This cytokine has two receptors that it can bind to; TNFαR1 is constitutively expressed, whereas TNFαR2 expression is mostly seen in immune cells (Zafari et al., 2022). It starts and propagates several responses, including the creation of other cytokines and chemokines, and is produced during the initial inflammatory response. Also, TNFα is a promoter of inflammation, tumour dissemination, and angiogenesis (Kruglov et al., 2008; Terzić et al., 2010). TNFα activation causes the cellular inhibitor of apoptosis protein-1 (cIAP1) and induces NF-κB to activate an antiapoptotic pathway (Zafari et al., 2022). TNFα can also augment NF-κB activation in a variety of cell types by binding to TNF-Rp75 or TNF receptor p55 (TNF-Rp55) (Liu, 2005).

TNFα's role in the initiation of inflammatory disorders is noticeable in IBD (Noti et al.,2010). TNFα levels are elevated in circulating blood and in inflamed intestinal mucosa of patients with IBD (Matsuda et al., 2009). TNFα is a major pro-inflammatory cytokine

in IBD-like chronic inflammatory conditions, which drives downstream immune responses that result in IBD. It has been reported that colonic macrophages produce the majority of TNFα in active IBD (Tracey & Cerami, 1994). TNFα levels in the intestine are inversely related to IBD severity and activity, and IBD can be effectively treated with anti-TNFα therapy administered systemically (Gareb et al., 2020). Single nucleotide polymorphisms (SNPs) in the promoter of TNFα have been linked to an elevated incidence of IBD. They may be genetically predisposed to colitis-associated CRC (Garrity-Park et al.,2008). Garrity-Park et al. (2008) reported that the SNPs in the promoter region of TNFα might be associated with an increased risk for IBD and possibly lead to a genetic predisposition towards developing colitis-associated CRC. A recent study found that IBD patients treated with anti-tumour necrosis factor drugs (225,090 with Crohn's disease and 188,420 with ulcerative colitis) were less likely to develop CRC. Studies have shown that anti-TNF drugs can reduce the incidence of CRC in patients with IBD (Alkhayyat et al., 2021). Popivanova (2008) have shown that TNFα acts as a critical mediator of the initiation and progression of colitis-associated colon carcinogenesis in models of experimental colitis, and blocking TNFα reduces the formation of colorectal tumours in mice lacking the TNFα receptor, p55 (TNF-Rp55 knockout). Also, Baars et al. (2011) observed a significant protective effect of anti- TNFα therapy in 173 IBD-associated CRC patients from 78 hospitals. Moreover, TNFα blockade was found to attenuate the development of colitis and CRC in a recent animal study (Yang et al., 2020).

The effects included anti-inflammatory effects, reduced DNA damage following colonic crypt regeneration, changes in gut microbiota, and functionally attenuated CRC development. Furthermore, a higher level of TNFα is associated with more advanced

stages of CRC (stage III/IV cancers) (Kasprzak, 2021). Additionally, in comparison to earlier stages of disease and controls, patients with stage IV disease had the highest level of TNFα. Patients with low serum levels of TNFα had significantly better median survival than those with high levels (p=00015). TNFα serum levels correlate with cancer stage and progression, and elevated TNFα levels are associated with poor prognosis (Stanilov et al., 2014). A study has found that patients undergoing surgery with high plasma TNFα levels have increased mortality from CRC-specific cancer (Olsen et al., 2017). Also, high-grade CRC and disease progression have been correlated with overexpression of TNFα mRNA (Al Obeed et al., 2014). As with TNFα serum levels, higher levels were associated with patients with higher-stage disease, while lower levels were associated with patients with early disease (Limanu et al., 2021). These findings imply that targeting TNFα could be beneficial in the treatment of CRC.

1.6.2.2 IL-6

IL-6 is produced by a variety of cell types, including macrophages, fibroblasts, endothelial cells, T cells, B cells, and numerous tumour cells, and works as a differentiation factor in B-cells (Tanaka et al., 2014). IL-6 appears to be produced primarily by macrophages and monocytes during acute inflammation and by T cells during chronic inflammation (Naugler & Karin, 2008). The activation of numerous transcription factors, including NF-κB, regulates IL-6 production in these cells. This transcription factor-mediated control of IL-6 expression allows for a very unspecific increase of IL-6 during practically every kind of inflammation (Waldner et al.,2012). IL-6 is also involved in the formation of human cancers such as lymphoma, glioma, breast, ovarian, pancreatic, prostate, renal, and CRC. Increased IL-6 expression has been found

in patients with a variety of cancers, including both sporadic and colitis-associated CRC, and has been linked to a poor prognosis (Waldner et al.,2012). Furthermore, IL-6 expression is elevated in IBD patients (Atreya & Neurath, 2008).

High levels of IL-6 have been linked to increased tumour stage, size, metastasis, and poor survival in CRC patients (Knüpfer & Preiss, 2010). Because of the relationship between IL-6 expression and CRC prognosis, as well as the elevated expression of IL-6 in patients with IBD, IL-6 is hypothesised to operate as a link between chronic inflammation and tumour formation. The IL-6 signaling system and its numerous components have been presented as a possible target for CRC therapy (Waldner et al.,2012).

1.6.2.3 IL-4

IL-4 is a pleiotropic cytokine produced by activated T helper type 2 (Th2) lymphocytes, as well as mast cells, basophils, epithelial cells, and B cells. It has been discovered that IL-4 binds to the IL-4Rα and IL-13Rα1 chains in cancer cells, producing functional receptors. Under normal physiological conditions and in cancer, IL-4 regulates immune responses and the immunological microenvironment. Upregulation of major histocompatibility complex class (MHC) class II and CD23 on monocytes, inhibition of inflammatory cytokine production, induction of an anti-CD40-dependent IgG class switch, and induction of IgG and IgM synthesis in B cells are all examples of their normal biological roles (Suzuki et al.,2015). Through a direct anti-apoptotic impact, IL-4 appears to increase the survival of tumour cells. IL-4 is not only produced by many cultured tumour cell lines, but its interaction with IL4-Rα leads to the up-regulation of anti-

apoptotic molecules, according to several studies in prostate, bladder, colon, breast, lung, and fibrosarcoma cancer models (Conticello et al., 2004; Li et al., 2005).

1.6.2.4 IFN-γ

The ability of IFN-γ to 'interfere' with viral replication in infected cells distinguishes it as a pleiotropic cytokine (Lengyel, 1982). CD8, CD4 Th1 cells, and Natural Killer (NK) cells are the main producers. IFN-γ has numerous inflammatory effects, including macrophage activation, synergy with cell stimulator cytokines, increased cell antibody synthesis, and increased natural killer cell activity (Lengyel, 1982). IFN-γ plays a vital role in the clearance of intracellular pathogens by activating macrophages, resulting in increased MHC class I and II expression, enhanced phagocytosis, and the generation of nitric oxide, IL-12, and superoxide formation (Boehm et al.,1997). However, it was discovered in a study by Wang et al. (2015) that a lack of interferon-gamma or its receptor accelerates the development of CRC. The tumour microenvironment was also found to have powerful anti-tumour efficacy against CRC, which anti- VEGFR-interferon-α2 may control (Li et al.,2017).

1.6.3 Effect of Cytokines on Steroidogenic Enzymes and Steroid Sulfatase

Very little is known about the impact of cytokines on the expression and activity of steroidogenic enzymes. At the level of the adrenal glands, ovaries, and testes, cytokines can interact and control steroidogenesis, impacting the function and development of these glands in a complicated and systemic manner (Bornstein et al.,2004). A wide range of cytokines are secreted by stromal and immune cells infiltrating solid tumours in humans. In turn, these cytokines may regulate the activity of immune cells as well as cancer cells

(Simard & Gingras,2001). Establishing a relationship between cytokines expression, influence on steroidogenesis (primarily sex steroid) and disease progression, survival, and response to therapy remains a significant challenge in understanding the mechanisms of these cytokines in the tumorigenic process in hormone-dependent cancers. Many cytokines can interact with many systems, but only a few have been linked to steroidogenic pathway regulation.

Cytokines (IL-8, IL-11, IL-1, IL-6, TNFα, IFN-γ, and TGF-β) play a key role in hormone secretion regulation by regulating particular enzyme steps in steroidogenesis in a variety of cell types, including adrenal, breast, ovarian, and breast tumour cells (Herrman et al.,2002). Pro-inflammatory cytokines suppress the most important early steps in steroidogenesis, resulting in a more pro-inflammatory environment (loss of androgens and cortisol). Repair and growth factors, on the other hand, trigger some of the early stages of steroidogenesis, implying that these mediators have anti-inflammatory properties (Herrman et al.,2002). Local intracrine production of sex hormones from inactive precursors secreted by the adrenals, such as 4-androstenedione (4-dione) and dehydroepiandrosterone (DHEA), is critical for the regulation of peripheral target tissue growth and function. Because of their tissue and cell-specific expression and substrate specificity, the numerous forms of human HSD17Bs and 3β-HSD provide each cell with the required mechanisms to control the quantity of intracellular active androgens and oestrogens (Turgeon et al., 1998). Studies have demonstrated that IL-6 and TNFα promote tumour invasion, progression, and metastasis through their effects on carcinogenesis (Simard & Gingras, 2001; Philip et al., 2004). Additionally, their ability to stimulate oestrogen biosynthesis makes them risk factors for endometrial cancer (Simard & Gingras,2001; Purohit et al., 2002).

Usually, cytokines like TNFα and IL-6 are considered to be important factors in CRC (West et al., 2015). TNFα induced inflammation appears to play a key role in promoting innate and adaptive immune responses in CRC, which appears to be a significant factor in tumorigenesis (Mercogliano et al., 2021). IL-6 is a fibroblast-derived stimulatory factor that has been shown to play a paracrine role in the regulation of E_2 levels in breast cancer tissue (Adams et al., 1991). Also, a study by Turgeon et al. (1998) demonstrated that IL-6 regulates the expression of numerous enzymes involved in sex steroid production and inactivation in breast cells, as well as regulate STS (Purohit et al., 1996) and P450 aromatase (Zhao et al., 1995) activities. TNFα decreased the E_1/ E_2 ratio; more E_2 was formed as a result of the E_1/ E_2 equilibrium shifting. The conversion of weakly active oestrone (E_1) into highly potent oestradiol (E_2) may play a role in breast cancer development. This change in the E_1/ E_2 ratio could be due to TNFα modulating the expression of HSD17B1 in breast cancer, which catalyses the conversion of E_1 into E_2 (Zhang et al., 2012). Similarly, a study by Kamel et al. (2012) has demonstrated that TNFα stimulation of breast cancer cells (MCF-7) can decrease the E1/E2 ratio by altering gene and enzyme expression involved in E2 activation, as well as regulate STS and P450 aromatase (Purohit et al., 1997). The inflammatory cytokines TNFα and IL6 promote the production of 17hydroxysteroid dehydrogenase in human breast cancer cells (Adams et al., 1991; Duncan et al., 1994). In liver cancer tissues of rats, HSD17B4 is overexpressed. The levels of TNFα and IL-6, as well as their expression, were positively correlated (Pan et al., 2018). As a result, pro-inflammatory cytokines could increase E2 levels in CRC by affecting the expression of enzymes that produce E2.

Only a few studies have been done on the role of cytokines in the control of STS activity. In various cancers, the proinflammatory cytokines TNFα and IL-6 have been demonstrated to alter STS enzyme kinetics (Mueller et al., 2015). STS is regulated in a variety of tissue types as part of the inflammatory response. TNFα has been found to increase STS expression and activity in breast MCF7 cells through post-translational modification of the enzyme or increased substrate availability (Newman et al.,2000). Both TNFα and IL-6 cytokines may increase the STS enzyme's post-translational modifications. IL-6 and TNFα, on the other hand, may influence membrane permeability, increasing substrate availability and, as a result, enzyme activity (Purohit et al., 2002). Furthermore, in response to IL-6 and TNFα, STS activity was raised without an alteration in STS mRNA levels in the prostate cancer cell line LNCaP (Suh et al., 2011), suggesting that it caused post-translational modification of STS. Also, STS mRNA is induced by TNFα in chronic liver disease (Jiang et al., 2016). When comparing CRC to normal colonic mucosa, an increase in STS expression was associated with higher oestrogen concentrations (Sato et al., 2009). This shows that locally generated oestrogens (E_1 and E_2) may play a role in disease progression. To support this, increased oestrogen concentrations in CRC tissue were substantially linked with a combination of high STS and low SULT1E1 expression (Sato et al., 2009).

CRC expresses the organic anion transporter polypeptides (OATPs) involved in sulfated oestrogen uptake. Several cytokines regulate mRNA expression for OATPs, which are crucial for E1S uptake. TNFα has been studied for its effect on organic anion transport polypeptides (OATPs). By treating primary human hepatocytes with TNFα or IL-6 for 48 hours, Le Vee et al. have found that transporters, such as OATP1B1, OATP1B3 and

OATP2B1, are downregulated. Also, HCT116 cells were examined in vitro by Varma et al. (2018). and the results indicate that TNFα stimulates OATP2B1 mRNA expression, protein expression, and E1S uptake. It might be the STS activity increases in response to TNFα because of more E1S substrate availability.

1.7 Hypothesis and aims:

CRC is the third most common cancer and one of the highest mortality tumours worldwide. There is often an infiltration of cytokine-producing immune-inflammatory cells, which may interfere with steroid metabolism. Sex steroids, particularly oestrogens, influence CRC incidence and prognosis. Through OATPs, circulating E1S is desulfated to E1 by ST). HSD17Bs can reduce E1 into biologically active E2. My group has previously shown STS activity and HSD17B expression in CRC are altered to favour E2 production, which stimulates proliferation.

In CRC, however, it is unclear what regulates STS activity and HSD17Bs expression and activity. In breast and prostate cancer, inflammatory mediators, such as TNFα, increase STS activity. Therefore, my hypothesis is that specific inflammatory cytokines influence steroid metabolism in CRC, in turn altering cell proliferation by altering STS activity, HSD17Bs, and OATPs expression in CRC and driving E2 uptake and synthesis pathways.

Thesis Aims:

This thesis has four aims that will address the hypothesis outlined above. These are to:

1: Measure the effect of inflammatory cytokines on STS activity in a range of CRC cell lines.

2: Determine the effects of inflammatory cytokines on the expression of HSD17Bs and how this impacts oestrogen metabolism in CRC cell lines.

3: Determine the effects of inflammatory cytokines on the expression of specific oestrogen-uptake OATPs in CRC cell lines.

4: Examine the effects of inflammatory cytokines on oestrogen metabolism by LC-MS/MS.

5: Compare all cell-based data to online publically available datasets such as The Cancer Genome Atlas.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Culture

The HCT116, Colo205, Lovo, and SW620 cell lines were obtained from the American Type Culture Collection in the United States. HCT116 (Colorectal carcinoma cells from primary tumours and distant metastases in an adult male) and Colo205, SW620, and Lovo (colorectal adenocarcinoma from a metastatic site in a male) (ATCC,2016). Table 2.1.

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Table 2.1: Summary of ATCC cell lines. (ATCC,2016)

Cell Line	Type	Gender	Description
HCT116	Dukes' type D Colorectal carcinoma	Male	derived from adult male
Colo205	Dukes' type D Colorectal adenocarcinoma	Male	derived from metastatic site: ascites (Dukes' type D, colorectal adenocarcinoma) 70 years
Lovo	Dukes' type C grade IV Colorectal adenocarcinoma	Male	derived from metastatic site: left supraclavicular region, 56 years
SW620	Dukes' type C Colorectal adenocarcinoma	Male	derived from metastatic site: lymph node, 51 years

Although all four cell lines are derived from human colon carcinomas, there are some inherent differences between them. CRC cell lines exhibit different genetic and phenotypic characteristics, including different gene expressions, growth rates, treatment response, and metastatic capacity. HCT116 cells harbour mutated *KRAS*, *PIK3CA*, *APC*, and *P53* genes. Colo205 cells have mutations in *BRAF* and *APC* genes. In LoVo cells, there was *KRAS* gene mutation, wild-type *p53* gene, *SMAD2* and *APC*. SW620 cells have *APC*, *KRAS*, and *P53* mutations (Mouradov et al., 2014; Ahmed et al., 2013). Also, CRC cell lines exhibit a variety of growth rates and morphologies; COLO205 and HCT116 were the fastest-growing cultures, doubling in approximately 20-24 hours. While all are epithelial-like, they can differ in shape (e.g., cobblestone versus rounded).

Cells were seeded in 75 cm2 tissue culture flasks (T75, Corning Ltd, UK) in HyClone RPMI-1640 media (Sigma-Aldrich, Poole, UK) containing 50 ml 10% foetal bovine serum (FBS, Sigma-Aldrich, Poole, UK) and 5 ml 1% penicillin-streptomycin (10,000 units/100 ml, Invitrogen, Paisley, UK), incubations are carried out at 37°C with 5% CO2 in a humidified incubator. Sterile techniques should be used throughout all stages of cell culture to prevent contamination, such as mycoplasma contamination in cell cultures, which can be detected through specific staining methods, PCR assays, or commercial test kits. Regular testing for mycoplasma is essential for the integrity and reliability of cell cultures.

Every day, cells were reviewed to see whether the media needed to be changed. When the cells were 80% confluent, they were trypsinised with Triple-X (Sigma-Aldrich) applied, and the cells were incubated at 37°C for 2-5 minutes and split 1:8 into a new T75

flask. Passage numbers used were 40-60 for HCT116 cells, 40-55 for Colo205 cells, 5-15 for LoVo cells and 5-20 for SW620 cells. Cells were counted at 100 x magnification after 10 µl of cell suspension were pipetted into a haemocytometer for seeding. The number of cells per µl was calculated using the average of three counts.

When conducting cell experiments, it is essential to have replicates to assess the results' variability and reliability. An experiment can be replicated biologically (separate cultures) or technically (repeated measurements within the same sample). The results are then analysed statistically to determine their significance and reproducibility.

2.2 Cytokines treatment

Cytokines (TNFα, IL-6, IFNγ and IL-4) were reconstituted in phosphate buffered saline (PBS) (PBS, Oxoid, and Hampshire, England) containing 0.1% human albumin serum (HAS) (Sigma) and stored in working aliquots at -80^{o}C. All cytokines were purchased from (PeproTech, London, UK).

The HCT116, Colo205, Lovo, and SW620 cells were seeded into 6-well plates at 100,000 cells per well and treated with different concentrations of cytokines (2.5 ng/ml, 5 ng/ml,7.5 ng/ml,10 ng/ml, 20 ng/ml, 40 ng/ml) of TNFα, (10 ng/ml, 20 ng/ml, 40 ng/ml) of IL-6, (10 ng/ml, 20 ng/ml, 40 ng/ml) of IFNγ and (5 ng/ml, 10 ng/ml, 20 ng/ml) of IL-4. Cells were incubated for 24 h and 48 h at 37^{o}C. After 24-48 hours, the media was removed, and either an STS assay or RNA extraction was performed to determine the expression of steroidogenic enzymes in CRC cell lines treated with various cytokines. Untreated cells from all cell lines served as controls.

2.3 Protein Extraction and Quantification

Cell protein quantification was performed using the bicinchoninic acid (BCA) assay (Thermo Scientific Pierce Microplate BCA Protein Assay Kit), which allows for the measurement of total protein in cell lysate samples. In each well of a 96-well plate, 5 µl of the sample (in duplicate) was added. In separate wells, a pre-prepared standard of bovine serum albumin (BSA) in RIPA buffer was also included (at 0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2 mg/ml).

In a 1:50 dilution, Solution B (% cupric sulphate) was added to Solution A (0.1M sodium hydroxide, sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate) with this solution, then 200 µL added to each well and then incubated at 37^{o}C for 30 minutes. The reduced (cuprous) cation is then reacted with BCA, forming a water-soluble BCA/copper complex and turning the solution purple. This colourimetric change can be measured at 560 nm using a Wallac Victor3 1420 multilabel counter, and protein concentration can be calculated using the BSA standard curve.

2.4 RNA Extraction

RNA was extracted from cells using an RNeasy® kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. This kit can isolate RNA from cells by using a column purification method. The cells (100,000 cells) per well were treated with TNFα for 24 hours and -48 hours, as described previously. Media was removed from each well, and the cells lysed by adding 350 µl (6-well plate) to the RLT Buffer. After this, 350 µl of 70% ethanol was added, and the lysate was homogenised, mixed by pipetting, and

transferred to an RNeasy spin column. The column was then centrifuged at ≥ 8000 rpm for 15 seconds, after which the flow-through was discarded. 700 µl of RW1 Buffer was then added to the spin column, and again, this was centrifuged at ≥ 8000 rpm for 15 seconds with the flow-through discarded. After this, 500 µl of the RPE buffer was twice added, followed by centrifugation at > 8000 rpm for 15 seconds and 2 minutes, respectively, with again the flow-through discarded. Finally, to elute the RNA, 30-50 µl of RNase-free water was added to the column, centrifuged at > 8000 rpm for 1 minute, and the flow through collected in a 1.5ml Eppendorf. The quality and quantity of total RNA in ng/µl were determined by using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) measurements of absorbance at 260 (nucleic acids). Purity was quantified using A 260/280 nm. Additionally, 260/230 nm gives information about other contaminants, with a good result being 2.0-2.2. RNA was stored at -80ºC until reverse transcribed. The RNA extraction was performed in triplicate (N=3) for each experimental condition.

2.5 Reverse Transcription

To synthesize single-stranded cDNA from total RNA, the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City CA, USA) was used. For the reverse transcription reaction, each tube contained up to 2 µg of RNA per sample. The amount of RNA was equal in all the reactions, so the efficiency of the RT step was equal between samples. The sample RNA concentration was standardised to 700 ng/ul. RNA (2 µl) was reverse transcribed in a 20 µl reaction using random primers. Up to 2 µg of total RNA was added to 10 µL of the RT master mix containing 2 µL 10× RT Buffer,0.8 25× dNTP

Mix (100 mM),2 µL 10× RT Random Primers, 1 µL MultiScribe™ Reverse Transcriptase and 4.2 µL Rnase-free H_2O. The reverse transcription reaction was run in Eppendorf PCR tubes in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA) for 10 minutes at 25 °C (primer annealing), 120 min at 37 °C (reverse transcription), 5 min at 85 °C and cooled at 4°C (inactivation) and then the cDNA was stored at -20°C.

2.6 Quantitative Polymerase Chain Reaction

Reverse transcription followed by real-time PCR was used for the systematic measurement to quantify gene expression for oestrogenic enzymes and OATPs were measured via qRT-PCR in HCT116, Colo205, Lovo, and SW620 cells after treatment with inflammatory cytokines (TNFα, IL-6, IL-4 and IFN-γ) at various concentrations (5,10, 20, and 40 ng/ml), and different incubation times (24-48 hours) by using pre-optimised specific TaqMan™ (Applied Biosystems, Poole, UK) gene expression assays and RPLPO as a reference gene. The TaqMan® gene expression assay used for the qPCR, RPLPO (4310879E), HSD17B1 (Hs00166219_g1), HSD17B2 (Hs00157993_m1), HSD17B4 (Hs01069908_m1), HSD17B7 (Hs04937189_g1), HSD17B12 (Hs00275054_m1), OATP1A2 (Hs00366488), OATP1B1 (Hs00272374), OATP1C1 (Hs00213714), OATP2B1 (Hs00200670), OATP3A1 (Hs00203184), OATP4A1 (Hs00249583). All genes of interest TaqMan assay probes were labelled with 6-carboxyfluorescein (FAM), with the RPLPO probe labelled with VIC® fluorophore. The SensiFAST™ Probe Lo-ROX Kit was used, and a 96-well plate loaded with 1 µl cDNA in a final volume of 10 µl by using 5 µl master mix, 0.5 µl TaqMan probe and 3.5 µl of Rnase-free water. The qRT-PCR reactions were carried out in a 7500 ABI real-time

polymerase chain reaction system (Applied Biosystems, Quarrington, UK). The reaction program was one cycle at 95ºC 2-5 minutes (polymerase activation), followed by 40 cycles of 95ºC for 10 seconds (denaturation), then 60ºC for 20-50 seconds (Annealing/extension). The cycle threshold (Ct) was used for statistical analysis.

ΔCt was calculated by subtracting the CT value of the RPLPO from the CT value for the gene of interest in each sample. The fold change was calculated by using 2-ΔCt. If levels of expression were undetermined, an arbitrary Ct of 40 was used to allow for numerical analysis.

Controls have been used in real-time PCR reactions to be sure that the signal obtained from experimental samples representing the amplicon of interest was a no-reverse transcriptase control (no-RT), which contains all reaction components except the cDNA sample.

2.7 Cells treatment prior to LC-MS/MS

CRC cells HCT116, Colo205, LoVo and SW620 were seeded at 200,000 cells per well in 6-well plates for 24 hours. Then replace the media with 10% sFBS RPMI phenol-free media. Control wells were prepared for each cell, and the 40ng/ml of TNFα was added to specific wells and incubated for 24 hours at 37°C. Later, cells were incubated in a plain medium or medium containing 100 nM of E1S, E1 and E2 for 24 hours at 37°C. Media was collected and frozen at -20oC until extraction and analysis using LC-MS/MS. Finally, the protein was quantified using the BCA assay.

2.8 Mass Spectrometry Extraction

Mass spectrometry is an analytical technique that identifies unknown compounds within a sample, quantifies known materials, and determines molecules structures and chemical properties of different molecules. In this complete process, the sample is converted into gaseous ions, either fragmented or not, and then their mass-to-charge ratios (m/z) and relative abundances are determined. The technique focuses on the effect of ionizing energy on molecules. A gas phase chemical reaction forms ionic and neutral species by consuming sample molecules (Premier Biosoft, 2024). Both electron impact (EI) and chemical ionization (CI) have been used in MS analysis. The halogenated derivatization reagents improved sensitivity as a result of electron capturing. The majority of GC–MS/MS methods capable of detecting oestrogens at low concentrations use negative ion mode, despite positive ionization being used with ion-trap technology to achieve a lower limit of quantitation (LOQ) of 13–21 pg/mL. The mass spectrometer's resolution determines its ability to distinguish between different ions. A high resolution is essential for effectively separating compounds from their metabolites (Prokai-Tatrai et al., 2010).

Also, derivatization improves sensitivity by adding a permanently or readily charged group, permitting a higher ionization efficiency and increased sensitivity by MS. This, in turn, permits a shorter extraction protocol and a smaller sample amount (Faqehi et al., 2016). Moreover, effective chromatographic separation is crucial, which can be achieved by using columns like the Aquity UPLC BEH Phenyl column, which can provide efficient separation during analysis (Bertelsen et al., 2020).

Mass spectrometers, when used with liquid chromatography, require minimal, but usually some preliminary, pretreatment before analysis. Biological samples are typically not

directly compatible with HPLC analysis due to their complexity and protein content. In biological samples, proteins are irreversibly adsorbed to the stationary phase, resulting in a substantial loss of column efficiency and an increase in backpressure. Before LC/MS analysis, samples may be prepared by protein precipitation (PPT), solvent extraction (liquid: liquid; LLE), and solid-phase extraction (SPE) (Nováková and Vlčková, 2009). Since its development in the 1980s, solid-phase extraction (SPE) has become one of the most widely used sample preparation techniques (Dimpe and Nomngongo, 2016). This method is advantageous because of its simplicity, selectivity, enrichment factors, ease of automation, and ability to use various adsorbents (Żwir-Ferenc and Biziuk, 2006). Hydrophobic E1 and E2 and hydrophilic E1S were extracted simultaneously by this method. E1S (Estrone 3-sulfate sodium) and internal standard 17β-Estradiol-2, 3, 4- 13C3 were all purchased from Sigma-Aldrich, Dorset, UK.

Sample preparation for **extraction by** LC-MS/MS analysis starts with the preparation of the calibration series by adding different amounts of the serum steroid stock (0 to 250 µl) to different amounts of the cell media (1000 to 750 µl), then 10 µl of the internal standard have been added to the tubes (In the analysis, an internal standard is used for calibration and quantification). After mixing the calibration series thoroughly and adding 2 ml of solvent MTBE (tert-butylmethyl ether, Sigma-Aldrich, St. Louis, MO, USA), the top layer was transferred to the 96 plates LC-MS/MS plate (Thermo Fisher Scientific, Paisley, UK or Porvair Sciences Limited, Wrexham, UK), and samples were evaporated for 20 minutes under nitrogen at 55^{o}C. Next, add 200 µl of the mixer (methanol and water 1:1, 50:50). Finally, after the samples were mixed and centrifuged, they were ready to be run on the LC-MS/MS system. Based on a linear standard curve containing the steroids of

interest in concentrations ranging from 0.5 to 500 mg/ml, concentrations were quantified. Following extraction, samples were handed over to the SMAC team for further LC analysis.

2.9 Data Collection

The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) is a collaborative initiative between the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) to improve data exchange and accelerate cancer research (Tomczak et al., 2015). The Cancer Genome Atlas (TCGA) project has molecularly defined approximately 20,000 primary cancers and matched normal samples from 33 cancer types, including colon adenocarcinoma (COAD) (National Cancer Institute, 2023).

Colon cancer (COAD) is a dangerous gastrointestinal malignancy. The presence of the TCGA database enables us to more readily undertake gene expression profiling and data extraction on colon cancer patients worldwide, as well as to more easily find the relationship between genes and colon cancer survival prognosis (Chen et al., 2020).

OncoLnc (http://www.oncolnc.org/) is a tool for downloading clinical data along with expression data for mRNAs, miRNAs, or long non-coding RNAs (lncRNA), as well as for interactively examining survival correlations. In addition to lncRNA expression data from MiTranscriptome beta and RNA-SEQ expression for mRNAs and miRNAs from The Cancer Genome Atlas (TCGA), OncoLnc also includes survival data for 8,647 patients from 21 cancer studies conducted by TCGA. Participants that store this data may

divide patients based on gene expression, download the data for additional analysis, or produce Kaplan-Meier graphs (Anaya, 2016). To generate the survival curves through the OncoLnc website, after typing the favourite gene, COAD's survival data was chosen, and then a 50/50 split or a 25/25 split was requested. This will show you the gene's survival at its greatest and lowest expression levels.

FireBrowse is a website created by the Broad Institute that allows everyone to view and study data collected by the Cancer Genome Atlas (TCGA). Colorectal adenocarcinoma (COAD) is one of the cancer types that the TCGA has researched, and information on it is available on FireBrowse (Das et al., 2020). TCGA clinical data and expression data were manually downloaded from the Broad Institute (TCGA data version 2016_01_2800) via the firebrowse.org website. The first step is to select the COADREAD mRNASeq data and mRNASeq clinical. This dataset contains more than 600 CRC samples, which allows gene expression analysis. The code to download the data is illuminahiseq_rnaseqv2_RSEM_genes_normalized (MD5) can be accessed here: https://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/COADREAD/20160128/. Then, after downloading the COADREAD data and extracting it, the samples are divided into normal tissue and tumour tissue according to the number of samples. The TCGA data were then normalized using log2 transformations. To study gene expression in the TCGA data, Mann-Whitney (U tests), as well as Kaplan Meier survival curves, were applied. Finally, we can draw correlation graphs for TNFα and IL-6 compared to HSD17Bs, OATPs, STS and any favourite gene. The graphs were created to visualize the analysis results and compare TNFα and IL-6 to HSD17Bs, OATPs, STSs, and any favourite genes.

2.10 Statistical Analysis

Statistics were performed with GraphPad Prism V5.0C software (GraphPad Software, San Diego, CA, USA). Most of the experiments involved multiple comparisons and were analysed using one-way ANOVAs with Tukey post-tests. When comparing two independent groups whose dependent variables are not normally distributed, the Mann-Whitney U test is used. P-values were assigned to determine the probability of finding the observed and to evaluate if there was significance ($P < 0.05$) between the groups. A p-value < 0.05 was deemed significant. Results are expressed as mean ± standard error of the mean unless otherwise stated. Analysis related to The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD), RNA-Seq dataset (n=433) to generate Kaplan-Meier survival curves (log-rank method) and correlation graphs comparing normal and cancerous colon tissue expression of TNFα, the HSD17Bs, OATPs, and STS.

CHAPTER 3. REGULATION OF STS BY CYTOKINES

3.1 Introduction

Inflammation can contribute to the development of many diseases, including cancer. CRC is associated with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) (Muthusami et al., 2021). At several stages of tumour growth, including initiation, development, malignant conversion, invasion, and metastasis, inflammatory reactions are crucial (Grivennikov et al., 2010). Numerous inflammatory cytokines, including TNFα, interleukin-1 beta (IL-1β), IL-6, IL-11, IL-13, IL-23, and IL-33, cause inflammatory reactions that lead to dysplasia in a variety of tissue types. This, in turn, encourages the development of cancer in all of its stages (Muthusami et al., 2021). Studies have linked the production of TNFα to the development of cancer, which is proven to be primarily caused by chronic inflammation processes (Patel, H.J. and Patel, B.M, 2017; Grabarek et al., 2021). The concentration of a variety of cells at the site of a solid malignancy is the result of local inflammation, and these cells are directly involved in encouraging tumour growth. Similar to the majority of other solid tumours, colon carcinomas are invaded by a variety of cells, including tumour-associated macrophages (TAMs). Human colon cancer cell proliferation and migration are promoted by activated macrophage-conditioned medium containing tumour necrosis factor TNFα and IL-6 (Peddareddigari et al., 2010).

In many malignant tissues, including breast cancer, STS levels are typically increased, and STS activity has been discovered in both healthy and cancerous tissues. The STS enzyme is responsible for oestrogen metabolism via the sulfatase pathway. The principal hormone substrates for STS are E1S, DHEAS, pregnenolone sulfate, and cholesterol sulfate, and thus, this enzyme represents one of the essential pathways to regenerate

biologically active steroids (Mueller et al., 2015). STS converts the hydrophilic E1S to the hydrophobic E1, which is then converted to the active oestrogen E2 via HSD17B (Sato et al., 2009; Foster, 2013). Oestrogens influence the occurrence and development of CRC, which shows a selective preference for E2 production and dysregulated oestrogen metabolism (Potter, 2018). The expression of STS mRNA and STS activity is higher in cancerous tissues compared to normal tissues, and the circulating oestrogen concentrations are likely to play a significant role (Mueller et al., 2015). STS mRNA expression was significantly higher in malignant breast tissue than in normal breast tissue, consistent with a high level of STS activity in cancerous breast tissue (Utsumi et al., 2000; Suzuki et al., 2003; Foster, 2021). High levels of STS expression in breast cancer are associated with poor prognoses in predicting relapse-free survival (Foster, 2021).

Also, among ovarian cancer patients, higher STS activity is associated with shorter progression times and shorter cancer-free survival (Chura et al., 2009). Also, the ability to locally metabolize oestrogen exists in both the human ovarian surface epithelium (OSE) and epithelial ovarian cancer (EOC) cells. By promoting STS and suppressing oestrogen sulfotransferase, inflammatory cytokines boost the local production of E2 in the body (Ren et al., 2015). Furthermore, compared to normal endometrial tissues, endometrial cancer tissue exhibits higher STS enzymatic activity (Abulafia et al., 2009). STS activity was 12 times higher in malignant endometrial tissues compared to normal endometrial tissues (Yamamoto et al., 1993).

Cancer development, including colon cancer, breast, prostate, and endometrial cancer, has been related to inflammation and oestrogen metabolism. On the impact of inflammation on oestrogen metabolism in connection to cancer, numerous studies have

been done. A study by Reed and Purohit (1997) hypothesised that cytokines control the production of oestrogen in breast cancer, and cytokines IL-6 and TNFα are crucial in controlling the production of tumour oestrogen. TNFα may increase the production of the gp130 protein, which is a component of the IL-6 signalling pathway, which in turn increases the capacity of IL-6 to induce oestrogen synthesis. The activities of aromatase, oestradiol-17β-hydroxysteroid dehydrogenase, and oestrone sulfatase are all stimulated by IL-6 and TNFα in breast cancer, and these two substances can also work in concert to increase these activities (Purohit et al., 2002). Also, Frasor et al. (2008) studies have demonstrated that inflammatory mediators, such as prostaglandins and cytokines, increase the expression of aromatase and the consequent local synthesis of oestrogens in the breast. STS expression and activity have been investigated by Newman et al. (2000) about inflammation in breast cancer, and they found that the cytokines interleukin 6 (IL-6) and tumour necrosis factor (TNFα) increase steroid sulfatase activity in breast cancer cells without increasing STS gene expression. This may be due to post-translational modifications or increased substrate availability. Also, the proinflammatory cytokines IL-6 and TNFα alter STS enzyme kinetics in addition to oestrogen's ability to regulate STS activity; as a result of IL-6 and TNFα, MCF7 cells increase STS activity without altering STS mRNA levels because STS undergoes a post-translational modification that increases STS activity (Purohit et al., 1996, Reed and Purohit 1997, Mueller et al., 2015).

In prostate cancer, oestrogen's role in prostatic inflammation has been demonstrated. Findings by (Suh et al., 2011) suggest that human prostate cancer cells, as well as human breast cancer cells, are susceptible to TNFα mediated induction of human STS through transcriptional activation via a mechanism involving PI 3-kinase/Akt pathway. It has

significant effects on oestrogen-mediated carcinogenesis when STS gene expression is induced by inflammatory cytokines like TNFα. Others have shown that STS is a novel NF-κB target gene that is activated in the livers of individuals with hepatitis and cirrhosis (Jiang et al., 2016). These results clearly imply that the inflammatory activation of STS contributed to the oestrogen excess seen in chronic liver disease. These results further revealed an STS-mediated negative feedback loop to suppress inflammation, in which NF-κB responsive activation of STS raises active oestrogen levels, which reduces NF-κB -mediated inflammation. Numerous pre-neoplastic cells and tumour tissue have been shown to express TNFα in an enhanced manner. It may serve as a helpful indicator of the success of treatment and the progression of disease. In patients with prostate and breast cancer, its levels in serum have been reported to decrease with reduced therapy (MA et al., 2017).

It has been proposed that inflammation, especially TNFα, may affect oestrogen metabolism via HSD17Bs and STS, which is enhanced in CRC and results in oestrogen-driven tumour development. In order to discover the role of inflammatory cytokines TNFα, IL-6, IFN-γ and IL-4 on STS activity in CRC, in this chapter, CRC cell lines have been treated with varied amounts of cytokines, STS activity assays and proliferation assays were used to characterize the influence of cytokines on oestrogen metabolism in CRC cell lines. Oestrogen metabolite quantification was also done using LC-MS/MS.

3.2 Materials and Methods

3.2.1 The Cancer Genome Atlas (TCGA) Data Collection and Analysis

The TCGA data collection and analysis were performed as described in section 2.9.

3.2.2 Cell Culture

HCT116, Colo205, LoVo, and SW620 CRC cell lines were cultured as described in section 2.1.

3.2.3 Cytokines treatment

Cytokines (TNFα, IL-6, IFN-γ and IL-4) were reconstituted in phosphate buffered saline (PBS) (PBS, Oxoid, and Hampshire, England) containing 0.1% human albumin serum (HAS) (Sigma) and stored in working aliquots at -80^{o}C. All cytokines were purchased from PeproTech, London, UK.

HCT116, Colo205, LoVo, and SW620 cells were seeded into 6-well plates at 100,000 cells per well and treated with different concentrations of cytokines (2.5 ng/ml, 5 ng/ml,7.5 ng/ml,10 ng/ml, 20 ng/ml, 40 ng/ml) of TNFα, (10 ng/ml, 20 ng/ml, 40 ng/ml) of IL-6, (10 ng/ml, 20 ng/ml, 40 ng/ml) of IFN-γ and (5 ng/ml, 10 ng/ml, 20 ng/ml) of IL-4. Cells were incubated for 24 h and 48 h at 37^{o}C. After 24-48 hours, the media was removed, and STS assay (see Section 3.2.3) was performed to determine the expression of steroidogenic enzymes in CRC cell lines treated with various cytokines. Untreated cells from all cell lines served as controls.

The experiments of our previous group work led to the selection of the TNFα and IL-6 concentrations and 48-hour time points.

3.2.4 STS Activity Assay for intact CRC Cell Lines

The STS assay has previously been described (Purohit et al., 1997). TNFα, IL-6, IFN-γ, and IL-4 were applied to the cells (100,000 cells per well) for 24 hours - 48 hours. The conversion of E1S to E1 was determined to measure STS activity. Cells were incubated in 1000 µl appropriate cell medium containing the STS substrate adjusted to a final concentration of 20 µM with unlabelled E1S. The STS substrate was pre-prepared by using 200 µL of (E1S 10 mM) (3.3 mg of estrone 3-sulfate sodium salt) purchased from (Sigma Aldrich, contains ~35% Tris as stabilizer) in 100% ethanol, 10 µL of the [6,7-^{3}H] E1S (4×10^{5} dpm, Perkin-ElmerLS, Boston, MA, USA) and by air-drying, the ethanol was removed. Then, 1000 µL of HBSS (Hanks Balanced Salt Solution, Gibco) was added. The final step was to add 1 ml of the previous mixture to 49 ml of RPMI media. In some experiments, the E1S substrate was prepared by adding 10 µL of the STS substrate [6,7-^{3}H] E1S to 50 ml of RPMI media due to the low STS activity and conversion.

After 18 hours of incubation at 37°C, the product, E1, was separated from E1S by partitioning with toluene by transferring 500 µL of the media to a glass tube followed by 4 ml of toluene and vortexed for 30 seconds. Subsequently, 1 ml (top layer) of the toluene was removed and added to 4 ml of optiphase hisafe 3 (PerkinElmer in scintillin vials (Fisher Scientific, UK). The ^{3}H radioactivity was then measured with a liquid scintillation counting spectrophotometer (Tri-Carb 2500 TR, Packard). CRC cells that had not been treated with cytokines served as positive controls. Negative controls were ^{3}H-E1S extracted with toluene to assess any potential non-enzymic loss of the E1S sulfate group and a PBS blank. A total count of the non-toluene extracted substrate was also counted, which represented maximum conversion and allowed the hydrolysed E1 concentration to be calculated. Finally, at the end of the experiments, all media from the 6-well plates were

removed, and 500 µl of RIPA buffer mixed with protease inhibitor (10 µl of protease inhibitor to 1ml of RIPA buffer) was added to lyse the cells. Protein lysate was collected and stored at -80^{o}C after the wells were scraped. Later, the protein was quantified using the BCA assay (described in Chapter 2). The results of STS activity are expressed as pmol of the product formed/mg protein/hour (pmol/mg/h). The STS activity assay was performed in triplicate (N=3) for each experimental condition.

3.2.5 STS Activity Assay for lysated CRC Cell Lines.

In order to assess whether the cell wall and, thus, E1S uptake impacted STS substrate availability, some STS assays were performed on lysated CRC cells. For this, cells were seeded into 6 well plates (100,000 cells per well) and allowed to plate down over 24 hours. After this time, the cells were treated with TNFα and IL-6 for 24 hours - 48 hours. All media from the 6 well plates was removed, and 500 µl of RIPA buffer mixed with protease inhibitor (10 µl of protease inhibitor to 1ml of RIPA buffer) was added to lyse the cells. Protein lysate was collected after the wells were scraped. Later, the protein was quantified using the BCA assay. Then, 200 µl of the protein lysate was transferred to glass tubes, followed by adding 500 µl of the STS substrate [6,7-^{3}H] E1S and incubated at 37^{o}C for 2 hours. Then, 4 ml of toluene was added, and the top layer of the toluene aliquot was removed and added to 4 ml of optiphase hisafe 3. ^{3}H radioactivity was then measured with a Liquid scintillation counting spectrophotometer. CRC cells that had not been treated with cytokines served as positive controls. Negative controls were ^{3}H-E_1S extracted with toluene to assess any potential non-enzymic loss of the E_1S sulphate group and a PBS blank. A total count of the non-toluene extracted substrate was also counted.

The results of STS activity were expressed as pmol of the product formed/mg protein/hour (pmol/mg/h). The STS activity assay was performed in triplicate (N=3) for each experimental condition.

3.2.6 Protein Extraction and Quantification

Protein Extraction and Quantification were performed as described in section 2.3.

3.2.7 Proliferation assay

Cell proliferation was evaluated using a non-radioactive proliferation assay by measuring the incorporation of bromo-deoxyuridine (BrdU) into the cells using the BrdU kit (Exalpha Biologicals Inc., Shirley, MA), used according to the manufacturer's instructions. Assays were performed by adding 500 cells/well, 100 µl/well of HCT116, Colo205, LoVo and SW620 cell suspension into 96 well plates (Tissue culture microtiter) for 24 hours at 37°C before the assay. Control wells were also prepared to include positive and negative control (cells with normal RPMI+FBS media and cells with phenol-free RPMI+sFBS). They were processed in the same way as experimental samples. After 24 hours, the wells media was replaced with 100 µl 10% sFBS RPMI phenol-free media with 40ng/ml of TNFα to specific wells, followed by incubation for 24-48 hours at 37°C. Later, cells were incubated in a control sFBS medium or an sFBS medium containing 100 nM of E1S, E1 and E2 for 24,48 and 72 hours at 37°C. After incubation times, 500X BrdU solution was added to each well (20 µl/well) directly in the incubation medium, and cells were kept in the incubator for 2-24 hours at 37°C. After this incubation, the media was

removed, and cells were fixed and denatured by adding 200 µl/well Fixative/Denaturing Solution. An Anti-BrdU Detector Antibody was then added (100 µl/well) and incubated for 1 hour at room temperature, followed by 100 µl/well of Peroxidase Goat anti-Mouse IgG incubated for 30 min at room temperature. Then, the chromogenic substrate tetramethylbenzidine (TMB) was added (100 µl/well) for 30 minutes at room temperature in the dark, followed by the addition of ELISA Stop Solution to every well. Finally, the amount of BrdU was quantified by the Wallac Victor3 1420 multilabel counter and the absorbance was read at 450/550 nm. The total net OD was calculated per sample (OD450 − OD540). Data are expressed as a percentage of control values. The proliferation assay was performed in triplicate (N=3) for each experimental condition.

3.2.8 Cells treatment prior to LC-MS/MS

The treatment prior to LC-MS/MS was performed as described in section 2.7.

3.2.9 Mass Spectrometry Extraction

Mass spectrometers were performed as described in section 2.8.

3.2.10 Statistical Analysis

Statistics were performed as described in section 2.10.

3.3 Results

3.3.1 Survival curves and COADREAD correlation of colon adenocarcinoma patient.

As the hypothesis for this thesis aims to examine the potential role of inflammatory cytokines in altering steroid metabolism in CRC, initial data was obtained from the TCGA datasets. MRNA expression correlations were ascertained for TNFα and IL-6 compared with STS expression, which were originally determined using the COADREAD dataset. Kaplan-Meier survival curves were also determined using this same data source.

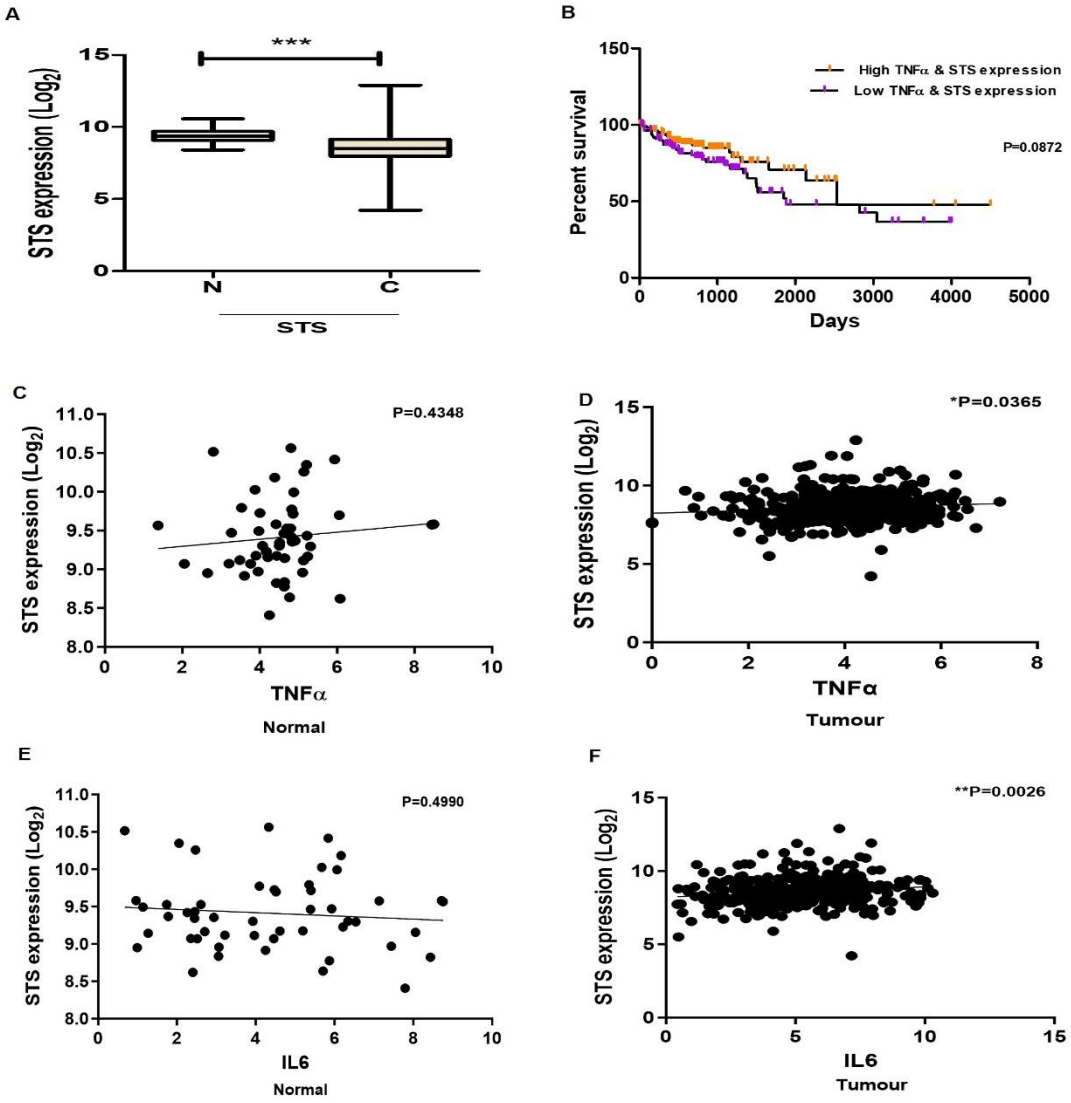


Figure 3.1: Kaplan-Meier survival curves and COADREAD correlation graphs in normal colon and CRC tissue. Data was taken from the TCGA dataset n=433 (normal=51 and CRC=382). (A) STS expression in normal and CRC patients. N- Normal and C-CRC. For the analysis, two-tailed Mann-Whitney (U tests) was used. (B) A non-significant higher survival rate was observed in patients with tumours containing high amounts of TNFα and high STS mRNA. (C&D) Correlation between TNFα in CRC tissue and STS expression in normal and CRC tissue. (E&F) Correlation between IL-6 in CRC tissue and STS expression in normal and CRC tissue. *P< 0.05, **P < 0.01 and ***P < 0.001.

Analysis of the TCGA dataset has been used to study STS expression in normal and CRC patients and to investigate whether STS mRNA expression is associated with CRC. To do this, the COADREAD TCGA cancer dataset containing STS mRNA expression from 382 CRC patient's samples was analysed and compared to 51 normal colorectal epithelial samples. Results show there was a reduction in STS mRNA expression in CRC patients compared to normal tissue (Figure 3.1A).

This COAD data was also analysed to determine Kaplan-Meier survival curves to examine the potential impact of high TNFα and high STS expression on patient survival compared to low TNFα and STS expressions (Figure 3.1B). Patients with tumours containing high amounts of TNFα and STS mRNA exhibited a noticeably higher but non-significant survival ($p = 0.087$) when compared to those with tumours containing both low levels of TNFα and STS mRNA.

Data was next analysed to determine mRNA expression correlations for TNFα and IL-6 compared with STS. In normal colon tissue, TNFα expression did not correlate to STS expression (Figure 3.1C). However, in CRC, a positive correlation was observed between STS and TNFα expression (Figure 3.1D, $p = 0.037$, $n = 382$). These results were similar when analysing another inflammatory cytokine, IL-6. There was no significant correlation of IL-6 with STS in normal colon tissue (Figure 3.1E). However, in CRC tissue, increased IL-6 expression correlated with increased STS expression (Figure 3.1F, $p = 0.026$, $n = 433$).

3.3.2 The Effect of Cytokines on STS Activity in intact HCT116 and Colo205 Cell Lines

As the TCGA COADREAD data analysis (see above) suggested that increased TNFα and IL-6 expression may lead to an increase in STS, it was decided to test these effects in CRC cell lines. Thus, the effects of TNFα and IL-6 on STS activity were assessed in HCT116 and Colo205 cells. Furthermore, other inflammatory cytokines, IFN-γ, IFN- and IL-4, were also tested.

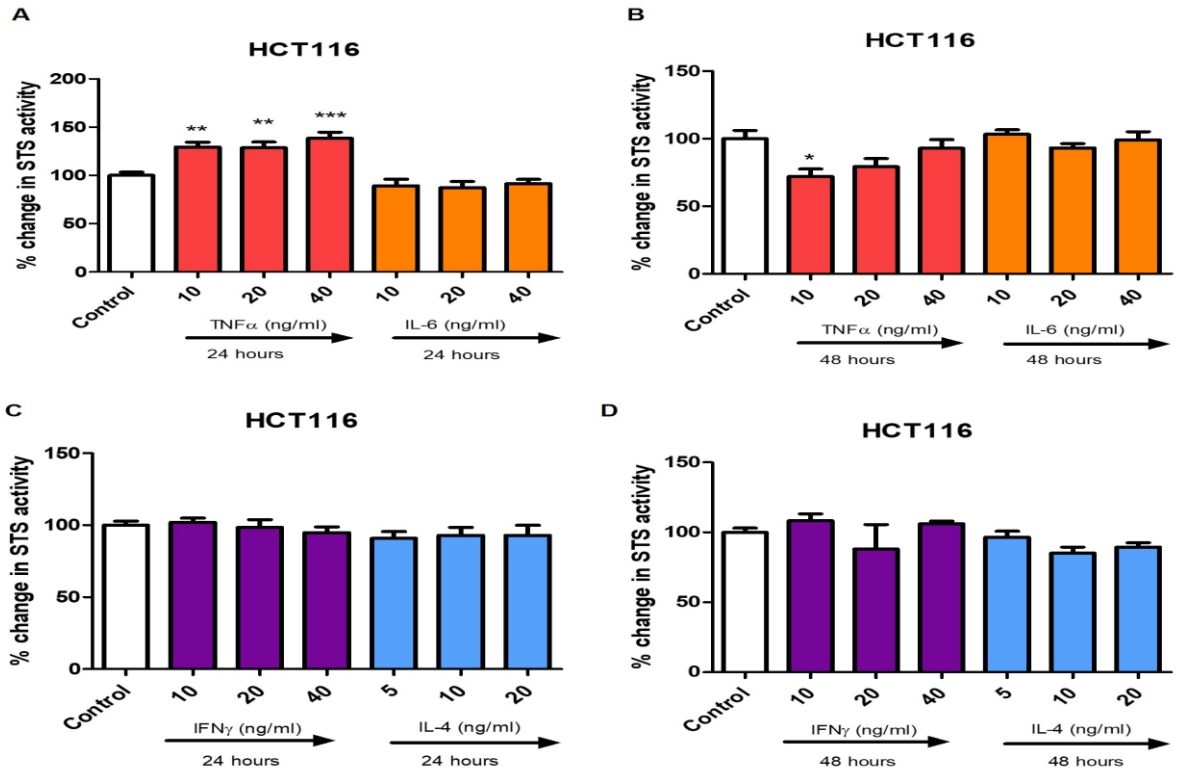


Figure 3. 2: The effect of various cytokines on STS Activity in intact HCT116 cells. STS activity was measured in HCT116 cells with (A) TNFα & IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 hours. (B) TNFα & IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours. (C) IFN-γ & IL-4 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 hours. (D) IFN-γ & IL-4 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours. Data represents % change in STS activity ± SD, n = 3. Statistical analysis is a one-way ANOVA with Tukey post-test, *** $p < 0.001$ **$p < 0.01$ compared to control.

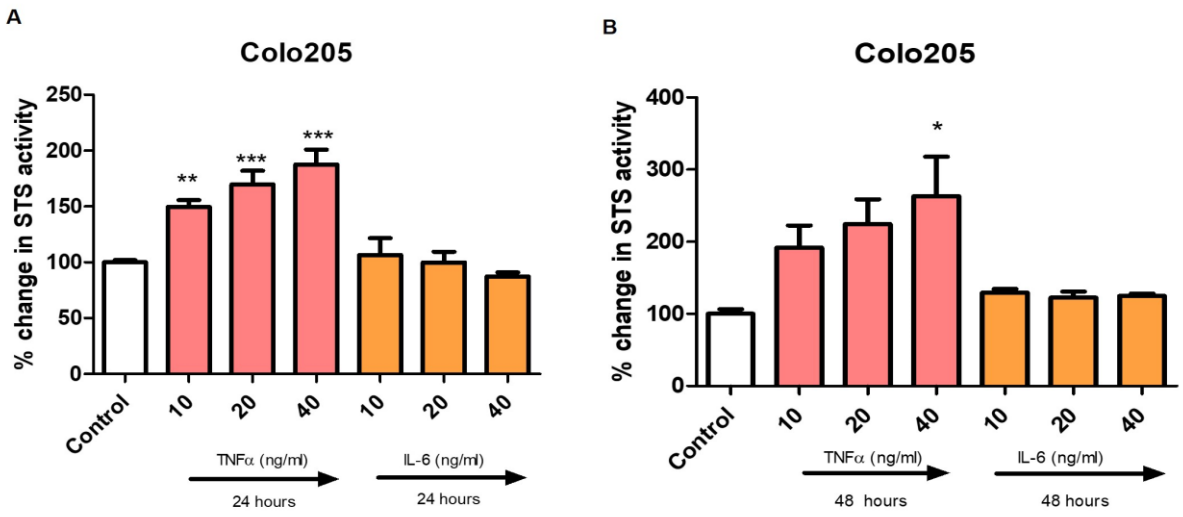


Figure 3.3: The effect of the cytokines TNFα and IL-6 on STS Activity in intact Colo205. STS activity was measured in Colo205 cells with (A) TNFα & IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 hours. (B) TNFα & IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours. Data represents % change in STS activity ± SD, n = 3. Statistical analysis is a one-way ANOVA with Tukey post-test, *P< 0.05, **P < 0.01 and ***P < 0.001 compared to control.

Therefore, CRC cells were treated with varying concentrations of cytokines for 24 - 48 hours, followed by an STS activity assay. Cell lines were treated for 24-48 hours with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml), IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml), IFN-γ (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-4 (5 ng/ml, 10 ng/ml, 20 ng/ml). Figure 3.2A shows STS activity was significantly increased in HCT116 cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 hours to 129% ($P < 0.01$), 128% ($P < 0.01$) and 138% ($P < 0.001$) respectively compared to the control. IL-6, IFN-γ, and IL-4 treatment of HCT116 cells for 24 hours did not significantly alter STS activity compared to control (Figure 3.2A, 3.2C). After 48 hours, TNFα at 10ng/ml significantly ($p < 0.05$) lowered STS activity compared to control (Figure 3.2B). There was no change in STS activity in intact HCT116 cells treated with IFN-γ and IL-4 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours (Figure 3.2D). However, higher doses of TNFα after 48 hours had no effect. Furthermore, STS activity was significantly increased in Colo205 cells treated with TNF α (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 hours to 149% ($P < 0.01$), 169% ($P < 0.001$) and 187% ($P < 0.001$) respectively compared to the control (Figure 3.3A and 3.3B). However, IL-6 treatment for 24 or 48 hours did not significantly alter STS activity compared to control.

3.3.3 The effect of high dose TNFα on STS activity at 4 hours treatment and in different CRC cell lines

As only TNFα showed any effects on STS activity in both HCT116 and Colo205 cells, it was decided to examine further TNFα effects on STS activity in other CRC cell lines. It was also determined to ascertain whether the impact of TNFα on STS activity could be induced rapidly over 4 hours compared to later time points.

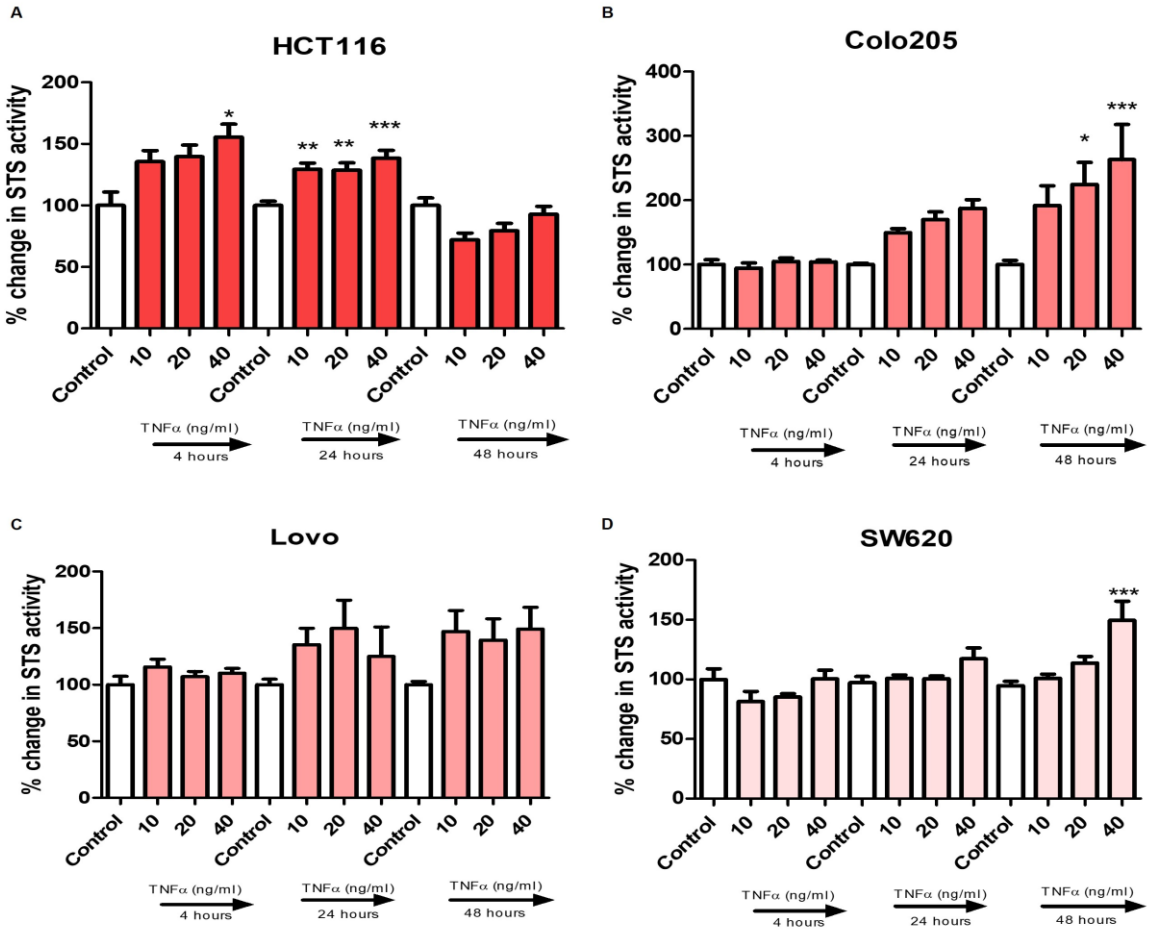


Figure 3.4: The effect of TNFα on STS activity in intact LoVo, Colo205, HCT116 and SW620 cells. STS activity was measured in HCT116 (A), Colo205 (B), LoVo (C) and SW620, cell lines treated with inflammatory mediators TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 4, 24 and 48 hours. Data represents STS activity n = 3, mean ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test, *P< 0.05, **P < 0.01 and ***P < 0.001 compared to control.

All CRC cell lines (HCT116, Colo205, LoVo, and SW620) were treated for 4, 24 and 48 hours with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml). STS activity was significantly increased in intact HCT116 cells treated with TNFα 40 ng/ml for 4 (155 %, $P < 0.01$). With 24 hours of incubation, there was a significant increase in HCT116 cells after 10 ng/ml, 20 ng/ml, and 40 ng/ml with (129%, 128% and 139 %) respectively, compared to the control (Figure 3.4A). STS activity was also significantly increased in intact Colo205 cells treated with TNFα (20 ng/ml ($p < 0.05$) and 40 ng/ml ($p < 0.001$) for 48 hours with a percentage (224 % and 263 %) (Figure 3.4B). LoVo cells showed a non-significant increase in STS activity after adding TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 4, 24 and 48 hours (Figure 3.4C), whereas SW620 cells only showed a significant increase in STS activity after 40 ng/ml of TNFα treatment for 48 hours with 149%, $p < 0.001$ (Figure 3.4D).

3.3.4 The effect of low dose TNFα on STS activity assay in intact CRC cell lines

As relatively high doses of TNFα increased STS activity in some CRC cell lines, it was decided to examine whether lower TNFα doses had similar effects. Thus, low-dose TNFα treatment was performed on intact HCT116, LoVo, Colo205, and SW620 cells. All cell lines were treated with TNFα (2.5 ng/ml, 5 ng/ml, 7.5 ng/ml) for 4, 24 and 48 hours.

Figure 3.5A shows STS activity was significantly increased in intact HCT116 cells treated with TNFα at 5 and 7.5 ng/ml for 4 hours (140% and 166 % compared to the control). In Colo205 cells, STS activity showed a non-significant increase across all time points and all doses in response to TNFα treatment (Figure 3.5B). In LoVo cells, a non-significant increase in STS activity was observed after adding TNFα (2.5 ng/ml, 5 ng/ml, 7.5 ng/ml) for 4 hours but not at other time points (Figure 3.5C). Whereas in SW620 cells,

there was only a non-significant increase in STS activity after 4 hours of 7.5 ng/ml of TNFα treatment (Figure 3.5D).

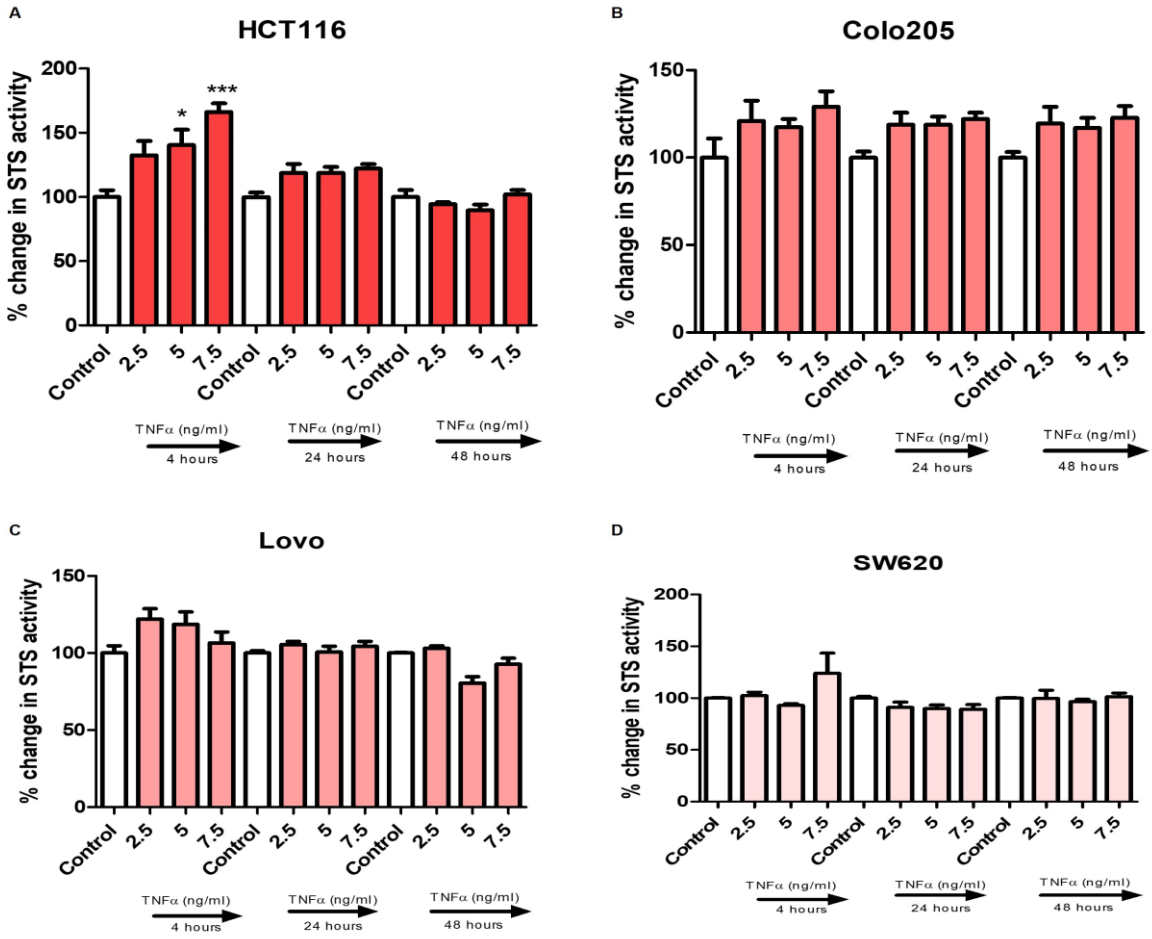


Figure3.5: The effect of a lower dose TNFα on STS activity in intact HCT116, Colo205, LoVo, and SW620 cells. STS activity was measured in HCT116 (A), Colo205 (B), LoVo (C) and SW620, cell lines treated with TNFα treatment (2.5 ng/ml, 5 ng/ml, 7.5 ng/ml) for 4, 24 and 48 hours. Data represents STS activity n = 3, mean ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test, *P< 0.05 and ***P < 0.001 compared to control.

3.3.5 The effect of TNFα on STS activity in lysated CRC cell lines

As STS activity was increased in some CRC cell lines in intact cells, it was important to test whether this was a real change in STS enzyme activity or a potential change in the ability of CRC cells to take up the E_1S substrate across the cell wall. Thus, STS activity of the cell lysate of CRC cell lines was measured. All cell lines were originally treated for 24-48 hours with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml), after which time they were lysated using RIPA buffer. Figure 3.6A shows there were no changes in STS activity in lysated HCT116 cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. In Colo205 cells, STS activity was increased after 48 hours of incubation with TNFα (40 ng/ml). However, this change was not significant (Figure 3.6B). STS activity was significantly increased in LoVo cells treated with TNFα (20 ng/ml, 40 ng/ml) after 48 hours (195% and 169% compared to the control, respectively) (Figure 3.6C). In SW620 cells, there is a non-significant increase in STS activity after 48 hours of treatment of TNFα at all doses tested (Figure 3.6D).

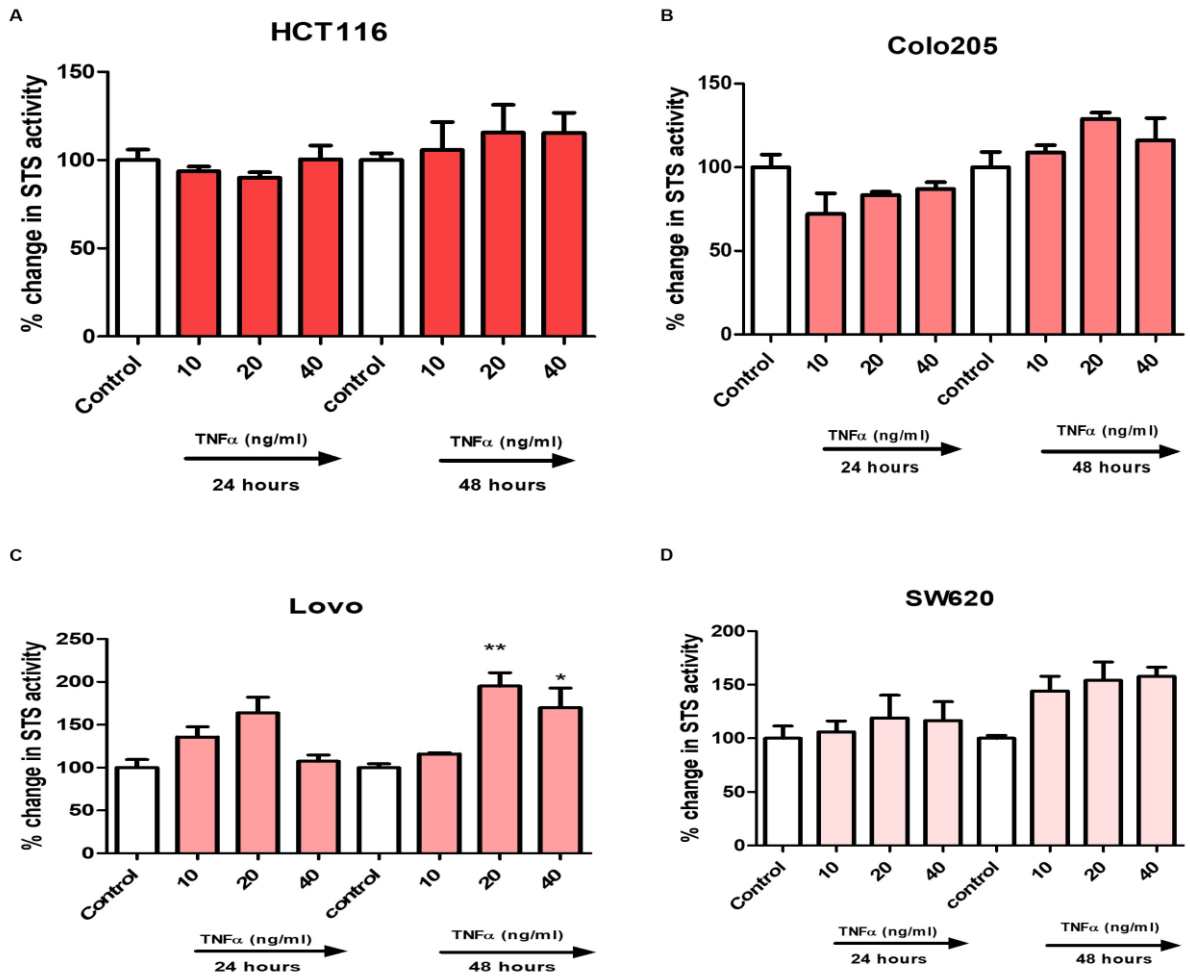


Figure 3.6: The effect of high doses of TNFα on STS activity in HCT116, Colo205, LoVo, and SW620 CRC cells that have been lysated**.** STS activity measured in lysated cells including, HCT116 (A), Colo205 (B), LoVo (C) and SW620, cell lines treated with TNFα treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Data represents STS activity n = 3, mean ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test, *P< 0.05 and **P < 0.01 compared to control.

3.3.6 Effect of TNFα in combination with oestrogens on HCT116 and Colo205 proliferation

CRC development and proliferation are affected by oestrogen. Steroid sulfatase (STS) hydrolyses the sulfated forms of biologically active oestrogens, oestrone (E1) and oestradiol (E2), to produce their active forms. Cellular factors, such as post-translational modifications, as well as the overall physiological state, influence STS activity. Thus, it is important to use a cellular environment to provide insight into how it works. Due to our findings that only TNFα affected STS activity in both HCT116 and Colo205 cells, we decided to further examine the effects of TNFα on proliferation in both HCT116 and Colo205 cells after treatment with 100 nM of E1S, E1 and E2. Cell proliferation of HCT116 and Colo205 was performed using a BrdU cell proliferation assay. Therefore, CRC cells were treated with TNFα (40 ng/ml) for 24 - 48 hours, with 100 nM of E1S, E1 and E2, respectively, for 48 hours.

Figure 3.7A shows a significant increase in HCT116 cells treated with E2 (100 nM) to 141% ($P < 0.01$) and a significant increase in HCT116 cells treated with 100 nM of E1S, E1 and E2 for 48 hours & TNFα (40 ng/ml) for 24 hours to 168% ($P < 0.001$), 152% ($P < 0.001$) and 154% ($P < 0.001$) respectively compared to the control. Also, Figure 3.7B shows a significant increase in HCT116 cells treated with E1S (100 nM) for 48 hours & TNFα (40 ng/ml) for 48 hours to 149% ($P < 0.01$). Similarly, Figure 3.7C shows a significant increase in Colo205 cells treated with 100 nM of E1S, E1 and E2 for 48 hours & TNFα (40 ng/ml) for 24 hours to 265% ($P < 0.001$), 235% ($P < 0.001$) and 193% ($P < 0.05$) respectively compared to the control. Also, Figure 3.7D shows a significant increase in Colo205 cells treated with E1S (100 nM) for 48 hours & TNFα (40 ng/ml) for 48 hours to 239% ($P < 0.01$).

CRC development and progression have been linked to several nuclear receptors that regulate multiple signalling pathways and transcription factors, altering cell behaviour. As nuclear receptors play an increasingly important role in CRC, their ability to influence a variety of tumour-related processes, including proliferation, differentiation, homeostasis, migration, invasion, and cell death. These receptors such as Estrogen Receptors (ERs), Estrogen-related Receptors (ERRs), Androgen Receptors (ARs), Progesterone Receptors (PRs), Peroxisome Proliferator-activated Receptors (PPARs) help in the development and progression of CRC through the regulation of various transcription factors and signalling cascades (Mulholland et al., 2005, Degirolamo et al., 2011, Manickasamy et al., 2024).

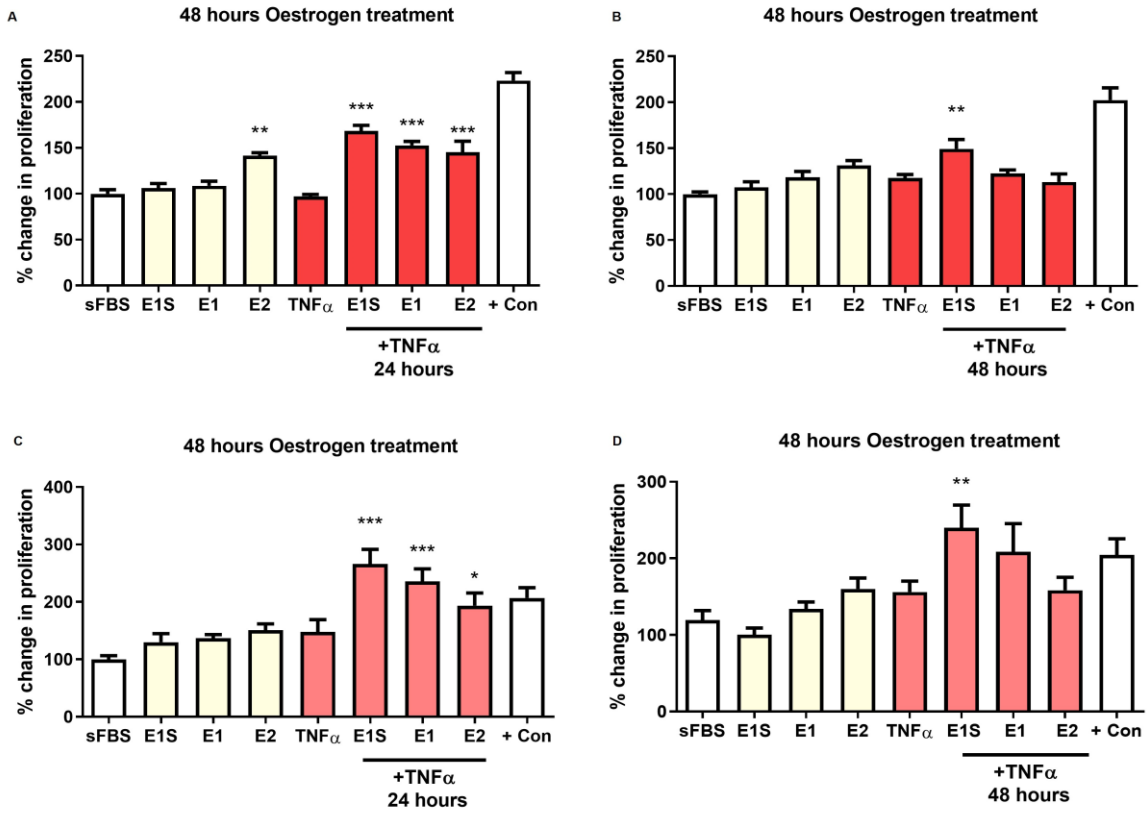


Figure 3.7: The effect of TNFα on oestrogen-induced proliferation in HCT116 and Colo205 cells. HCT116 and Colo205 Cells were treated with 40ng/ml of TNFα alone and 40ng/ml of TNFα with 100 nM of E1S, E1 and E2 for 24 hours (A&C) and 48 hours (B&D). HCT116 and Colo205 cells were treated with 100 nM of E1S, E1 and E2 for 48 hours (A, B, C & D). Cells that had not been treated with TNFα and oestrogen served as positive (RPMI+FBS media) and negative controls (phenol free RPMI+sFBS). Data are expressed as a percentage of control values. Data represents % change in proliferation ± SD, n = 4. Statistical analysis is a one-way ANOVA with Tukey post-test, *** $p < 0.001$ **$p < 0.01$ compared to control.

5.3.7 Oestrogen metabolism in CRC cells

The data above suggests that TNFα may affect the activity of the STS enzyme. LC-MS/MS was now used to examine the effects of TNFα treatment on oestrogen metabolism. HCT116, Colo205, LoVo, and SW620 were cultured and treated with 40ng/ml of TNFα for 24 hours. Then, cells were incubated in media containing 100 nM of E1S for a further 24 hours. The resultant media was then extracted and analysed using LC-MS/MS.

Both the HCT116 and Colo205 cells treated with TNFα (40ng/ml) have shown an increase in HST116 and a significant increase in Colo205 (*P<0.05) in the E1 concentration (2.34 ± 0.46 nM and 2.83 ± 0.34 nM) compared to untreated cells (1.1 ± 0.30 nM and 0.986 ± 0.62 nM) (Figure 3.8A), indicating increased STS activity in TNFα treated cells. LoVo cells treated with E1S followed by TNFα (40ng/ml) show a decrease in the E1 concentration (0.98 ± 0.34 nM) compared to untreated cells (1.1 ± 0 nM), and there was no change in the E1 concentration in SW620 cells compared to the control. None of these changes are significant.

When E2 concentrations were measured after E1S (100nM) and TNFα (40ng/ml) treatment, differences between groups were evident (Figure 3.8B). Both the HCT116 and Colo205 cells treated with TNFα have a decrease in the E2 concentration (1.46 ± 0.79 nM and 6.73 ± 4.36 nM) compared to untreated cells (4 ± 2.34 nM and 19.5 ± 22.69 nM). LoVo cells treated with TNFα show an increase in the E2 concentration (5.2 ± 4.5 nM) compared to untreated cells (2.69 ± 3 nM).

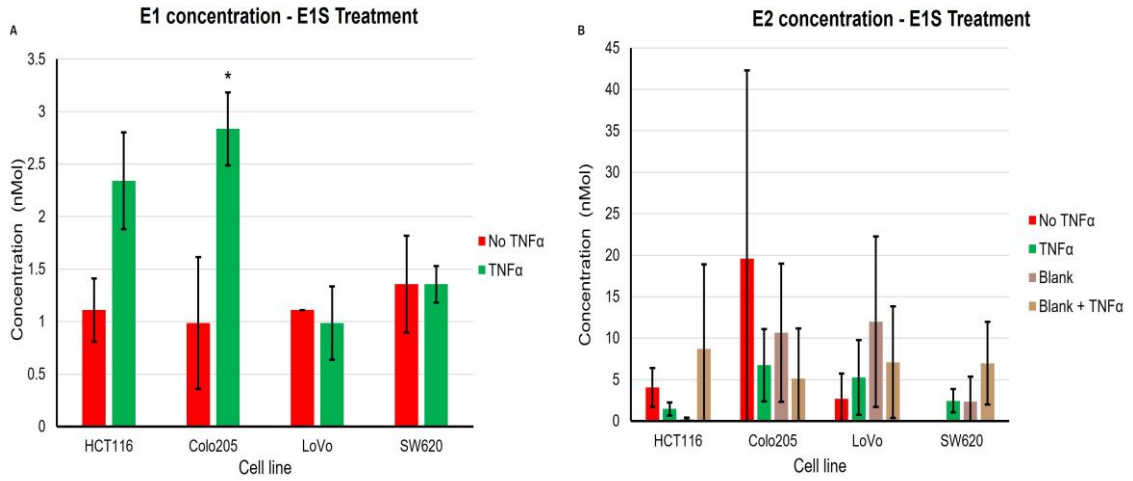


Figure 3.8: The effect of TNFα on oestrogen metabolism after E1S treatment in CRC cells. HCT116, Colo205, LoVo and SW620 cells were treated with TNFα (40 ng/ml) and 100 nM of E1S for 24 hours. Statistical analysis is a one-way ANOVA with Tukey post-test *P< 0.05 compared to control. n= 3 independent experiments.

3.4 Discussion

3.4.1 STS Activity in Colorectal Cancer: Implications

Many endocrine-related malignancies exhibit a major shift in steroid metabolism. Desulfation activity has been shown to be upregulated, while sulfation pathways are downregulated in many cancers. This promotes steroid desulfation and its consequent conversion to more active molecules (Mueller et al., 2015). Oestrogen dependency is a significant contributor to the development of CRC, and the enzyme STS is able to control the intratissue oestrogen levels in tumour cells. However, little is known regarding STS expression and activity during CRC development. Although previous results suggest that elevated STS expression is a poor prognostic sign in CRC patients, suggest that the role of STS activity and oestrogens in CRC is not yet fully understood (Sato et al., 2009; Gilligan et al., 2017). The regulation of STS expression and activity in CRC has been explored. It is shown here that STS activity is upregulating in some CRC cell lines in response to TNFα and other cytokines (IL-6, IFN-γ, and IL-4) did not impact STS activity, suggesting the regulation of STS is primarily a response to TNFα activity. The increase in STS activity was mainly observed in TNFα -treated intact CRC cells with limited changes in STS activity in cell lysates. This suggests that TNFα may alter substrate cell wall uptake mechanisms in CRC cells, allowing STS access to greater substrate availability.

It appears that STS activity is elevated in CRC at an early stage of the disease, but it is not yet known if this is better or worse for the prognosis of the disease (Gilligan et al. 2017). STS, which hydrolyses E1S and E2S to their active forms through the OATP4A1

transporter, is highly expressed in the CRC cell lines Caco2, HCT116, and HT29 (Gilligan et al. 2017).

3.4.2 TNFα Regulates STS Activity in CRC

The production of TNFα by infiltrating macrophages in CRC is linked with increased tumour diameter (Numata et al.,1991). Patients with CRC had significantly greater serum levels of IL-17A and TNFα than patients with UC, colon polyps, and healthy volunteers across the 5 groups. The CRC group had considerably greater TNFα levels than any of the control groups, and these proinflammatory cytokines may serve as CRC biomarkers (Ma et al.,2022).

A recent study by (Ghafoor, 2023) showed while there was no significant difference in the levels of TNFα at stages I or II between the Breast cancer patients and the healthy control group, there was a significant difference in the levels at stage III ($P< 0.05$) between the Breast cancer patients and the control group. TNFα serum levels were also significantly higher in invasive Breast Cancer patients than in healthy controls, and they were also connected with the Breast Cancer stage (Soria et al., 2011). Also, according to studies, a strong tumour's invasiveness and a bad prognosis are associated with high serum levels of IL-6, IL-8, and TNFα in breast cancer patients (Alokail et al., 2014).

STS activity was increased in HCT116, Colo205, LoVo, and SW620 cells by TNFα. The STS activity of HCT116 and Colo205 cells was significant (Figures 3.2 & 3.3). Also, LCMS results of HCT116 and Colo205 cells treated with TNFα (40ng/ml) showed an

increase in the E1 concentrations compared to untreated cells incubated in media containing 100 nM of E1S (Figure 3.8A). In response to TNFα treatment, STS activity was increased. These findings are consistent with previous findings in other cell lines *in vitro* on prostate and breast cancer, which have suggested that TNFα may partly control STS activity. TNFα and IL-6 stimulation increased STS activity in MCF7 breast cancer cells, whereas TNFα and IL-6 enhanced both endogenous and transfected STS activities. This finding argues that IL-6 and TNFα can increase STS activity by post-translationally altering the enzyme or by increasing substrate availability in conjunction with the absence of control of STS mRNA. STS activity is increased by the cytokines IL-6 and TNFα without the aid of promoter/enhancer elements, demonstrating that changes in membrane fluidity or organic anion transporters indirectly influence STS activity by converting cysteine to formyl glycine in the active site, allowing for greater absorption of the hydrophilic substrate (Newman et al., 2000). TNFα effects have been investigated both *in vitro* and *in vivo* to determine how inflammation controls STS. When TNFα was applied to CRC cells, the STS activity was measured using ^{3}H-E1S desulfation. TNFα treatment resulted in an increase in STS activity in CRC cells, suggesting that this response is present in all cancer types and that this elevation in STS activity may be more of a typical physiological response to inflammation than just a cancer-related trait (Gilligan et al., 2015).

Also, a further study showed that STS activity in HCT116 cells was considerably raised by TNFα at concentrations of 20 ng/ml and 40 ng/ml, respectively, with ($P<0.05$) and ($P<0.001$). However, IL6 and IL4 did not influence STS activity. These results demonstrate that in CRC, TNFα enhances both E1S uptake by OATP2B1 and its

following hydrolysis by STS (Varma et al., 2018). According to Gilligan et al. (2017) research, cell protein from cell lysates had considerably higher STS activity, which suggests that E1S cell membrane transporting may be the limiting factor in oestrogen hydrolysis. Therefore, E1S transport and subsequent oestrogen activity may be significantly influenced by the patterns of OATP expression in CRC. The two E1S transporters with the highest levels of expression in CRC cell lines are OATP4A1 and OATP2B1. We examine the STS activity in both intact and cell lysates to investigate how inflammation affects oestrogen metabolism. HCT116, Colo205, LoVo and SW620 cells were chosen from CRC cells. After being treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours, both lysated HCT116 and Colo205 showed an increase in STS activity, whereas lysated LoVo and SW620 demonstrated an increase in the STS activity after being treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

The STS regulation mechanism in breast cancer is poorly understood. It is possible that a post-transcriptional alteration of the enzyme was how IL-6 and TNFα together enhanced STS activity and increased enzyme activity. Also, it's likely that TNFα and IL-6's capacity to increase STS activity in MCF-7 cells is the result of a post-transcriptional effect. It's conceivable that the cytokines alter membrane permeability to improve the availability of E1S substrate (Newman et al.,2000).

3.4.3 Effect of Oestrogen and TNFα in CRC Proliferation

Oestrogen metabolism in the colon is poorly understood. However, prognostic relevance has been shown for the expression of the oestrogen metabolism-related enzymes HSD17Bs, STS, and SULT1E1. Furthermore, it appears that ERβ modulates protective

effects, although it remains unclear which metabolite of oestrogen, E2 or E1, is more active (Foster et al., 2013).

Various studies using animal models revealed a decreased risk of CRC when oestrogens were present. A study by Weyant et al. (2001) suggested that endogenous oestrogens suppress Apc-associated tumour development, and E2's ability to prevent tumours is linked to a rise in ERβ and a decrease in ERα expression in the target tissue. Also, the oestrogenic involvement in CRC is that oestrogen initially protects against CRC, but once developed, it may enhance proliferation and tumour growth (Foster et al., 2013). In a study done by (Wada-Hiraike et al., 2006) on ERB-knockout mice, its loss causes an increase in proliferation, loss of differentiation, and decrease in apoptosis in the colon epithelium, indicating a critical role for this receptor in the regular organization and structural maintenance of the colon. E2 (10–1000 nmol/L) stimulation of colon cancer cell lines results in the expression of mainly ERB, with a dose-dependent activation of apoptosis (Marino, 2014). E2 can activate the GPER, which has been shown to promote CRC proliferation (Bustos et al., 2017; Gilligan et al., 2017). Another research by Sasso et al., 2019 found that progesterone and oestradiol work together to decrease colon tumour development and promote apoptosis, most likely through ERβ activation.

According to research by Fiorelli and colleagues, E2 enhanced the proliferation of HCT8 cells but prevented the development of LoVo cells at physiological concentrations (1–100 pmol/L). The LoVo cell line, when exposed to E2, increases proliferation through a FASN-mediated pathway (Santolla et al., 2012). The local concentrations of active oestrogens and the subsequent effect will be determined by the CRC's capacity to take up

and subsequently metabolize E1S (Gilligan et al., 2017). The primary enzyme responsible for hydrolyzing E1S into E1 is steroid sulfatase (Mueller et al., 2015). According to Foster et al. (2006), the STS activity is known to directly promote the proliferation of oestrogen-dependent breast cancer. E1S stimulates the proliferation of colon cancer cells through the following series of reactions: uptake to the cells, desulfation by STS, conversion of E1 to E2 by 17-hydroxysteroid dehydrogenase, binding to nuclear oestrogen receptor, and control of gene transcription.

We examine the proliferation in CRC cells after treatment with 40ng/ml of TNFα and 100 nM of E1S, E1 and E2 for 24 and 48 hours. There was a significant increase in the proliferation of HCT116 and Colo205 cells treated with TNFα and E1S after 24 and 48 hours of incubation. Also, a significant increase in the proliferation of HCT116 cells treated with E2 after 24 hours and a non-significant increase in the proliferation in both HCT116 and Colo205 cells after 24 and 48 hours of incubation.

3.5 Conclusion

It has been demonstrated that elevated STS activity may lead to increased CRC growth after exposure to TNFα at varied concentrations and incubation times; lower to higher concentrations and shorter to longer incubation times were found to have a statistically significant relationship. Although there was some variation in the outcomes based on how each CRC cell line responded, HCT116 and Colo205 displayed the greatest outcomes. Understanding how expression is regulated is crucial since malignant colon tissues are likely to have high STS activity. In order to answer this, I investigated if the cytokine TNFα, which can activate STS, could be implicated. It's also possible that these cytokines

change membrane permeability, enhancing substrate availability and increasing STS. I have demonstrated that TNFα causes an increase in the STS and proliferation of CRC. This suggests potential therapeutic ways, such as the use of TNFα and STS inhibitors in CRC.

CHAPTER 4. REGULATION OF HSD17Bs BY TNFα and IL-6

4.1 Introduction

CRC is one of the most frequent cancers worldwide, and its occurrence is rising. There is an increased incidence of CRC among men than among pre-menopausal women, suggesting that 17β-oestradiol (E2) plays a protective role (Qiu et al., 2004; Brenner et al., 2007). According to a review by Vihko, the development of cancers of the colon, breast, uterine endometrial, or prostate was thought to be enabled by a decrease in oxidative activity and an increase in reductive activity toward oestrogens and androgens (Vihko et al., 2002). The occurrence of colorectal and endometrial cancer in humans has also been connected to oestrogen (Foster, 2013). Furthermore, it is anticipated that any modifications in the peripheral ratios of these enzymes and receptors will affect the development of CRC (Foster, 2013).

In colon cancer, the mRNA expression of HSD17B types 1 and 2 enzymes was studied by (Oduwole et al., 2003; Rawłuszko et al., 2011). HSD17B1 expression was investigated in CRC cell lines (HT29, SW707) by (Rawuszko et al.,2011); in CRC from the proximal colon, DNA methylation in the 5' flanking region of HSD17B1 was increased compared to normal colonic epithelium. As a result of these epigenetic differences, HSD17B1 levels were decreased significantly in cancers located in the proximal colon. A study by (Gilligan et al., 2017) showed that human CRC does not express HSD17B1 protein.

HSD17B2 and HSD17B4 mediated the conversion of E2 to E1, which is antiproliferative in human colon cancer cell lines and occurs in the colon. It was discovered that malignant colon tissue had larger concentrations of E1 than non-neoplastic colon tissue but not E2.

This discovery is in line with the high amounts of HSD17B2 expression found in this tissue (Foster, 2021). In most cases, HSD17B2 expression was downregulated in CRC. Also, in comparison to healthy colon tissue, colon cancer has reduced levels of HSD17B2 and HSD17B4 expression, which lowers E1 levels in the tumour. HSD17B7 is highly expressed in human ductal and breast cancer cell lines, and oestradiol significantly upregulates HSD17B7 expression in MCF-7 cells at the mRNA and protein levels (Shehu et al., 2011). CRC expresses the HSD17B7, and compared to healthy colon tissue, it is expressed more frequently in CRC (Gilligan et al., 2017). Finally, the expression of HSD17B12 has been associated with CRC (Gilligan et al., 2017).

TNFα is a crucial cytokine that encourages the growth of CRC (Braumüller et al., 2023). Also, the experimental data shown by (Zhang et al., 2012) reported that TNFα regulates the expression of HSD17B1. This enzyme effectively catalyses the conversion of E1 into E2, which might be used to explain the change in the E1/E2 ratio.

Both ERα and ERβ, with conflicting results on their roles in colon carcinogenesis, were found in CRC clinical samples, animal models, and several colon cancer cell lines (Mahbub, 2022). The two ERs have been shown to have opposing effects on CRC cells, with ERα increasing colon cancer cell proliferation and ERβ inducing colon malignant cells to undergo apoptosis (Das et al., 2023).

ERβ expression was significantly higher in normal epithelial cells than in colon adenocarcinoma cells in both genders (Konstantinopoulos et al., 2003). Cells from colon adenocarcinomas express significantly less ERβ, which correlates with their loss of

differentiation (Konstantinopoulos et al., 2003). Preclinical evidence indicates that oestrogen's protective effects are mediated through ERβ, and they also support a function for oestrogen and its receptors in the development and progression of CRC (Barzi et al.,2013). Additionally, it has been demonstrated that colon cancer patients with diminished ERβ have a worse prognosis (Foster, 2013), and malignant colon cells lose differentiation as ERβ expression declines, regardless of where the tumour is located (Konstantinopoulos et al., 2003).

Additionally, non-genomic effects have been reported via membrane-bound GPER1 activation (Levin, 2005). Colon cancer cells require the activation of GPER-mediated signaling in order to proliferate and survive (Das et al., 2023). CRC patients with low intestinal expression of GPER have a poorer survival rate than those with high intestinal expression of GPER (Liu et al., 2017). There is an association between higher GPER expression and poorer relapse-free survival in women with stage 3 and 4 CRC, but not in men, regardless of stage. The study by Liu et al. demonstrated that GPER is involved in numerous processes and pathways that affect CRC cell proliferation. The expression of GPER in both *in vitro* and *in vivo* studies has been confirmed to regulate proliferation, migration, and invasion of CRC cells through cell cycle, endoplasmic reticulum stress, and modulation of apoptosis.

In order to form E1, E1S must be hydrolysed, followed by E2 via HSD17Bs activity, and it is unclear how oestrogens act in CRC. A significant risk factor for CRC has been discovered as inflammation, and although HSD17B enzymes may be involved in this

process, their exact role is yet unknown. This chapter aims to investigate the relationship between HSD17B enzymes and ERs and inflammation, as well as their possible contribution to CRC. In order to discover these effects, qRT-PCR was used to determine the expression of steroidogenic enzymes in CRC cells treated with varied amounts of cytokines. An LC-MS/MS method was also used to quantify oestrogen metabolites.

4.2 Materials and Methods

4.2.1The Cancer Genome Atlas (TCGA) Data Collection and Analysis

The TCGA data collection and analysis were performed as described in section 2.9.

4.2.2 Cell Culture

The HCT116, Colo205, LoVo, and SW620 cells were cultured as described in section 2.1.

4.2.3 Cytokines treatment

Cytokines TNFα and IL-6 were reconstituted in phosphate buffered saline (PBS) (PBS, Oxoid, and Hampshire, England) containing 0.1% human albumin serum (HAS) (Sigma) and stored in working aliquots at -80ºC. All cytokines were purchased from (PeproTech, London, UK).

The HCT116, Colo205, LoVo, and SW620 cells were seeded into 6-well plates at 100,000 cells per well and treated with different concentrations of cytokines (10 ng/ml, 20 ng/ml, 40 ng/ml) of TNFα and (10 ng/ml, 20 ng/ml, 40 ng/ml) of IL-6. Cells were incubated for

24 h and 48 h at 37^{o}C. After 24-48 hours, the media was removed to run the RNA extraction, cDNA synthesis and real-time PCR to determine the expression of steroidogenic enzymes in CRC cell lines treated with various cytokines. Untreated cells from all cell lines served as controls.

4.2.4 RNA Extraction, cDNA Synthesis

RNA extraction followed by cDNA synthesis was performed as described in sections 2.4 to 2.5.

4.2.5 Real Time PCR

The RT-PCR has been described previously in section 2.6.

4.2.6 Cells treatment prior to LC-MS/MS

CRC cells HCT116, Colo205, LoVo and SW620 were seeded at 200,000 cells per well in 6-well plates for 24 hours. After this time, the media was replaced with 10% sFBS RPMI phenol-free media. Control wells were prepared for each cell line, and the 40ng/ml of TNFα was added to specific wells and incubated for 24 hours at 37°C. Later, cells were incubated in a plain medium or medium containing 100 nM of E1 and E2 for 24 hours at 37°C. Media was collected and frozen at -20^{o}C until extraction and analysis using LC-MS/MS. Finally, cells were lysed with RIPA buffer, and the protein concentration was quantified using the BCA assay.

4.2.7 Mass Spectrometry Extraction

Mass spectrometers were performed as described in section 2.8.

4.2.8 Statistical Analysis

Statistics were performed as described in section 2.10.

4.3 Results

4.3.1 The expression of HSD17B in TCGA COADREAD dataset

The HSD17Bs enzymes play an important role in the synthesis of sex steroid hormones, particularly active oestrogens and androgens (Bouguen et al., 2015; Oduwole et al., 2003). After E1S is desulfated, HSD17B1, 7, and 12, reduce it to E2, which is the most efficient for ER receptor binding (Zhu et al., 2006). In contrast, 2 and 4 catalyse the oxidation of HSD17Bs (Adamski et al., 1995; Vihko et al., 2002). Here, analysis of the TCGA COADREAD dataset has been used to study HSD17B expression and to investigate whether HSD17Bs mRNA expression is associated with CRC; the COADREAD TCGA cancer dataset containing HSD17Bs mRNA expression from 382 CRC patient samples was analysed and compared to 51 normal colorectal epithelial samples (Figure 4.1).

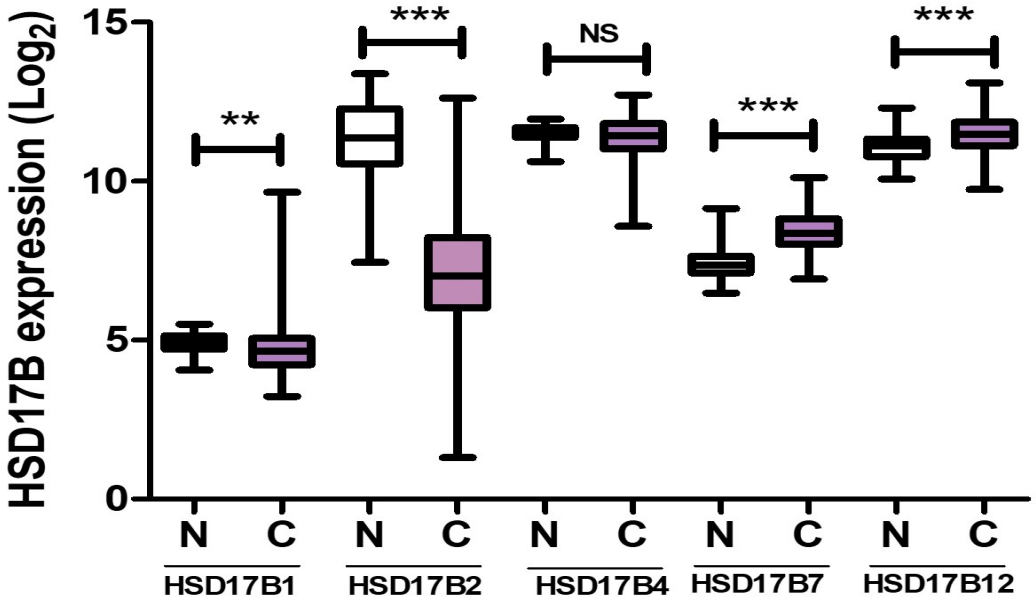


Figure 4.1: The expression of the HSD17Bs responsible for oestrogen metabolism in normal and CRC patients from COADREAD TCGA data. **P < 0.01 versus controls, ***P < 0.001 versus controls. N- Normal and C-CRC (N=51 and C=382). For the analysis, two-tailed Mann-Whitney (U tests) was used.

Patients with CRC had elevated expression of HSD17B7 and HSD17B12 (Figure 4.1). Conversely, HSD17B2 had very low mRNA expression in CRC compared to normal colon tissue. A significant increase in the expression of HSD17B7 and HSD17B12 mRNA was demonstrated when comparing CRC samples to the normal epithelial samples (***P < 0.001). There was a significant reduction in HSD17B2 mRNA expression in CRC samples ($p<0.001$) and a non-significant reduction in mRNA expression of HSD17B1. CRC and normal colorectal tissues did not show differences in HSD17B4 mRNA expression.

4.3.2 The survival outcomes for CRC patients with high or low HSD17Bs expressions

Survival outcomes for CRC patients were determined from TCGA COADREAD data to generate Kaplan-Meier survival curves. Specifically, these survival analyses focused on CRC survival data and the tumour's HSD17Bs and TNFα expression.

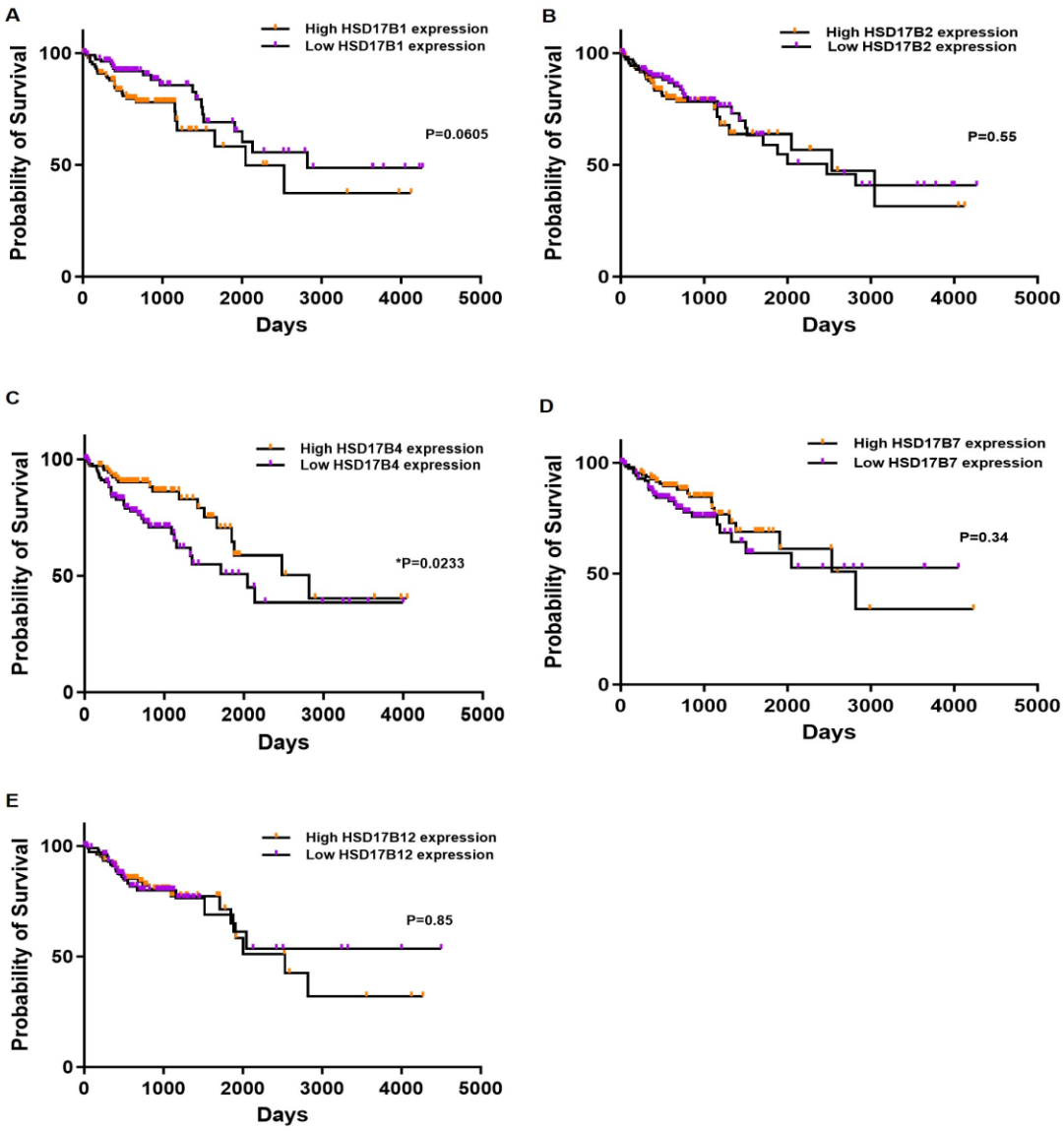


Figure 4.2: Survival outcomes for CRC patients HSD17Bs expression. TCGA COADREAD survival curves based on Kaplan-Meier analysis. The survival curves of high expression of HSD17B1 (A), or HSD17B2 (B), or HSD17B4 (C), or HSD17B7 (D) or HSD17B12 (E) compared to low HSD17B expression. Statistical analysis by using the log-rank test, *P< 0.05 compared to control.

As there were significant changes in HSD17B expression in CRC, it was next determined to analyse whether these effects impact survival outcomes for CRC patients. Therefore, TCGA COADREAD expression data and clinical survival data were analysed (Figure 4.2). Specifically, these survival analyses focused on CRC and the HSD17B genes. The survival outcome of CRC patients with low HSD17B1 expression has a high survival percentage. The survival of patients with high HSD17B4 expression has been improved. Patients with high HSD17B2, HSD17B7 and HSD17B12 expression had worse survival outcomes than those with low expression.

4.3.3 COADREAD analysis of TNFα expression correlation to HSD17Bs expression in CRC

As TNFα is known to alter the expression and activity of STS, it was hypothesised that this cytokine might also impact other oestrogen-metabolising enzymes. Thus, TCGA COADREAD, RNA-Seq dataset (n=433) was used to generate correlation graphs comparing normal and cancerous colon tissue expression of the HSD17Bs and TNFα (Figure 4.3). Analysis of COADREAD data demonstrated a significant correlation was seen between TNFα in CRC tissue and HSD17B4 (**P<0.004) (Figure4.3 F) and HSD17B12 (*P<0.016) (Figure4.3 J).

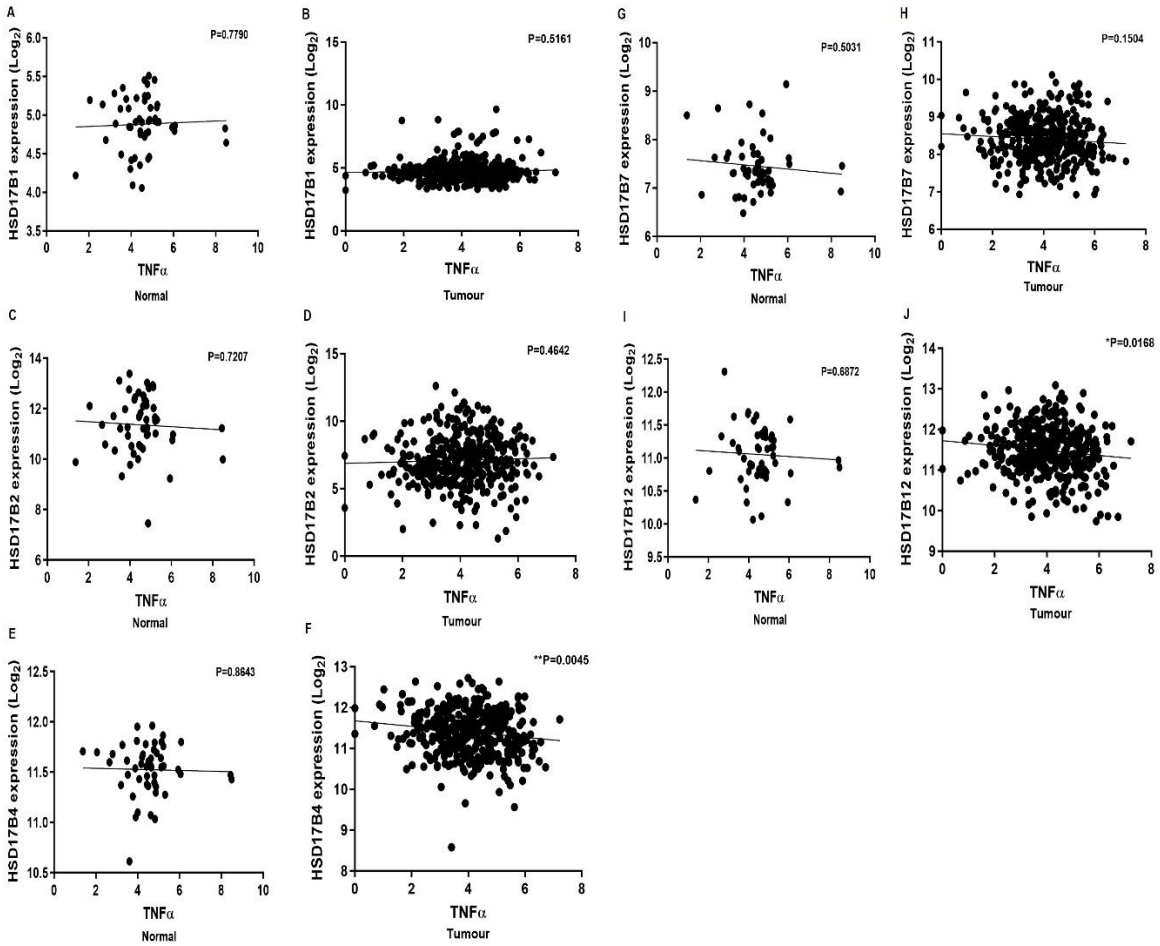


Figure 4.3: The COADREAD correlation of TNFα and HSD17Bs expression in CRC. Correlation between TNFα in CRC tissue and HSD17Bs expression in the COADREAD from the TCGA dataset. The correlation in normal and tumour between TNFα and HSD17B1 (A and B), HSD17B2 (C and D), HSD17B4 (E and F), HSD17B7 (G and H) and HSD17B12 (I and J). Correlation statistical analysis was done by using Prism, *** $p < 0.001$ **$p < 0.01$ compared to control. (n=433).

No correlation was seen between HSD17B1 and TNFα in normal tissue (P=0.77) (Figure 4.3A) and tumour CRC tissues (P=0.516) (Figure 4.3B). This was similar to HSD17B2, where no correlation was seen with TNFα in normal tissue (P=0.72) (Figure 4.3C) and tumour CRC tissues (P=0.46) (Figure 4.3D). However, a positive correlation was seen between HSD17B4 in tumour CRC tissues and TNFα (**P=0.0045) (Figure 4.3F), but no correlation was seen between these in normal tissue (P=0.86) (Figure 4.3E). For HSD17B7, no correlation was seen with TNFα expression in normal tissue (P=0.5) (Figure 4.3G) or tumour CRC tissues (P=0.15) (Figure 4.3H). Finally, a positive correlation was seen between HSD17B12 in tumour CRC tissues and TNFα (*P=0.016) (Figure 4.3I), and there was no correlation between HSD17B12 and TNFα in normal tissue (P=0.68) (Figure 4.3J).

4.3.4 The Effect of cytokines on HSD17B expression in CRC cell lines

It has previously been shown that in human CRC samples, there is a change in HSD17Bs expression (Rawłuszko et al., 2011; Gilligan et al., 2017). Little is known about what regulates this alteration in steroidogenesis (Vihko et al., 2002). To determine if TNFα or IL-6 affects steroidogenic enzymes, cell RNA was extracted from HCT116, Colo205, LoVo and SW620 treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24-48 hours. Since STS converts peripheral and circulating E1S to E1 by desulfating it, we then examined in the same human CRC samples whether cytokines altered the expression of the enzymes that oxidize E2 to E1 (HSD17B2 and HSD17B4) and reduce E1 to E2 (HSD17B1, HSD17B7, and HSD17B12).

Appendix I provides further results of the IL-4 and IFN effects on HSD17Bs expression in HCT116 cell lines.

4.3.4.1 The effect of cytokines (TNFα and IL-6) on HSD17B1 expression in HCT116 and Colo205 Cell Lines

HSD17B1 expression was measured by qRT_PCR in HCT116 (A) and Colo205 (B) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours (Figure 4.4).

There was no effect on HSD17B1 expression caused by TNFα treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours in both HCT116 and Colo205 cells (Figure 4.4). There was a non-significant increase in HSD17B1 expression caused by IL-6 treatment (10 ng/ml and 40 ng/ml) incubated for 48 hours and in Colo205 cells after treatment with IL-6 treatment (20 ng/ml) for 24 hours.

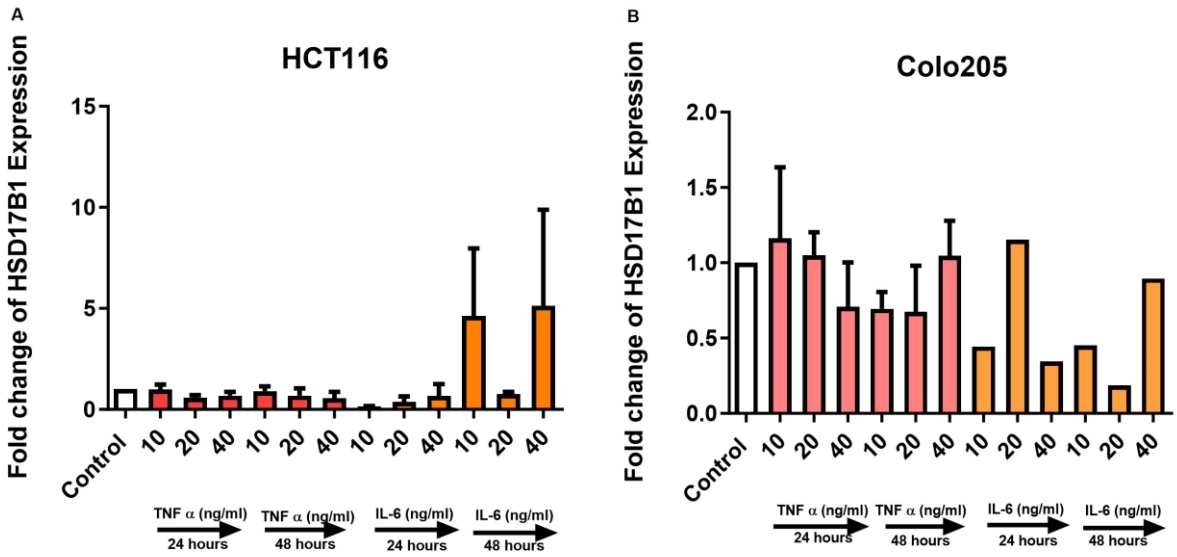


Figure 4.4: The effect of TNFα and IL-6 on HSD17B1 expression in HCT116 and Colo205 cells. HCT116 and Colo205 cells were treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24 & 48 hours. Statistical analysis is a one-way ANOVA with Tukey post-test. For the TNFα n=3 independent experiments and IL-6 n=1-2 independent experiments.

4.3.4.2 The Effect of TNFα on HSD17B1 expression in CRC Cell Lines

The mRNA expression of the HSD17B1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

There were no changes in HSD17B1 expression across all the cell lines (Figure 4.5). In general, HCT116 cells treated with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml) for 24 hours (0.98±0.48, 0.58±0.27 and 0.68±0.4 fold) and 48 hours (0.91±0.55, 0.67±0.74 and 0.56±0.5 fold decrease) have a non-significant decrease in the HSD17B1 expression, while LoVo cells showed a non-significant reduction in the HSD17B1 expression with TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 24 hours (0.47± 0.63, 0.34±0.46 and 0.38± 0.16 fold). As a result, TNFα does not affect the expression of HSD17B1. CRC patients with tumours containing high levels of TNFα and HSD17B1 mRNA had no difference from those with tumours containing low levels of TNFα and HSD17B1 mRNA (P = 0.98) (Figure 4.5 E).

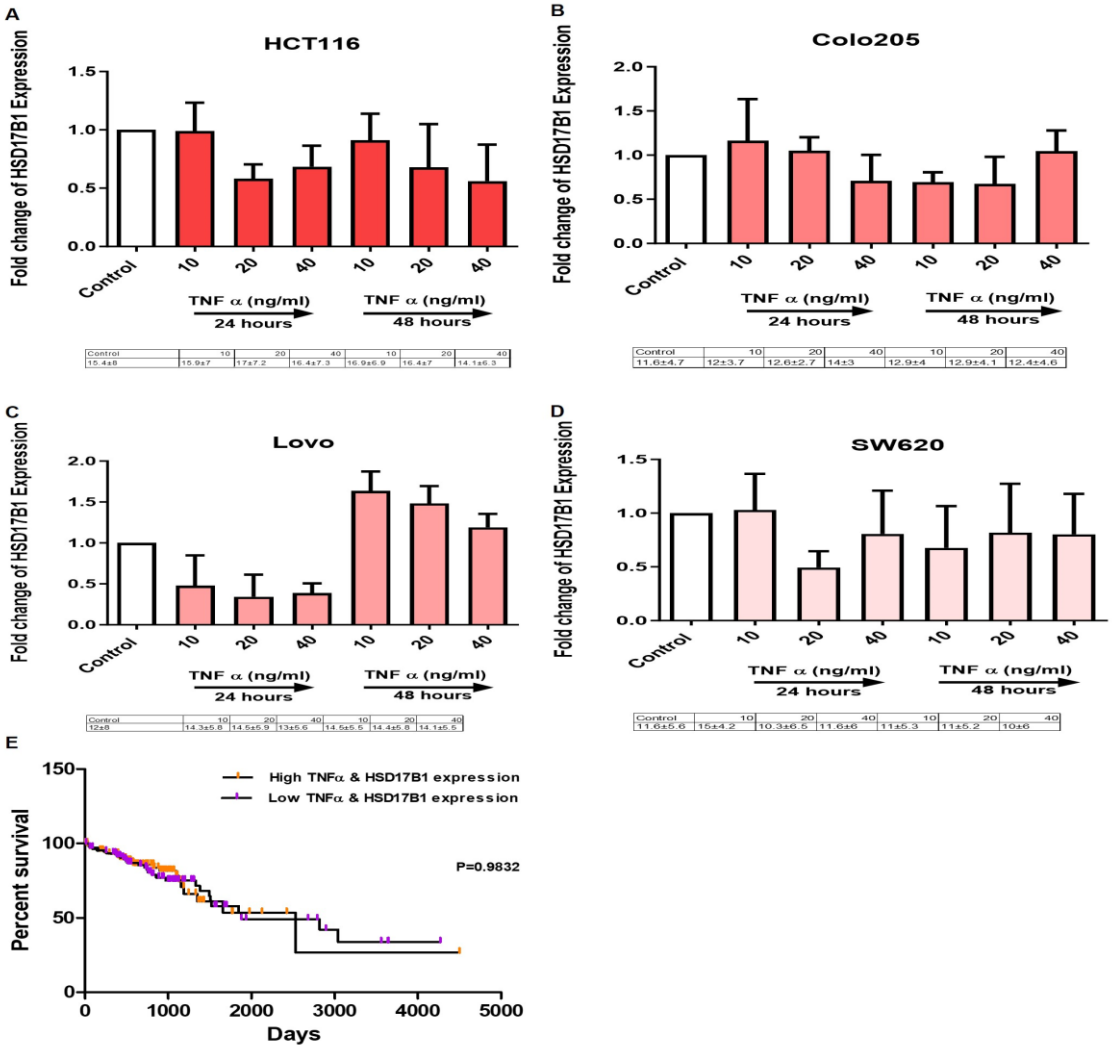


Figure 4.5: The effect of TNFα on HSD17B1 expression in HCT116, Colo205, LoVo and SW620 cell lines. The mRNA expression of the HSD17B1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ±SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of HSD17B1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

4.3.4.3 The effect of TNFα and IL-6 on HSD17B2 expression in HCT116 and Colo205

The mRNA expression of the HSD17B2 and RPLPO genes was measured by qRT-PCR in HCT116 (A) and Colo205 (B) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

Both HCT116 and Colo205 cells showed a reduction in HSD17B2 expression caused by TNFα and IL-6 treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Colo205 cells showed unchanged HSD17B2 expression caused by TNFα treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours (Figure 4.6).

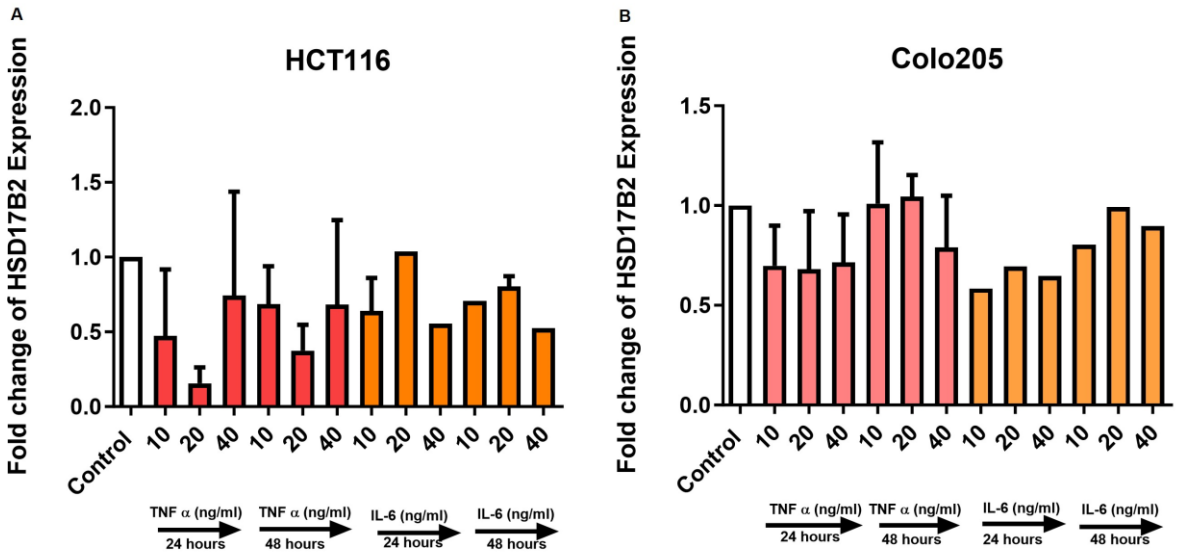


Figure 4.6: The effect of TNFα and IL-6 on HSD17B2 expression in HCT116 and Colo205 cells. HCT116 and Colo205 cells were treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24 & 48 hours. Statistical analysis is a one-way ANOVA with Tukey post-test. For the TNFα n=3 independent experiments and the IL-6 n= 1&2 independent experiments.

4.3.4.4 The effect of TNFα on HSD17B2 Expression in CRC Cell Lines

The mRNA expression of HSD17B2 and RPLPO was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

There was a non-significant decrease in HSD17B2 expression in HCT116 cells treated with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml) for 24 hours (0.47± 0.76, 0.15± 0.18 and 0.74± 1.2 fold) and 48 hours (0.68± 0.44, 0.37± 0.29 and 0.68± 0.97 fold decrease) that might be due to TNFα. Also, Colo205 and LoVo cells showed a non-significant reduction in HSD17B2 expression with TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 24 hours (0.69± 0.44, 0.68± 0.65 and 0.71± 0.53 fold of Colo205) and (0.87± 0.41, 0.77± 0.23 and 0.73± 0.28 fold of LoVo). Patients with tumours containing high amounts of TNFα and HSD17B2 mRNA exhibited low survival when compared to those with tumours containing low levels of TNFα and HSD17B2 mRNA (P=0.6) (Figure 4.7 E).

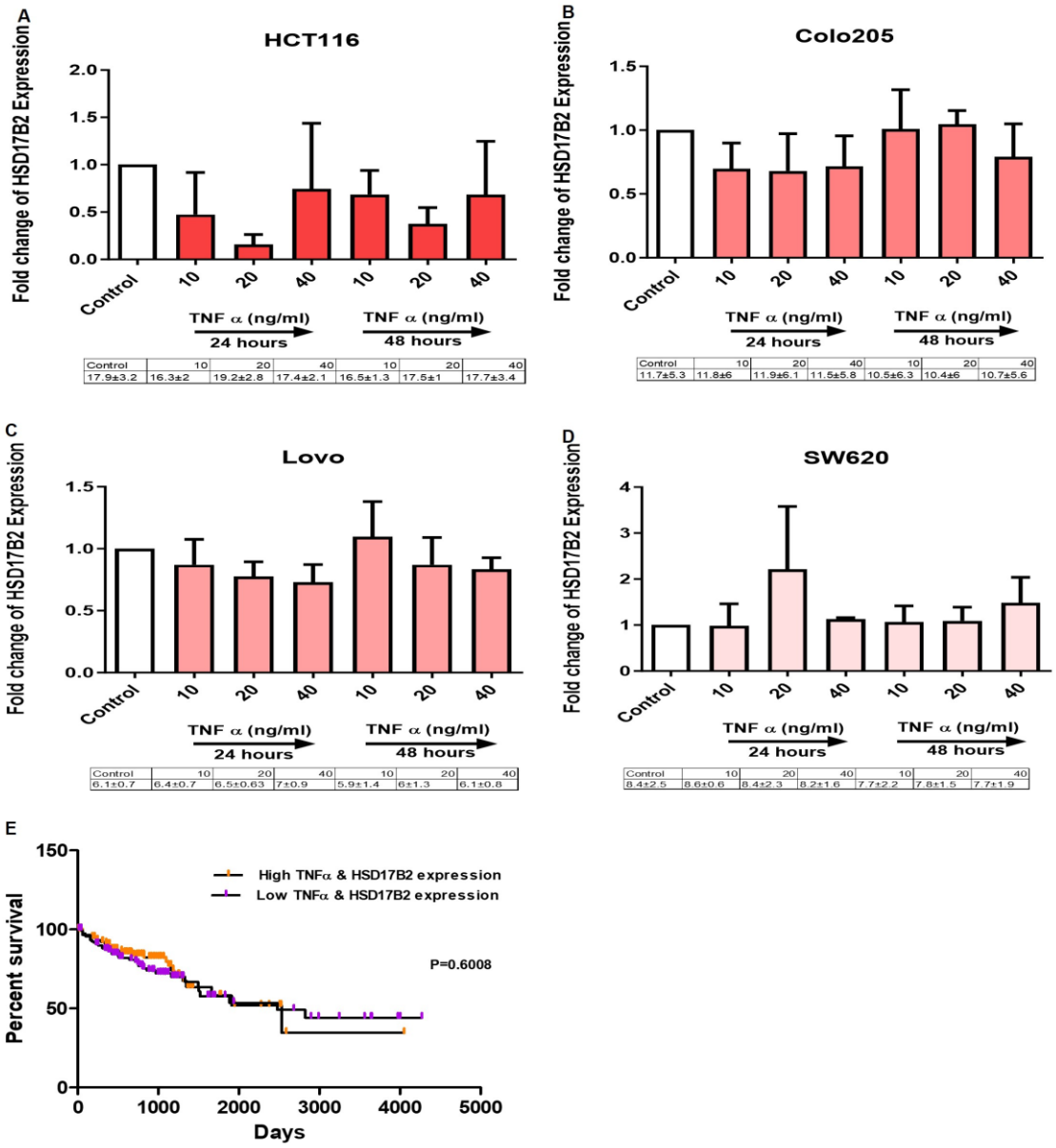


Figure 4.7: The effect of TNFα on HSD17B2 expression in HCT116, Colo205, LoVo and SW620 Cell Lines. The mRNA expression of the HSD17B2 and RPLPO genes was measured by qRT_PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of HSD17B2(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

4.3.4.5 The HSD17B2 gene expression in CRC stages and Sexes

According to previous studies (English et al., 1999; Oduwole et al., 2003; Gilligan et al., 2017), healthy colonic small intestines and surface epithelium express high levels of HSD17B2, whereas CRC cells and tissues show reduced levels of HSD17B2. The decrease in HSD17B2 expression has also been associated with colon cancer development (Vihko et al. 2002).

The expression of HSD17B2 was significantly reduced in all stages of the CRC compared to normal tissue (*** $p < 0.001$) (Figure 4.8A). Also, the expression of HSD17B2 was higher in normal tissue, especially in females and males (Figure 4.8B), with a significant reduction in both sexes in CRC patients (*** $p < 0.001$) (Figure 4.8B).

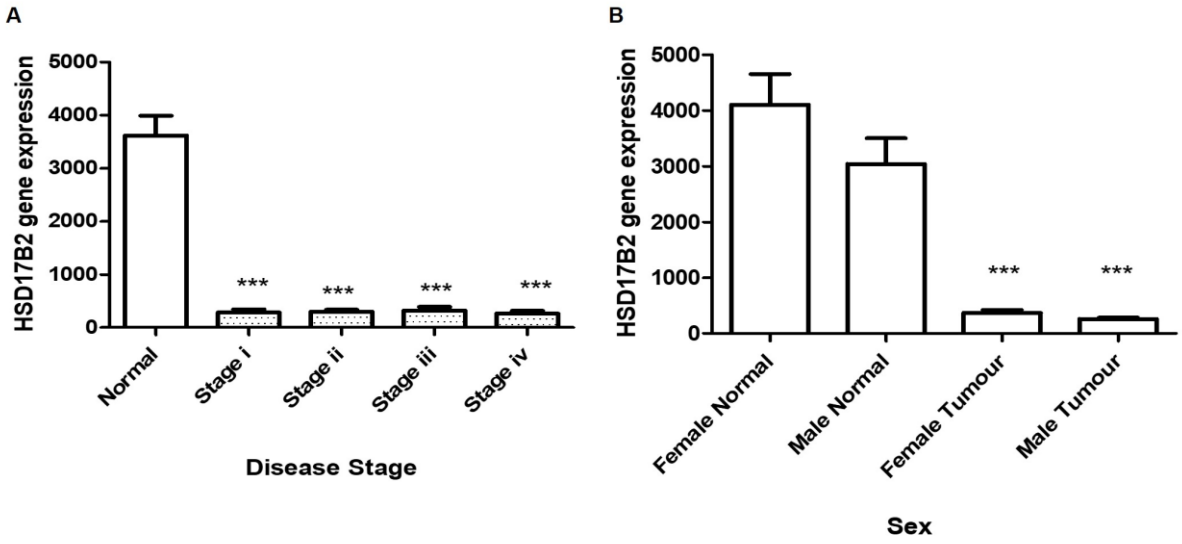


Figure 4.8: COADREAD correlation graphs, disease stages and sex differences of HSD17B2 expression in normal colon and CRC. Correlation between HSD17B2 expression, disease stages and sex differences in normal colon tissue and the TCGA COADREAD dataset (n=433). Statistical analysis is a one-way ANOVA with Tukey post-test. Prism was used for all statistical analysis. *** $p < 0.001$ compared to normal colon.

4.3.4.6 The effect of TNFα and IL-6 on HSD17B4 expression in HCT116 and Colo205

The mRNA expression of the HSD17B4 and RPLPO genes was measured by qRT-PCR in HCT116 (A) and Colo205 (B) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

There was a reduction in HSD17B4 expression caused by TNFα and IL-6 treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours in both HCT116 and Colo205 cells. HCT116 cells have an increase in the HSD17B4 expression after treatment with IL-6 (10 ng/ml and 40 ng/ml) for 48 hours. Also, Colo205 cells showed an increase in HSD17B4 expression caused by TNFα treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours and with IL-6 (10 ng/ml and 40 ng/ml) for 48 hours (Figure 4.9).

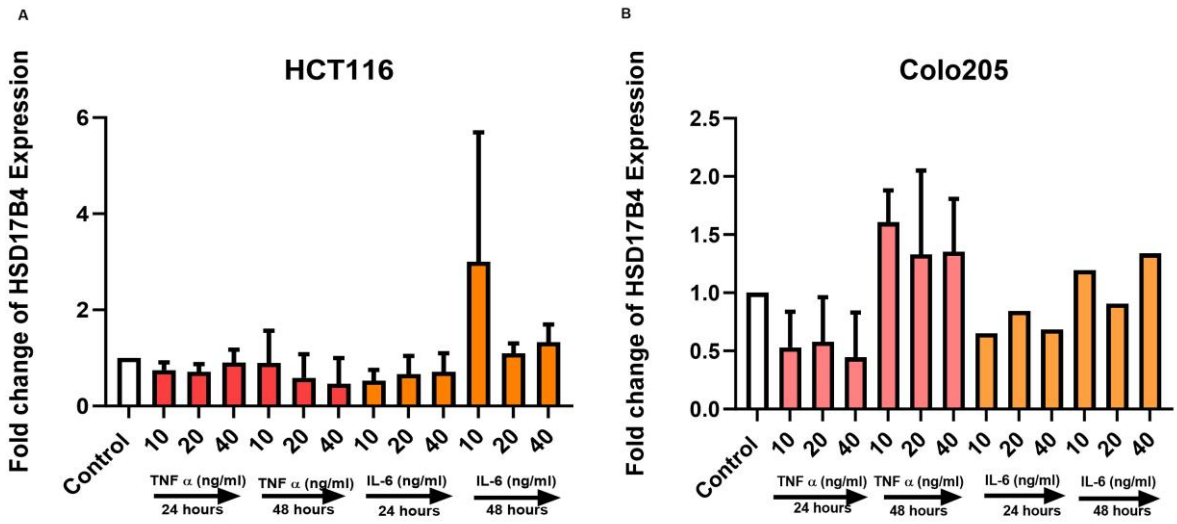


Figure 4.9: The effect of TNFα and IL-6 on HSD17B4 expression in HCT116 and Colo205 cells. HCT116 and Colo205 cells were treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24 & 48 hours. Statistical analysis is a one-way ANOVA with Tukey post-test. For the TNFα n=3 independent experiments and the IL-6 n= 1&2 independent experiments.

4.3.4.7 The effect of TNFα on HSD17B4 Expression in CRC Cell Lines

The mRNA expression of the HSD17B4 and RPLPO genes was measured by qRT_PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

There was a non-significant downregulation in HSD17B4 expression in HCT116 cells treated with TNFα (20 ng/ml and 40 ng/ml) for 48 hours (0.58 ± 0.49 and 0.46 ± 0.53 fold decrease). Also, Colo205 cells showed a non-significant reduction in HSD17B4 expression with TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 24 hours (0.52 ± 0.3, 0.57 ± 0.38 and 0.44 ± 0.38 fold) that might be due to TNFα. Patients with tumours containing high amounts of TNFα and HSD17B4 mRNA exhibited a high survival when compared to those with tumours containing low levels of TNFα and HSD17B4 mRNA (**P=0.0037), thus favouring E_2 to E_1 synthesis, have improved survival outcomes (Figure 4.10 E).

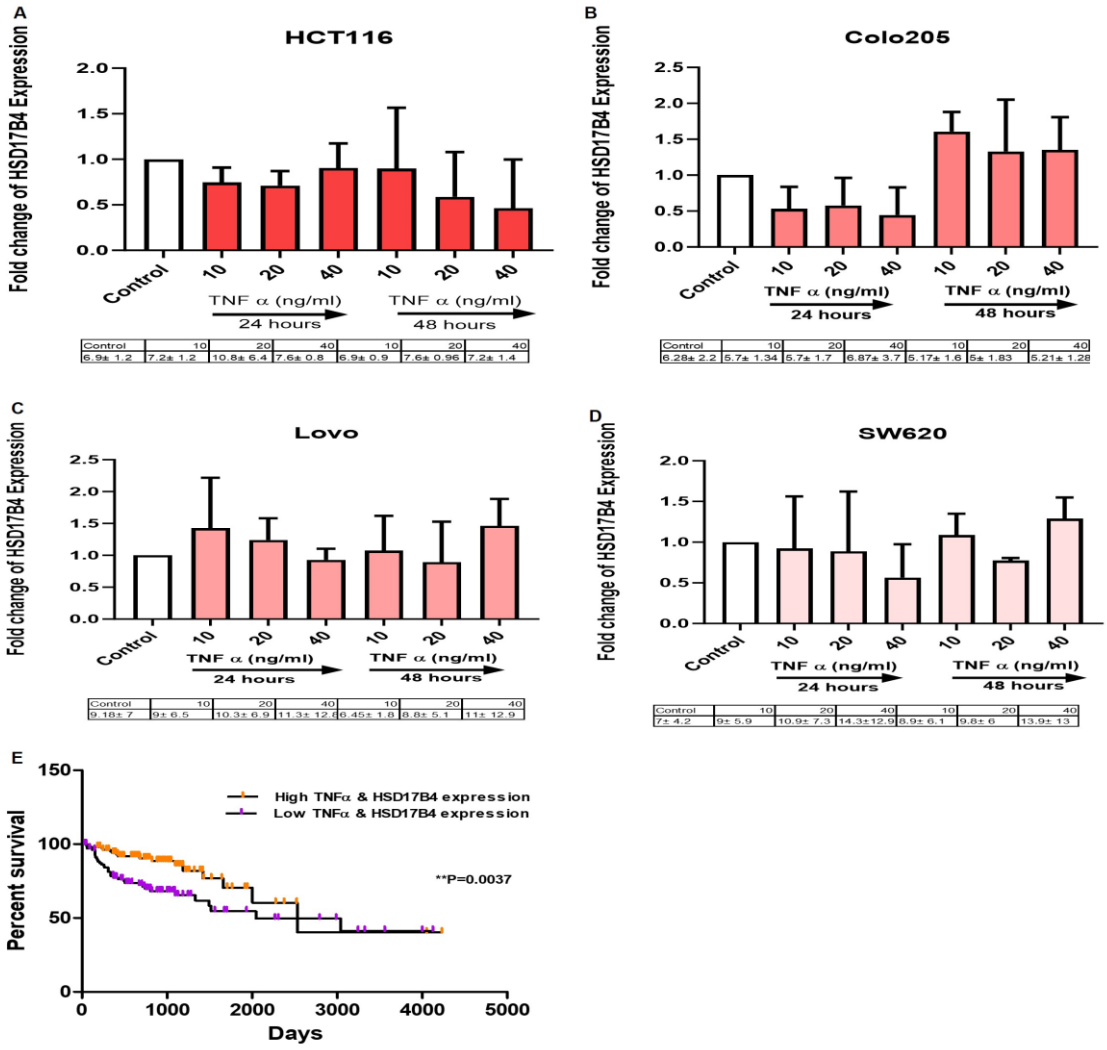


Figure 4.10: The effect of TNFα on HSD17B4 expression in HCT116, Colo205, LoVo and SW620 Cell Lines. The mRNA expression of the HSD17B4 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of HSD17B4(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments. **p < 0.01 compared to control.

4.3.4.8 The effect of TNFα and IL-6 on HSD17B7 expression in HCT116 and Colo205

The mRNA expression of the HSD17B7 and RPLPO genes was measured by qRT_PCR in HCT116 (A) and Colo205 (B) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

In HCT116 cells, TNFα (10 and 40 ng/ml) for 48 hours caused an increase in HSD17B7 expression, although this increase was not statistically significant. In Colo205 cells, after treatment with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml) for 24-48 hours, there was also a non-significant rise in HSD17B4 expression. In both cell lines, IL-6 treatment did not significantly alter HSD17B7 expression (Figure 4.11).

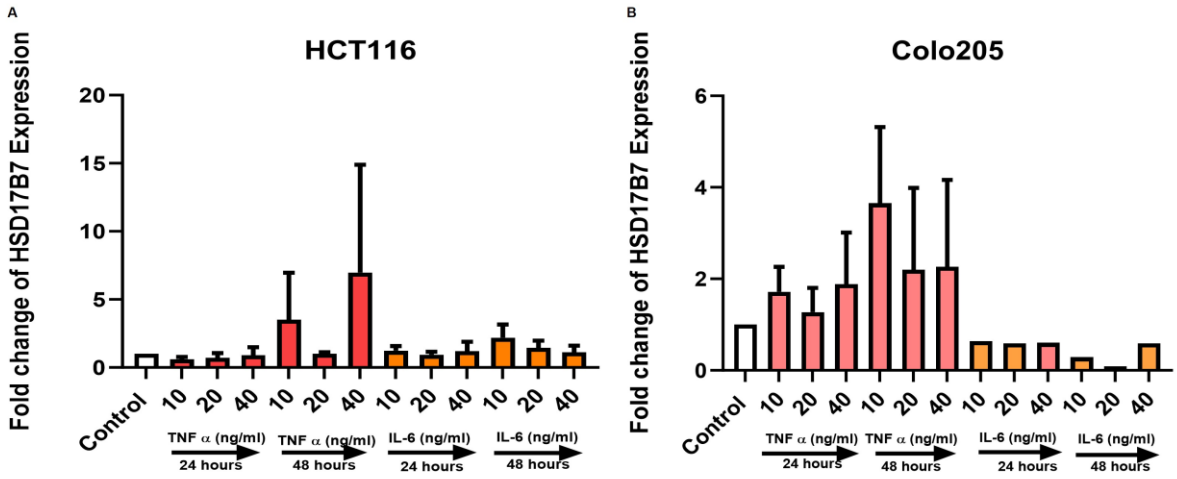


Figure 4.11: The effect of TNFα and IL-6 on HSD17B7 expression in HCT116 and Colo205 cells. HCT116 and Colo205 cells were treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24 & 48 hours. Statistical analysis is a one-way ANOVA with Tukey post-test. For the TNFα n=3 independent experiments and the IL-6 n= 1&2 independent experiments.

4.3.4.9 The effect of TNFα on HSD17B7 expression in CRC Cell Lines

The mRNA expression of the HSD17B7 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

Across all cell lines, there was a non-significant increase in HSD17B7 expression. The highest increase was observed in HCT116 and Colo205 cells treated with TNFα (10 ng/ml) for 48 hours (2.18 ± 0.97 and 3.6 ± 1.66 folds respectively). Furthermore, LoVo cells treated with (40 ng/ml) for 24 hours (1.6 ± 0.75 fold) and (10, 40 ng/ml) for 48 hours (1.77 ± 0.7 and 1.89 ± 0.7 fold) have a non-significant increase in the HSD17B7 expression. Also, the highest increase was observed in SW620 cells treated with TNFα (40 ng/ml) for 48 hours (3.2 ± 1.5 fold). Patients with tumours containing high amounts of TNFα and HSD17B7 mRNA exhibited low survival when compared to those with tumours containing low levels of TNFα and HSD17B7 mRNA (P=0.078) (Figure 4.12 E).

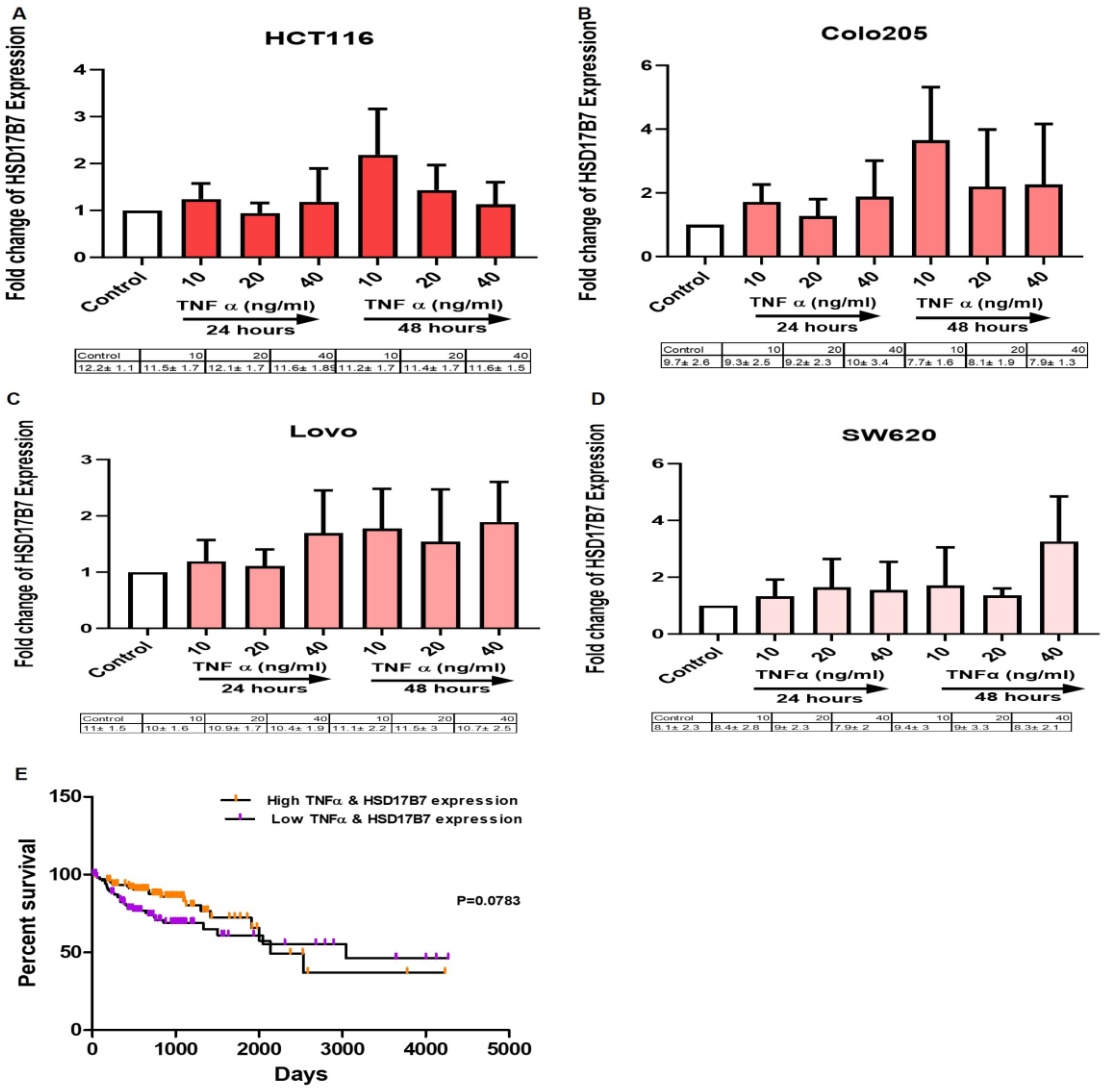


Figure 4.12: The effect of TNFα on HSD17B7 expression in HCT116, Colo205, LoVo and SW620 Cell Lines**.** The mRNA expression of the HSD17B7 and RPLPO genes was measured by qRT_PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of HSD17B7(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

4.3.4.10 The effect of TNFα and IL-6 on HSD17B12 expression in HCT116 and Colo205

The mRNA expression of the HSD17B12 and RPLPO genes was measured by qRT-PCR in HCT116 (A) and Colo205 (B) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) and IL-6 (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

There was an increase in the HSD17B12 expression caused by TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 48 hours in HCT116 cells and Colo205 cells after treatment with TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 24- 48 hours. HCT116 cells have an increase in the HSD17B12 expression after treatment with IL-6 (20 ng/ml) for 24 hours and with IL-6 (10 ng/ml and 40 ng/ml) for 48 hours. Also, Colo205 cells showed an increase in HSD17B12 expression caused by IL-6 treatment (10 ng/ml, 20 ng/ml, 40 ng/ml) for 48 hours (Figure 4.13).

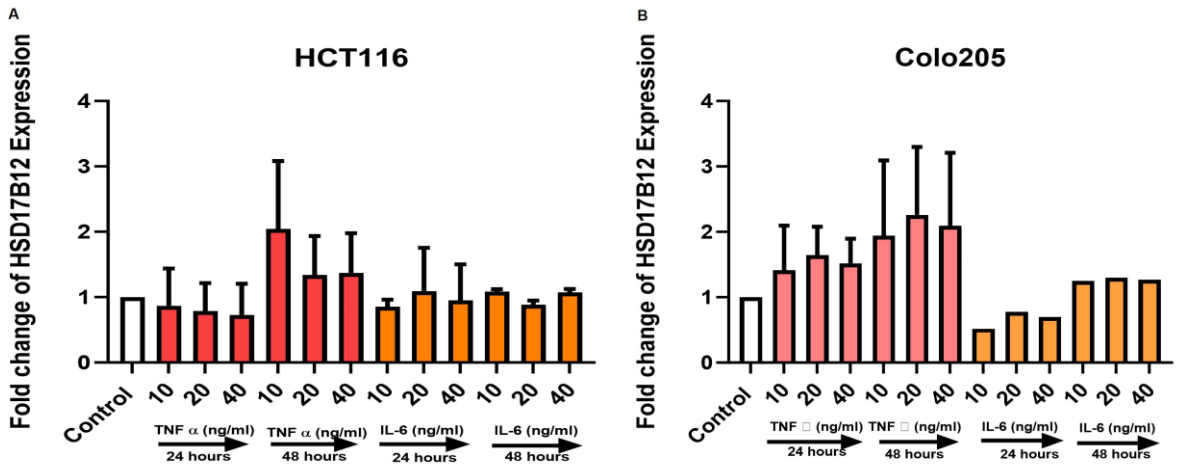


Figure 4.13: The effect of TNFα and IL-6 on HSD17B12 expression in HCT116 and Colo205 cells. HCT116 and Colo205 cells were treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24 & 48 hours. Statistical analysis is a one-way ANOVA with Tukey post-test. For the TNFα n=3 independent experiments and the IL-6 n= 1&2 independent experiments.

4.3.4.11 The effect of TNFα on HSD17B12 Expression in CRC Cell Lines

The mRNA expression of the HSD17B12 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

Across all cell lines, there was a non-significant increase in HSD17B12 expression. The highest increase was observed in HCT116 cells treated with TNFα (10 ng/ml) for 48 hours (2± 1 fold), while the Colo205 cells had the highest increase after treating the cells with TNFα (10 ng/ml, 20 ng/ml and 40 ng/ml) for 48 hours (1.9 ± 1.1, 2.5 ± 1 and 2 ± 1.2 fold). Furthermore, LoVo cells treated with (40 ng/ml) for 48 hours (1.59 ± 0.3 fold) have the greatest non-significant increase in the HSD17B12 expression. Also, the highest increase was observed in SW620 cells treated with TNFα (10,40 ng/ml) for 48 hours (2.3 ± 0.98 and 2.5 ± 1.1 fold). Patients with tumours containing high amounts of TNFα and HSD17B12 mRNA exhibited low survival when compared to those with tumours containing low levels of TNFα and HSD17B12 mRNA (P=0.21) (Figure 4.14 E).

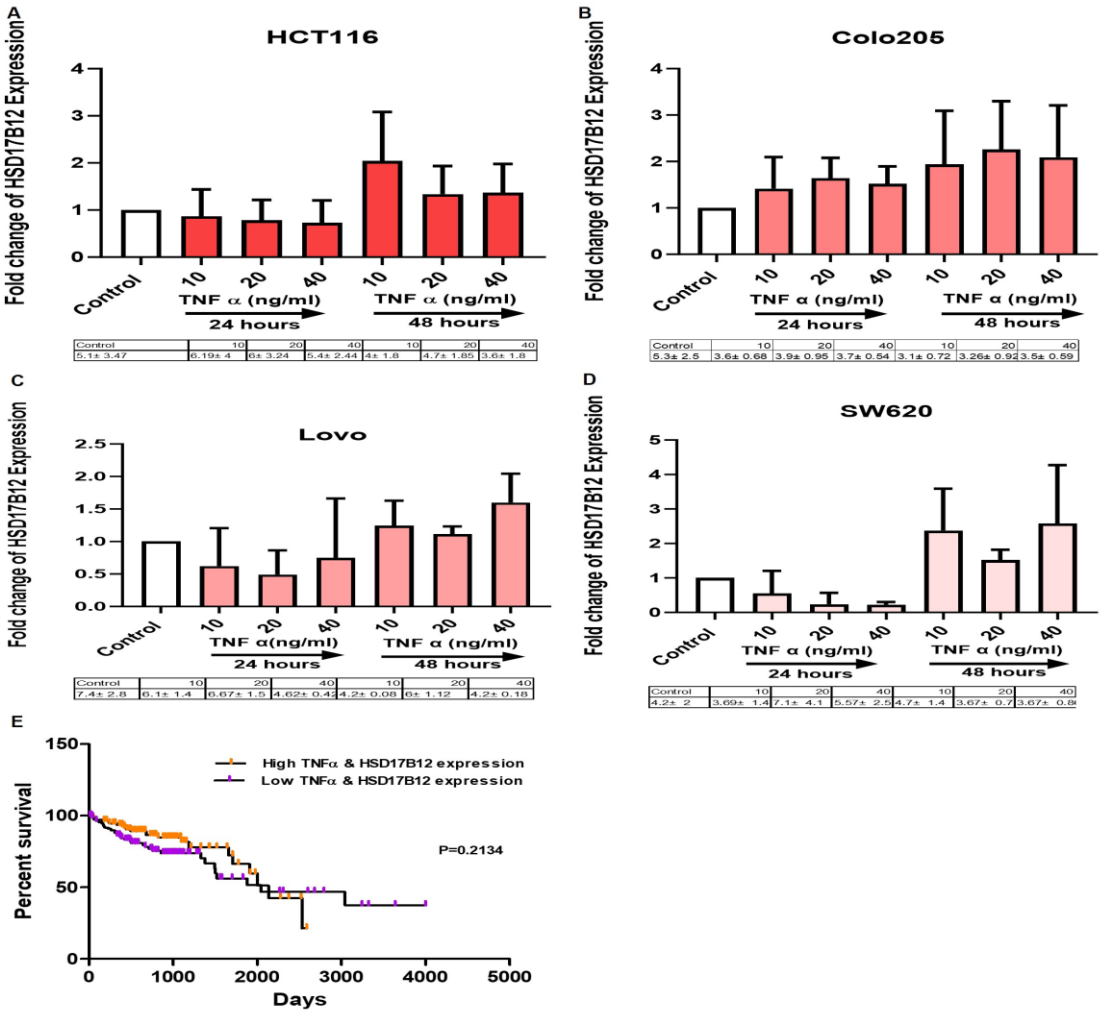


Figure 4.14: The effect of TNFα on HSD17B12 expression in HCT116, Colo205, LoVo and SW620 Cell Lines. The mRNA expression of the HSD17B12 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of HSD17B12(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

4.3.5 Oestrogen receptors expression and effect of TNFα

The nuclear receptors ERα (*ERS1*) and ERβ (*ERS2*), as well as the cell receptor GPER (GPER-30), bind available oestrogens after being metabolised to their active forms by the HSD17B enzymes analysed above (Cui et al., 2013).

4.3.5.1 The expression of ER in TCGA COADREAD dataset

The analysis of the TCGA COADREAD dataset has been used to study ERs expression and to investigate whether ERs mRNA expression is associated with CRC. The COADREAD TCGA cancer dataset containing ERα, ERβ and GPER mRNA expression from 382 CRC patient's samples was analysed and compared to 51 normal colorectal epithelial samples.

Patients with CRC had a significant reduction in the expression of ERα, ERβ and GPER (Figure 4.15) compared to the normal epithelial samples (***$P < 0.001$).

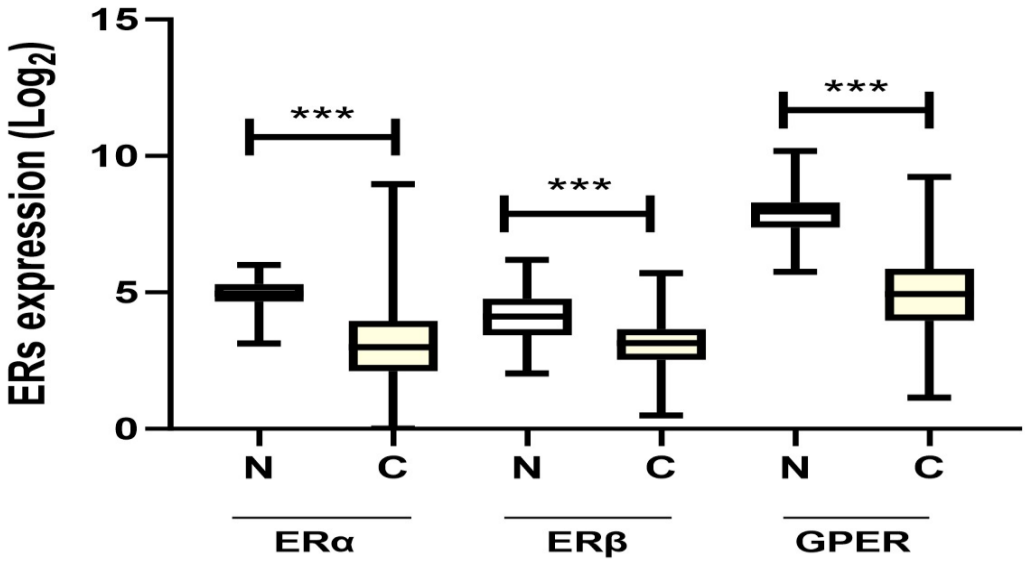


Figure 4.15: The expression of the ERs (ERα, ERβ and GPER) in normal and CRC patient from COADREAD TCGA data. ***P < 0.001 versus controls. N- Normal and C-Colorectal Cancer (N=51 and C=382). For the analysis, two-tailed Mann-Whitney (U tests) was used.

4.3.5.2 CRC patient survival and TNFα and ERα expression

Analyses of the TCGA COADREAD data show that ERα has very low or no expression in CRC. A high level of ERα expression, however, is associated with a significantly worse prognosis and progression-free survival (Gilligan et al., 2017; Ye et al., 2019; Banibakhsh et al., 2023).

There was no correlation (P=0.13) between ERα and TNFα expression in normal colon tumours (Figure 4.16A). However, a significant correlation was seen between ERα and TNFα in tumour CRC tissues (***P=0.0001) (Figure 4.16B). This suggests that TNFα expression may regulate ERα expression in CRC.

The survival outcome of CRC Patients with high ERα expression has worse survival outcomes (Figure 4.16C). When TNFα and ERα expression was analysed in regards to patient survival, there was no difference between outcomes if CRC tumours had high TNFα and ERα expression compared to low TNFα and ERα expression (Figure 4.16D). The expression of ERα was significantly reduced in all stages of the CRC compared to normal tissue (*** $p < 0.001$) (Figure 4.16E). Also, the expression of ERα was higher in normal tissue, especially in females over 50 and males under & over 50 (Figure 4.16F).

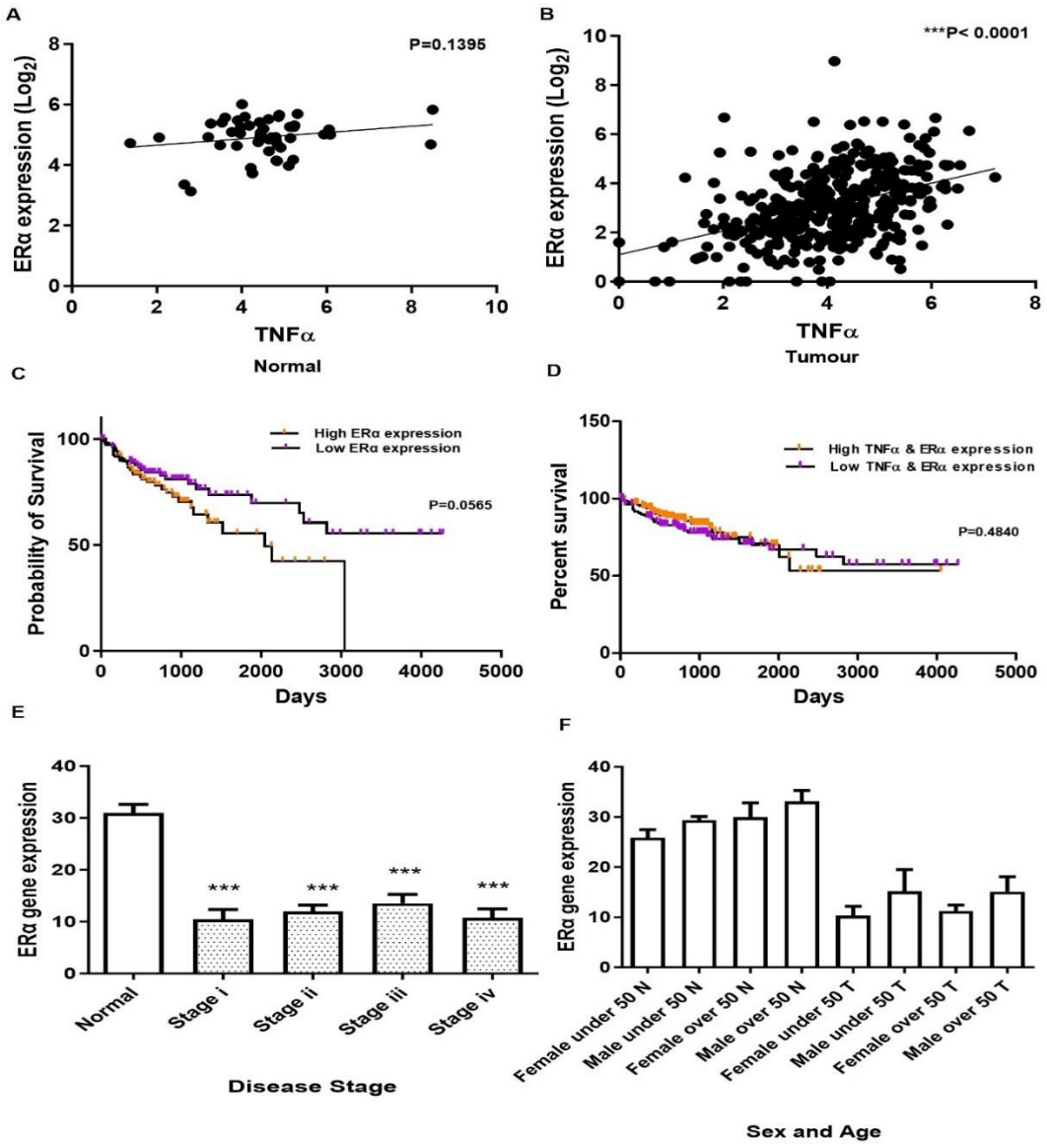


Figure 4.16: Correlation graphs, disease stages and sex differences and Kaplan-Meier survival curves of ERα in normal colon and CRC. Correlation between TNFα and ERα expression, disease stages and sex differences in normal colon tissue and the TCGA COADREAD dataset (n=433). TCGA survival curves were analysed using Kaplan-Meier survival analysis (log-rank method) and one-way ANOVA with Tukey post-test. Prism was used for all statistical analysis. *** $p < 0.001$ compared to normal tissue.

4.3.5.3 CRC patient survival and TNFα and ERβ expression

Previous research has shown that ERβ expression was significantly higher in normal epithelial cells than in colon adenocarcinoma cells in both genders (Konstantinopoulos et al., 2003). The expression and downregulation of ERβ are both observed in colon adenomas and colon cancer (Foley et al.,2000), which correlates with their loss of differentiation (Konstantinopoulos et al., 2003). However, little is known about the inflammatory regulation of ERβ in CRC.

There was no correlation (P=0.14) between ERβ and TNFα expression in normal colon tumours (Figure 4.17A). However, a significant correlation was seen between ERβ and TNFα in tumour CRC tissues (**P=0.002) (Figure 4.17B). This suggests that TNFα expression may regulate ERβ expression in CRC.

The survival outcome of CRC Patients with high ERβ expression has been improved (Figure 4.17C). When TNFα and ERβ expression was analysed in regards to patient survival, there was no difference between outcomes if CRC tumours had high TNFα and ERβ expression compared to low TNFα and ERβ expression (Figure 4.17D). The expression of ERβ was significantly reduced in all stages of the CRC compared to normal tissue (*** $p < 0.001$) (Figure 4.17E). Also, the expression of ERβ was higher in normal tissue, especially in females under 50 and males over 50 (Figure 4.17 F).

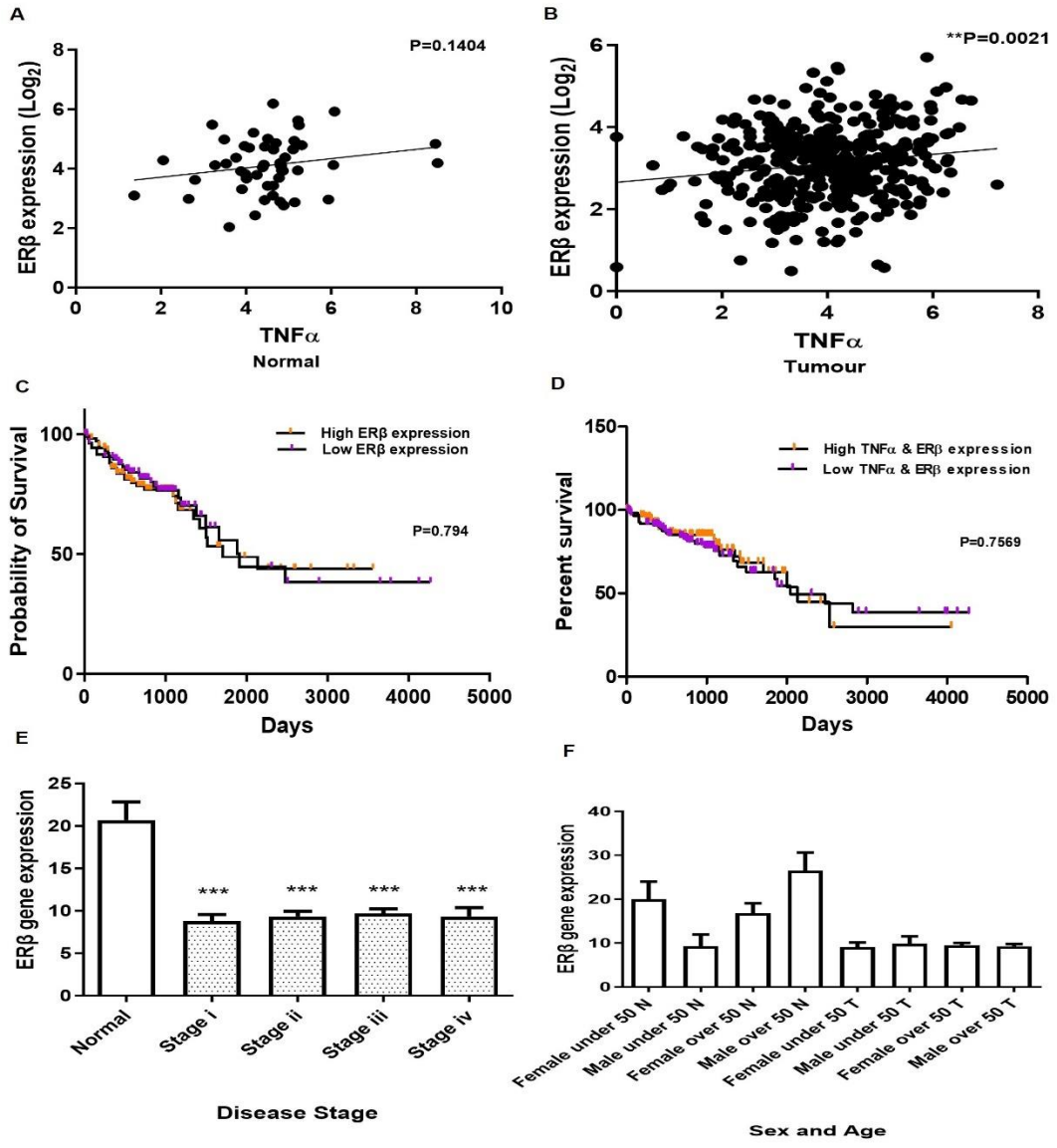


Figure 4.17: Correlation graphs, disease stages and sex differences and Kaplan-Meier survival curves of ERβ in normal colon and CRC. Correlation between TNFα and ERβ expression, disease stages and sex differences in normal colon tissue and the TCGA COADREAD dataset (n=433). TCGA survival curves were analysed using Kaplan-Meier survival analysis (log-rank method) and one-way ANOVA with Tukey post-test. Prism was used for all statistical analysis. *** $p < 0.001$ compared to normal tissue.

4.3.5.4 Survival curves and COADREAD correlation of TNFα and GPER

Normally, GPER is expressed in human colon samples and CRC (Gilligan et al., 2017); as the expression of GPER in CRC is higher than that of ERα and ERβ, this suggests that this nongenomic receptor is active in CRC (Banibakhsh et al., 2023).

There was no correlation (P=0.42) between GPER and TNFα expression in normal colon tumours (Figure 4.18A). Also, there was no correlation between GPER and TNFα in tumour CRC tissues (P=0.63) (Figure 4.18B). Thus, TNFα expression may not influence GPER expression in CRCs.

The survival outcome of CRC Patients with high GPER expression had worse survival outcomes compared with those with low GPER expression. (Figure 4.18C). When TNFα and GPER expression were analysed in regard to patient survival, there was no difference between outcomes if CRC tumours had high TNFα and GPER expression compared to low TNFα and GPER expression (Figure 4.18D). The expression of GPER was significantly reduced in all stages of the CRC compared to normal tissue (*** $p < 0.001$) (Figure 4.18E). Also, the expression of GPER was higher in normal tissue, especially in males under 50 (Figure 4.18 F).

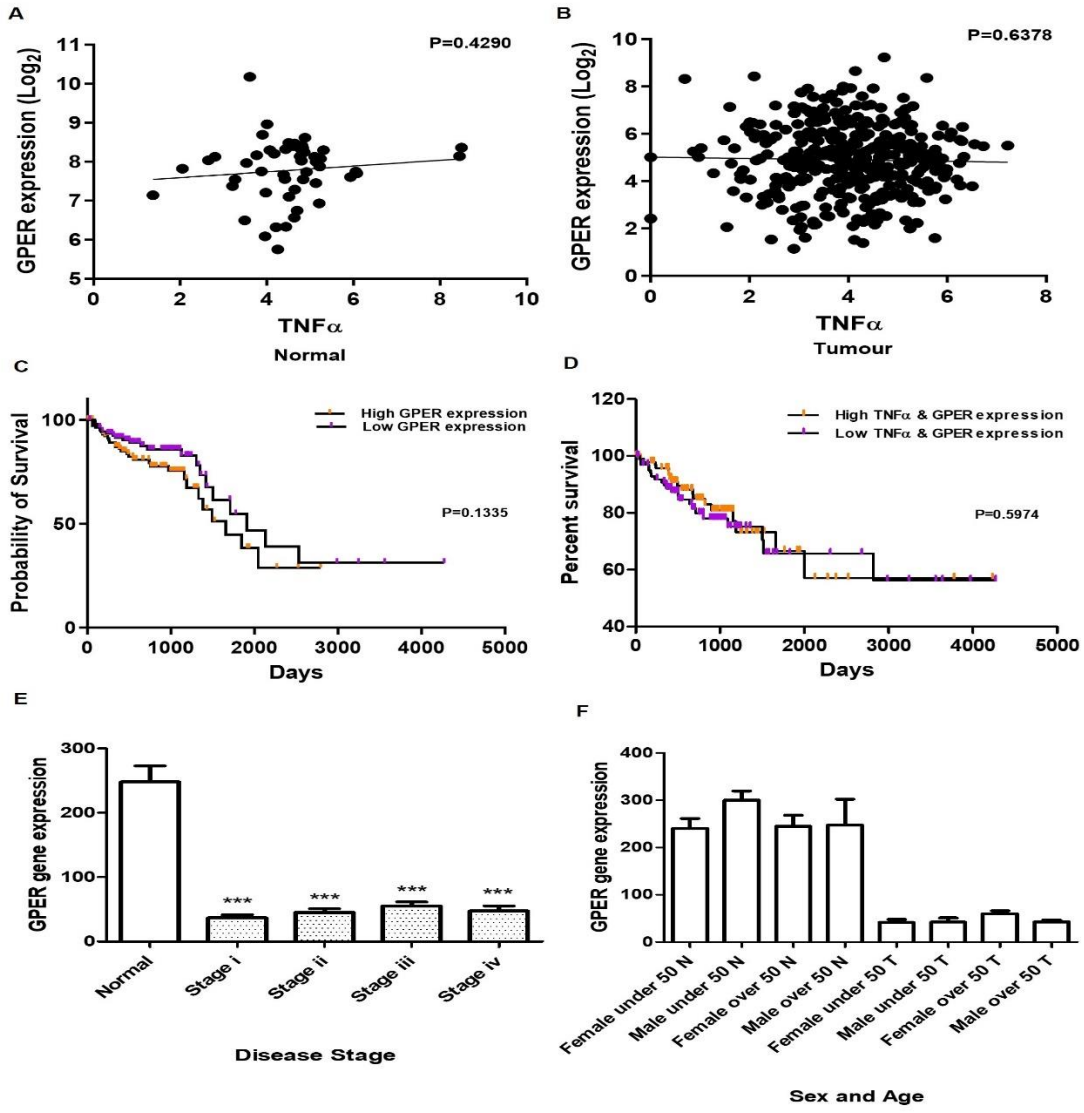


Figure 4.18: Correlation graphs, disease stages and sex differences and Kaplan-Meier survival curves of GPER in normal colon and CRC. Correlation between TNFα and GPER expression, disease stages and sex differences in normal colon tissue and the TCGA COADREAD dataset (n=433). TCGA survival curves were analysed using Kaplan-Meier survival analysis (log-rank method) and one-way ANOVA with Tukey post-test. Prism was used for all statistical analysis. *** $p < 0.001$ compared to normal colon.

4.3.6 Oestrogen metabolism in CRC cell lines.

The data above suggests that TNFα may impact the expression of HSD17B enzymes and ER status. However, it is important to perform functional enzyme studies to see whether overall oestrogen metabolism is altered in response to TNFα treatment. Thus, liquid chromatography-mass spectrometry was employed to determine how TNFα affects oestrogen metabolism in CRC cell lines. Cells were cultured and treated with 40ng/ml of TNFα for 24 hours. Then, cells were incubated in media containing 100 nM of E1 or E2 for a further 24 hours. The resultant media was then extracted and analysed using LC-MS/MS.

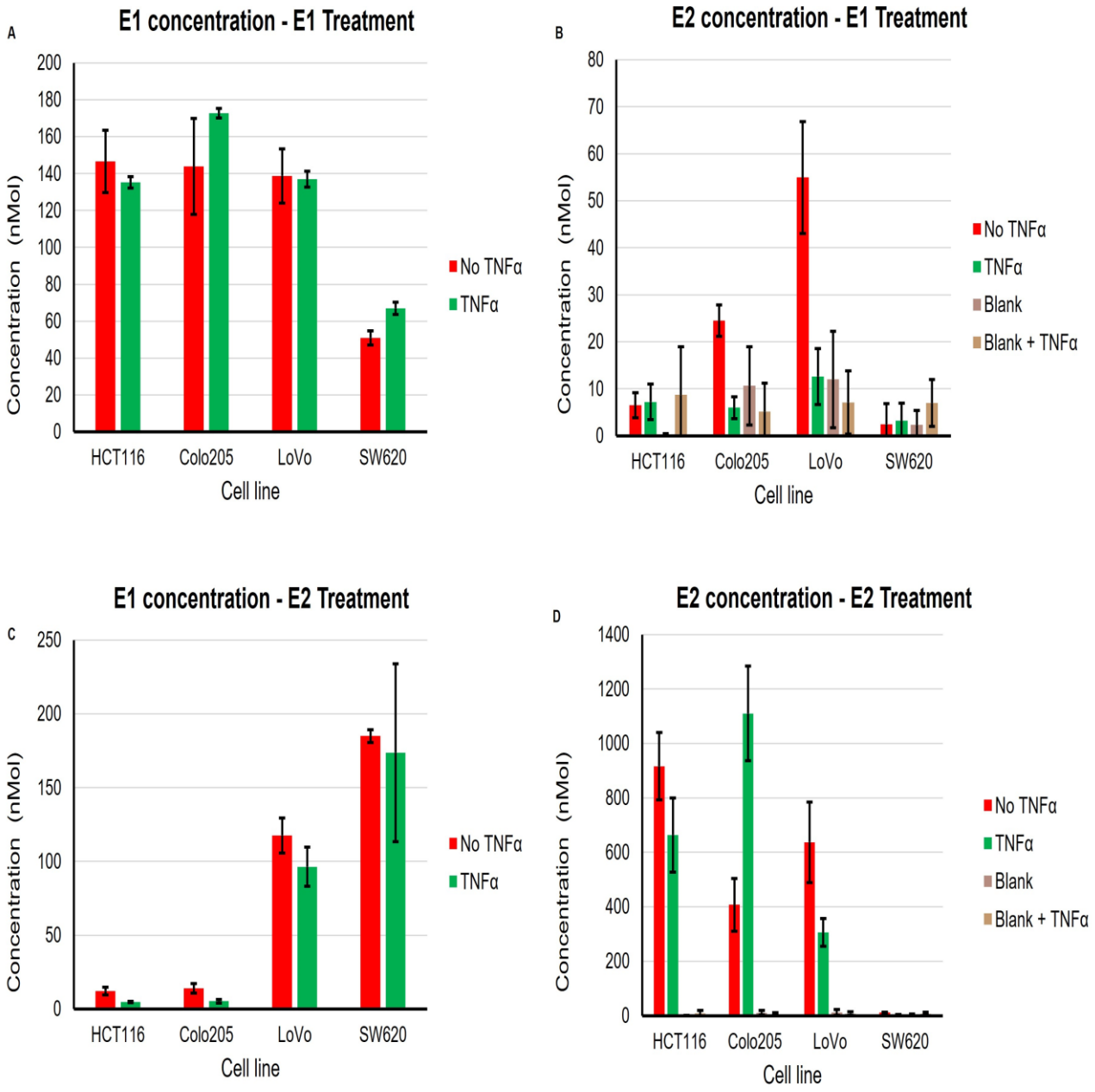


Figure 4.19: The oestrogen metabolism after E1 & E2 treatment in CRC cells. HCT116, Colo205, LoVo, and SW620 cells were treated with TNFα (40 ng/ml) and 100 nM of E1& E2 for 24 hours. Statistical analysis was a one-way ANOVA with a Tukey post-test. N = 3 independent experiments.

HCT116 cells treated with E1 followed by TNFα (40ng/ml) (Figure 4.19A) show a decrease in the E1 concentration (135.2 ± 3nM) compared to untreated cells (146.5 ±16.9 nM). While both the Colo205 and SW620 cells treated with TNFα (40ng/ml) have shown an increase in the E1 concentration (172.7 ± 2.57 nM and 66.9 ± 3.2 nM) compared to untreated cells (143.8 ± 26 nM and 50.9 ± 3.77nM). None of these changes are significant.

When E2 concentrations were measured after E1 (100nM) and TNFα (40ng/ml), treatment differences between groups were evident (Figure 4.19B). HCT116 cells treated with TNFα have a small increase in the E2 concentration (7.2 ± 3.7 nM) compared to untreated cells (6.4 ± 5.6 nM), suggesting an increase in HSD17B1, 7, or 12 activity. InColo205 and LoVo cells treated with TNFα show a decrease in the E2 concentration (5.9 ±2.3 nM and 12.6 ± 5.9 nM) compared to untreated cells (24.4 ± 16.2 nM and 54.9 ± 27.7 nM respectively), suggesting an change in HSD17B2 and HSD17B4 activity.

HCT116, Colo205, LoVo and SW620 cells treated with E2 (100nM) and TNFα (40ng/ml) (Figure 4.19C) show a decrease in the E1 concentration (4.6 ± 0.46 nM, 5.3 ± 1.2 nM, 96.4 ± 13.3 nM and 173.8 ± 60.2 nM) compared to untreated cells (12.2 ± 2.6 nM, 13.9 ± 3.32 nM, 117.6 ± 11.9 nM and 185 ± 4.36 nM respectively). Thus, TNFα treatment reduced the synthesis of E1 from E2, suggesting a decrease in HSD17B2 and HSD17B4 activity.

When E2 concentrations were examined in the same experiment (Figure 4.19D), HCT116 and LoVo cells treated with TNFα had decreased E2 concentrations (666 ± 135.58 nM

and 305 ± 50.99 nM) compared to untreated cells (916 ± 123.95 nM and 637 ± 148.5 nM respectively). This suggests in these cell lines that E2 was being metabolised to an unknown oestrogen metabolite. In Colo205, cells treated with TNFα had an increase in the E2 concentration (1109.7 ± 174 nM) compared to untreated cells (406.8 ± 96.7 nM).

4.4 Discussion

Few studies have looked at the enzymes responsible for oestrogen metabolism in CRC, and those that did not include important HSD17Bs and STS activities. Furthermore, although accumulating evidence that ERβ downregulation and the subsequent loss of this proapoptotic pathway mark an important point in the development of CRC, there remains a lack of understanding on how this occurs and whether other oestrogenic pathways are also altered in CRC. In this chapter, it has been shown that there are changes to the expression of HSD17B enzymes in response to inflammatory, mainly TNFα signals.

4.4.1 HSD17Bs and CRC

The complicated regulatory systems that govern the steroidogenic pathways and steroid production involve a variety of substances, including steroids, growth factors, cytokines, and pituitary trophic hormones (Atanassova and Koeva, 2012). The colon is one of many peripheral organs that can produce physiologically active isoforms of oestrogens (17β-oestradiol-E2) from circulating precursors (Fiorelli et al., 2002). Previous *in vitro* and *in vivo* studies revealed the expression of oestrogen-metabolizing enzymes in CRC cell lines and normal gut mucosa (Fiorelli et al., 1999; English et al.,2001; SANO et al., 2001; Fiorelli et al., 2002; Sato et al., 2012). The five human 17HSDs identified so far that are involved in oestrogen metabolism differ significantly in tissue distribution and substrate

selectivity (Labrie et al., 2000). Usually, HSD17Bs catalyse the last stage of steroid hormone production, and they influence the intracellular levels of inactive and active steroids based on their reductive or oxidative actions (Marchais-Oberwinkler, 2011). English et al. (1999) and Fiorelli et al. (1999) have reported that human colonic tissues and CRC cell lines express both aromatase and HSD17Bs and that the HSD17Bs oxidative activity correlates inversely with cell proliferation, indicating that a lack of oestrogen inactivation plays a role in the pathogenesis of CRC. Oestrone can be generated as a result of aromatase or STS activity, but it must be converted to oestradiol to acquire its full biological efficacy; HSD17B1 mediates this reaction (Purohit et al., 2002). In the ovaries and placenta, HSD17B1 largely converts the weak oestrogen E1 to the physiologically active oestrogen E2, whereas the HSD17B2 catalyses the oxidative conversion of E2 to E1, which is thought to be crucial in the peripheral inactivation of oestrogens (Labrie et al., 2000).

The role of enzymes that metabolise oestrogens in regulating tissue E2 concentration has also been studied in CRC development. Oduwole et al. found that the 5-year survival rate was poor in CRC patients with elevated amounts of the mRNA encoding HSD17B2, which oxidizes E2 to E1. Also, Previous research by Rawluszko et al. showed that primary colon cancer tissue had lower levels of HSD17B1. In a study by Gilligan et al., it was shown that human CRC tissue had significantly (P 0.01) lower levels of HSD17B2 mRNA than matched controls. There was no evidence of HSD17B1 mRNA. Comparing matched controls to female and male CRC tissue, HSD17B7 and HSD17B12 mRNA levels were considerably higher.

Here, it is shown through the analysis of COADREAD TCGA data (Figure 4.1) that CRC patients had significant reductions in HSD17B1 mRNA expression compared to normal tissue. Foster et al. (2013) have also demonstrated that HSD17B1 is not or is weakly expressed in CRC tissue. Also, mRNA expression of HSD17B2 was significantly reduced in CRC samples ($p<0.001$). These results are similar to those of previous studies (English et al., 1999; Oduwole et al., 2003; Gilligan et al., 2017). HSD17B2 is strongly expressed in the healthy colon's small intestine and surface epithelium, but its expression is reduced in CRC cells and tissues. Moreover, in this chapter, it is seen that HSD17B2 expression is significantly reduced in all stages of CRC (Figure 4.8A). Others have also demonstrated this (Vihko et al. 2002) and found that the decrease in HSD17B2 expression is associated with the development of colon cancer. Finally, here, it is also demonstrated that there is a significant increase in HSD17B7 and HSD17B12 mRNA expression observed in CRC samples compared with normal epithelial samples (***$P < 0.001$). Previously, Gilligan et al. (2017) reported that HSD17B7 is expressed more frequently in CRC than in healthy colon tissue.

When looking at the expression of HSD17Bs in CRC, these findings suggest that these enzymes may be implicated in the development and progression of CRC and that the downregulation of HSD17B2 and HSD174 and upregulation of HSD17B7 and HSD17B12 are important in CRC progression.

4.4.2 ER and CRC

The nuclear oestrogen receptors ERα and ERβ mediate the genomic effects of oestrogens and GPER for non-genomic effects (Revankar et al., 2005; Kirker et al., 2013). The two

ERs appear to frequently interact antagonistically, as shown in the regulation of cell proliferation, where ERα is pro-proliferative, and ERβ is anti-proliferative. The direct action of oestrogens through ERβ on CRC cells is increasingly supported by experimental evidence, which is supported by the differential expression of ER subtypes α and β in animal and human CRC tissues (Issa et al., 1994; Foley et al., 2000; Campbell-Thompson et al., 2001; v et al., 2001). E2 primarily interacts with the ER to cause apoptosis in CRC cells (Weige et al., 2009). A low level of ERβ expression is associated with advanced cancer stages and poor survival independently (Rudolph et al., 2012). In advanced CRC tumours, oestrogen effects may be transmitted via GPER since patients and cell lines studied for CRC lack ERα or ERβ. As colonic epithelia develop, the GPER receptor becomes the dominant ER (Gilligan et al., 2017). Moreover, Gilligan et al. (2017) found that oestrogen increases the proliferation of cancer cell lines that express GPER, but not ER, through a positive feedback loop in which GPER induces the expression of steroid sulfatase, which in turn triggers conjugated oestrogen, accelerating cancer progression.

Due to the low expression of ERα in healthy colonic mucosa, little is known about how it contributes to colon cancer (Campbell-Thompson et al., 2001; Hasson et al., 2014). Caiazza et al. (2015) report that ERβ expression in the colon mucosa gradually decreases with the development of CRC. Also, a gradual decline in GPER expression is associated with CRC stage and lymph node metastases in CRC patients (Liu et al., 2017). In this chapter, the results are consistent with previous studies. It is shown that the COADREAD TCGA cancer dataset containing ERα, ERβ and GPER mRNA expression from 382 CRC patient samples was analysed and compared to 51 normal colorectal epithelial samples. The patients with CRC had a significant reduction in the expression of ERα, ERβ and

GPER (Figure 4.15) compared to the normal epithelial samples (***P < 0.001). Also, oestrogen receptors ERα, ERβ, and GPER are all significantly reduced in CRC throughout all stages (***P=0.001). (Figure 4.16D, Figure 4.17D and Figure4.18 D).

4.4.3 TNFα Regulates HSD17Bs and ERs Expression

CRC development may be linked to an imbalance between pro-inflammatory and anti-inflammatory cytokines (Borowczak et al., 2022). In response to localized inflammation, gut immune cells produce TNFα, IL-1β, and IL-6, which stimulate complex cross-talk within the gut microenvironment and promote the development of CRC, immunosuppression, and anticancer drug resistance (Bhat et al., 2022). Oestradiol-17β-hydroxysteroid dehydrogenase, aromatase, and oestrone sulfatase are the enzyme complexes involved in the synthesis of oestrogens in peripheral tissues. When IL-6 and TNFα levels are high, IL-6 and TNFα increase the activities of the three enzymes (Purohit et al., 2002). A study by Gilligan et al. (2017) demonstrated that the TNFα and IL-6 treatment of Colo205 and Caco-2 cells for 48 hours resulted in a decreased HSD17B2 mRNA expression trend. Only Caco-2 cells treated with the combined therapy, however, showed significant alterations (*p 0.05). TNFα and IL-6 treatment did not affect the HSD17B7 or HSD17B12 mRNA in HCT116. The expression of HSD17B enzymes in CRC cell lines remained substantially unaffected, suggesting that TNFα and IL-6 do not control these enzymes.

There are no studies that directly link TNFα treatment with ERα and ERβ expression in CRC cells.

This chapter focused on determining if TNFα affects steroidogenesis through the oestrogen metabolic pathways in CRC cell lines. TCGA COADREAD, RNA-Seq dataset (n=433) was used to generate correlation graphs comparing normal and cancerous colon tissue expression of the HSD17Bs and TNFα (Figure 4.3). Analysis of COADREAD data demonstrated that there was no correlation between HSD17B1, HSD17B2, HSD17B7 and TNFα in normal tissue and tumour CRC tissues. Also, a positive correlation was seen between TNFα in CRC tissue and HSD17B4 (**P<0.004) (Figure 4.3 F) and HSD17B12 (*P<0.016) (Figure 4.3 J).

qRT-PCR results have demonstrated that there were no changes in HSD17B1 expression across all the cell lines (Figure 4.5). In general, HCT116 and LoVo cells treated with TNFα have a non-significant decrease in the HSD17B1 expression. As a result, TNFα does not affect the expression of HSD17B1. Also, there was a non-significant decrease in HSD17B2 expression in HCT116, Colo205 and LoVo cells treated with TNFα (Figure 4.7 A, B &C). Furthermore, a non-significant downregulation in HSD17B4 expression in HCT116 and Colo205 cells treated with TNFα (Figure 4.10 A&B) might be due to TNFα. While across all cell lines, there was a non-significant increase in HSD17B7 and HSD17B12 expression (Figure 4.12 & Figure 4.14).

These data support the previously published research, analysis by TCGA COADREAD, and previous studies (English et al., 2000; Gilligan et al., 2017) support my findings, which showed that there was no change in the expression of HSD17B1 in HCT116 treated with TNFα and the untreated cells and the HSD17B2 expression is downregulated in CRC

in human CRC. Also, LCMS results of HCT116, Colo205, LoVo and SW620 cells treated with TNFα (40ng/ml) showed a decrease in E1 concentrations compared to untreated cells incubated in media containing 100 nM of E2 (Figure 4.19C). In response to TNFα treatment, HSD17B2 and HSD17B4 activity are reduced, indicating a decreased formation of E1 from E2.

Alternative enzymes responsible for the conversion of E1 to E2, HSD17B7 and HSD17B12, were also examined, and both mRNAs were found to be expressed in CRC cell lines. Expression of HSD17B7 and HSD17B12 was non-significantly increased compared with matched normal controls after TNFα treatment for 24-48 hours in HCT116, Colo205, LoVo and SW620 (Figure 4.12 and Figure 4.14). This mimicked data was seen in the TCGA COADREAD dataset, where HSD17B7 and HSD17B12 were elevated in CRC compared to the control. However, a non-significant correlation was seen between TNFα expression and HSD17B7 expression in CRC tissue (P=0.15) (Figure 4.3H), and a positive correlation was seen between HSD17B12 in tumour CRC tissues and TNFα (*P=0.016, R^2=0.014) (Figure 4.3I). This suggests there may be a decrease in the E1/E2 ratio in response to TNFα, resulting in more E2 being synthesised in CRC tissue.

When these results were investigated further by measuring the conversion of oestrogens via LC-MS/MS, data suggests that in HCT116 cells, there was an upregulation of E1 to E2 synthesis, but a downregulation of this pathway in Colo205 and LoVo cells (Figure 4.19B). However, when the reverse reaction was investigated, TNFα decreased the

formation of E1 from E2, suggesting a decrease in HSD17B2 and HSD17B4 activity in response to TNFα.

Finally, A positive correlation was seen between ERα, ERβ and TNFα in tumour CRC tissues (***P=0.0001) (Figure 4.16B and Figure 4.17B). This suggests that TNFα expression may regulate in ERα and ERβ expression in CRC.

4.5 Conclusion

The available evidence suggests that there is a correlation between HSD17Bs enzymes and inflammation in CRC. The PCR results and analysis of COADREAD TCGA data have demonstrated that TNFα has been downregulating the HSD17B2 and HSD17B4 responsible for the conversion of E2 to E1 and upregulated the HSD17B7 and HSD17B12 responsible for the reverse reaction. Also, a significant correlation was seen between HSD17B4 and HSD17B12 in tumour CRC tissues and TNFα. Further research is needed to better understand this relationship and its implications for prevention and treatment strategies for CRC.

CHAPTER 5. REGULATION OF OATPs and E1S UPTAKE BY TNFα

5.1 Introduction

Organic anion transport polypeptides (OATPs) are necessary for E1S uptake into cells (Obaidat et al., 2012). The transport of oestrogen sulfates, especially E1S, into cells, is mediated by specific SLC transporters, such as SLC21 (Banerjee et al., 2012). Endogenous substances, including steroids, bile acids, and peptides, are known ligands of the OATP family (Hagenbuch and Meier, 2004; Hagenbuch and Gui, 2008). As part of this concept, OATP (SLC21) family transporters that contribute to cellular accumulation of E1S OATPs and the transport of E1S is effectively accomplished by six different SLCO/OATP (OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1, OATP4A1) (Mueller et al., 2015). They are expressed in a variety of tissues throughout the body and transport a variety of endo- and xenobiotics, including hormones and their conjugates, as well as several medicines, including several anticancer drugs (Ban et al., 2017). The dysregulation of steroid precursor uptake, hormone activation, actions, metabolism, or excretion can result in malignancy and benign disease (Rižner, 2019).

Recent research has shown that several OATPs are up or downregulated in many malignancies and that OATP expression may influence the development of cancer (Obaidat et al., 2012). Furthermore, in certain hormone-dependent cancers, OATPs correlate with differentiation, making them potential biomarkers for assessing treatment response and disease stage (Pressler et al., 2011). In many normal endocrine tissues, the expression of these "sulfated-hormone transporters" (OATP1B1, OATP1B3, OATP2B1, and OATP1A2) is low but is elevated in hormone-dependent cancers (Pizzagalli et al., 2002; Pressler et al., 2011; Mueller et al., 2015).

The expression patterns of OATP in CRC may play a key role in the transport of E1S and consequent oestrogen action. In CRC cell lines, OATP4A1 is most abundantly expressed, followed by OATP2B1 (Gilligan et al., 2017). A hypermethylated promoter region of OATP3A1 was found in CRC tissues and cell lines, whereas a hypomethylated promoter region of OATP4A1 was found, resulting in low mRNA expressions of OATP3A1 and high expressions of OATP4A1 in CRC tumours (Kleberg et al., 2012; Rawłuszko-Wieczorek et al., 2015). OATP4A1 is up-regulated in colon cancer, according to DNA microarray investigations (Ancona et al., 2006). OATP4A1 could be a useful indicator of a bad prognosis for CRC. Additionally, OATP4A1 is crucial for the growth, invasion, migration, and carcinogenesis of CRC cells (Ban et al., 2017). The expression patterns of OATP4A1 and OATP3A1 in different cancers like breast cancer, colon adenocarcinoma and ovarian carcinoma cell lines have been observed to be similar (Kullak-Ublick et al., 2001).

The OATP1B3 gene is overexpressed in several human cancer tissues and cancer cell lines derived from solid tumours as well, and the level of OATP1B1 expression in colon cancer was significantly associated with the degree of differentiation, and earlier tumour staging and improved tumour differentiation are associated with higher OATP1B3 expression in the colon. (Pressler et al., 2011). There are differences in the expression and function of OATP1B3 in different tumour cells and tissues. Several studies have shown that OATP1B3 expression is significantly higher in cancerous tissues than in normal tissues, including the colon (Lee et al., 2008; Lockhart et al., 2008). In most clinical tissue specimens examined, OATP1B3 is expressed (56%) and may have improved clinical outcomes, and the lower-stage CRC has the greatest OATP1B3 expression (Lockhart et

al., 2008). The majority of colorectal adenocarcinomas overexpress OATP1B3 at both the mRNA and protein levels, and this overexpression interferes with the p53 signalling pathway, providing experimental evidence that cancer cells with high OATP1B3 overexpression are more likely to survive (Lee et al., 2008).

This chapter will examine how inflammation, primarily TNFα, impacts OATP expression and potential activity. Previous studies have shown that patients with IBD, which is defined by increased colon inflammation, have significantly higher levels of OATP4A1 and OATP2B1 expression in their colons compared to healthy colon tissue (Wojtal et al., 2009). TNFα has been studied for its effect on organic anion transport polypeptides (OATPs). As a result of treating primary human hepatocytes with 100ng/ml TNFα or 10ng/ml IL-6 for 48 hours, Le Vee et al. demonstrated reduced mRNA levels of transporters, including OATP1B1, OATP1B3 and OATP2B1. An *in vitro* study by Varma et al., 2018 examined how different cytokines affect transport protein expression and activity in HCT116 cells. Results suggested that TNFα enhanced OATP2B1 mRNA, protein expression and E1S uptake into cells.

The links between inflammation and oestrogen uptake via OATPs in CRC are poorly understood, with membrane transport of steroid precursors and metabolites, in particular (E1S), having received little attention to date. Thus, this chapter focuses on the effect of TNFα on the expression of key oestrogen transporters (OATP1A2, OATP1B1, OATP1C1, OATP2B1, OATP3A1 and OATP4A1) in CRC cell lines, and how this inflammation impacts oestrogen uptake into cells.

5.2 Materials and Methods

5.2.1 The Cancer Genome Atlas (TCGA) Data Collection and Analysis

The TCGA data collection and analysis were performed as described in section 2.9.

5.2.2 Cell Culture

The HCT116, Colo205, Lovo, and SW620 cells were cultured as described in section 2.1.

5.2.3 Cytokines treatment

Cytokines were reconstituted in phosphate buffered saline (PBS) (PBS, Oxoid, and Hampshire, England) containing 0.1% human albumin serum (HAS) (Sigma) and stored in working aliquots at -80^{o}C. All cytokines were purchased from (PeproTech, London, UK).

The HCT116, Colo205, Lovo, and SW620 cells were seeded into 6-well plates at 100,000 cells per well and treated with different concentrations of cytokines (10 ng/ml, 20 ng/ml, 40 ng/ml) of TNFα. Cells were incubated for 24 h and 48 h at 37^{o}C. After 24-48 hours, the media was removed to run the RNA extraction, cDNA synthesis and real-time PCR to determine the expression of steroidogenic enzymes in CRC cell lines treated with various cytokines. Untreated cells from all cell lines served as controls.

5.2.4 RNA Extraction, cDNA Synthesis

RNA extraction followed by cDNA synthesis was performed as described in sections 2.4 to 2.5.

5.2.5 Real Time PCR

The RT-PCR has been described previously in section 2.6.

5.2.6 E1S Uptake Studies

CRC cells HCT116, Colo205, Lovo and SW620 were seeded at 100,000 cells per well in 6-well plates for 24 hours. TNFα (40ng/ml) was applied to the cells for 24 hours. Cells were incubated in phenol red free medium containing 10% sFBS and [6,7-^{3}H] E1S (4×10^{5} dpm) and were subsequently removed after 60 min. The cells were washed twice in PBS and lysed using RIPA buffer, and the intracellular 3H radioactivity was measured by liquid scintillation spectrometry. Cell protein content was also determined with a BCA assay. Results are expressed as E1S uptake pmol/mg protein.

5.2.7 Statistical Analysis

Statistics were performed as described in section 2.10.

5.3 Results:

5.3.1 OATPs expression in TCGA COADREAD dataset

Analysis of the TCGA COADREAD dataset has been used to determine OATP expression in normal and CRC patients. The dataset contained OATP mRNA expression from 382 CRC patient samples, which was compared to 51 normal colorectal epithelial samples (Figure 5.1).

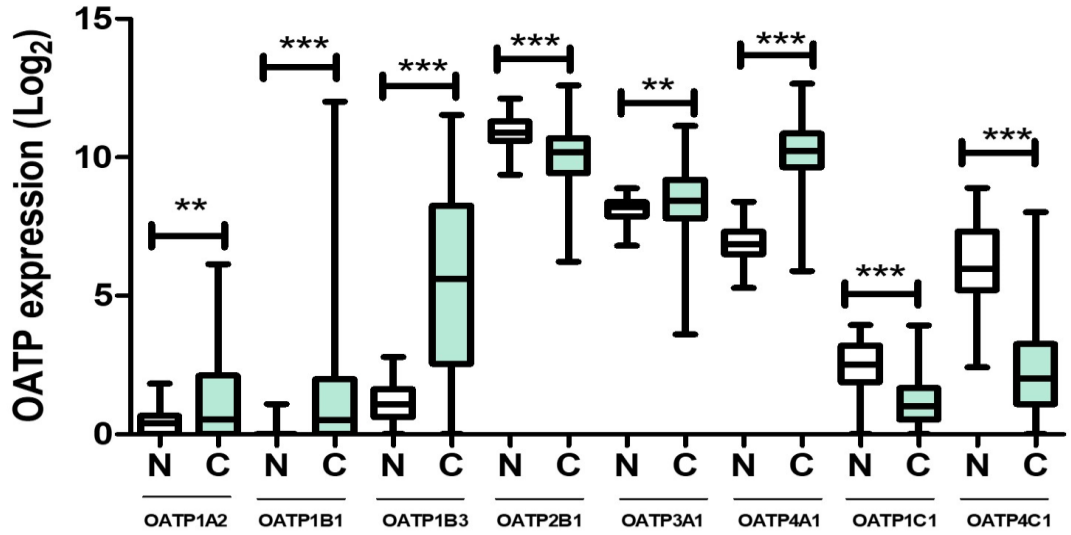


Figure 5.1: OATP expression of the six transporters responsible for E1S uptake from normal and CRC patient tissue from COAD TCGA data. **P < 0.01 versus controls, ***P < 0.001 versus controls. N - Normal and C - CRC (N=51 and C=382). For the analysis, two-tailed Mann-Whitney (U tests) was used.

Patients with CRC had high expression of OATP1B3, OATP2B1, OATP3A1 and OATP4A1 compared to other OATPs analysed. OATP1A2, OATP1B1, OATP1C1 and OATP4C1 had very low mRNA expression. There was a significant increase in CRC samples in the expression of OATP1A2 (**P < 0.01), OATP1B1 (***P< 0.001), OATP1B3 (***P< 0.001), OATP3A1 (**P < 0.01) and OATP4A1 (***P< 0.001) mRNA compared to the normal epithelial samples. There was a significant reduction in OATP2B1 (***P< 0.001), OATP1C1 (***P< 0.001) and OATP4C1 (***P< 0.001) mRNA expression in CRC samples compared to normal colon tissue.

5.3.2 The survival outcomes for CRC patients with high or low OATPs expressions

Survival outcomes for CRC patients were determined from TCGA COADREAD data to generate Kaplan-Meier survival curves. Specifically, these survival analyses focused on CRC survival data and the tumours' OATP expression.

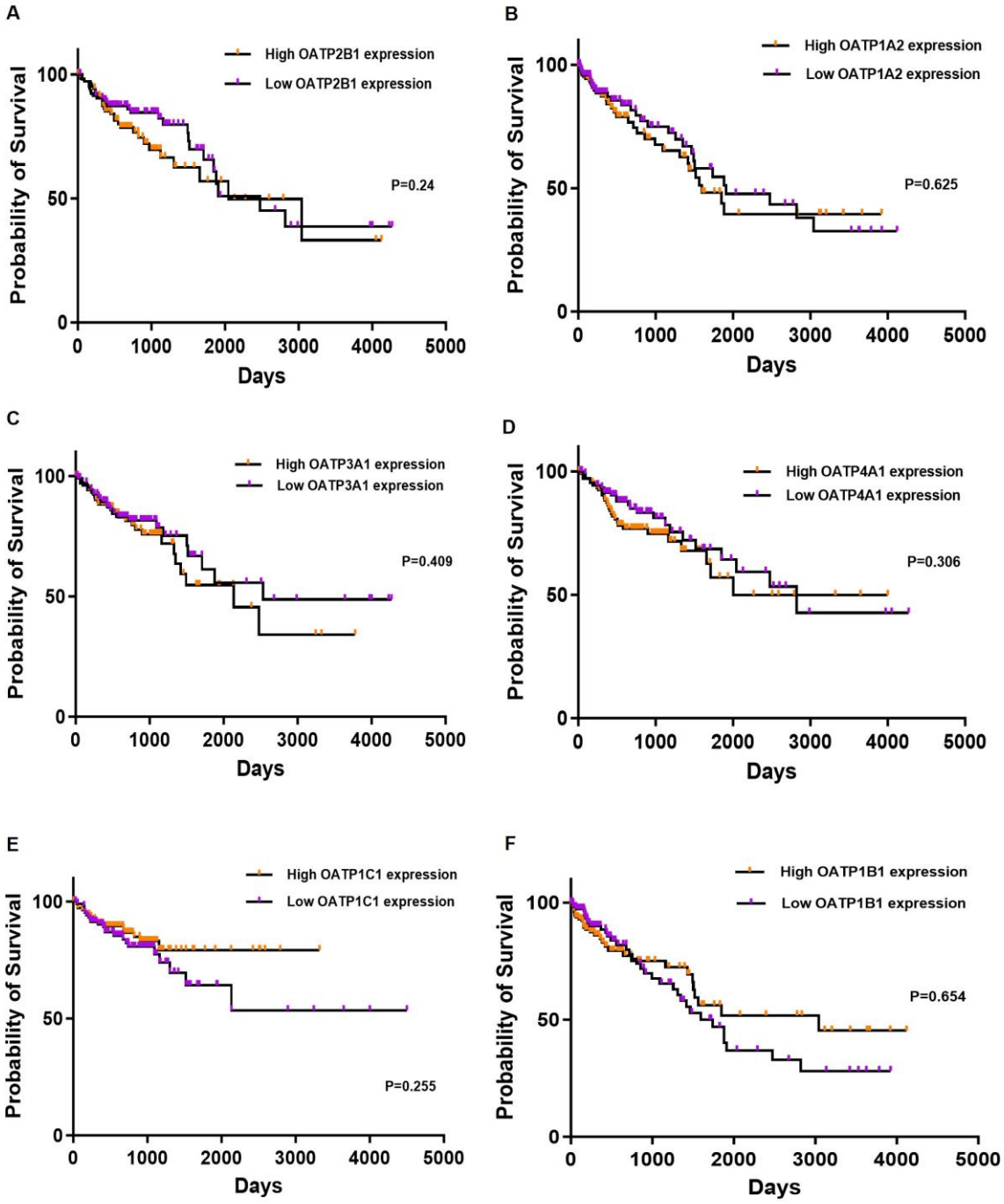


Figure 5.2: Survival outcomes for CRC patients and OATPs expression. TCGA COADREAD survival curves based on Kaplan-Meier analysis. The survival curves of high expression of OATP2B1 (A), OATP1A2 (B), OATP3A1 (C), or OATP4A1 (D), OATP1C1 (E), or OATP1B1 (F) compared to low OATP expression. Statistical analysis using the log-rank test.

As there were significant changes in OATP expression in CRC, it was next determined to analyse whether these effects impact survival outcomes for CRC patients. Therefore, TCGA COADREAD expression data and clinical survival data were analysed (Figure 5.2). Specifically, these survival analyses focused on CRC and the OATP genes. The survival outcomes of CRC patients with higher OATP1A2, OATP4A1, OATP1C1, and OATP1B1 expression have been improved. Patients with low OATP2B1 and OATP3A1 expression have high survival percentages.

5.3.3 The COAD correlation of TNFα and OATP expression in CRC

This chapter focuses on the potential role of TNα effect on oestrogen-uptake transporters OATPs. Thus, it was next determined to further analyse the TCGA COADREAD data to ascertain whether TNFα expression correlates with OATP expression in normal colon and CRC samples (Figure 5.3). Analysis of COAD data demonstrated a significant correlation was seen between TNFα in CRC tissue and OATP2B1 (***P<0.0001) (B), OATP3A1 (***P<0.0001) (F) and OATP1C1 (***P<0.0001) (J) expression, suggesting TNFα could play a role in upregulating these OATPs.

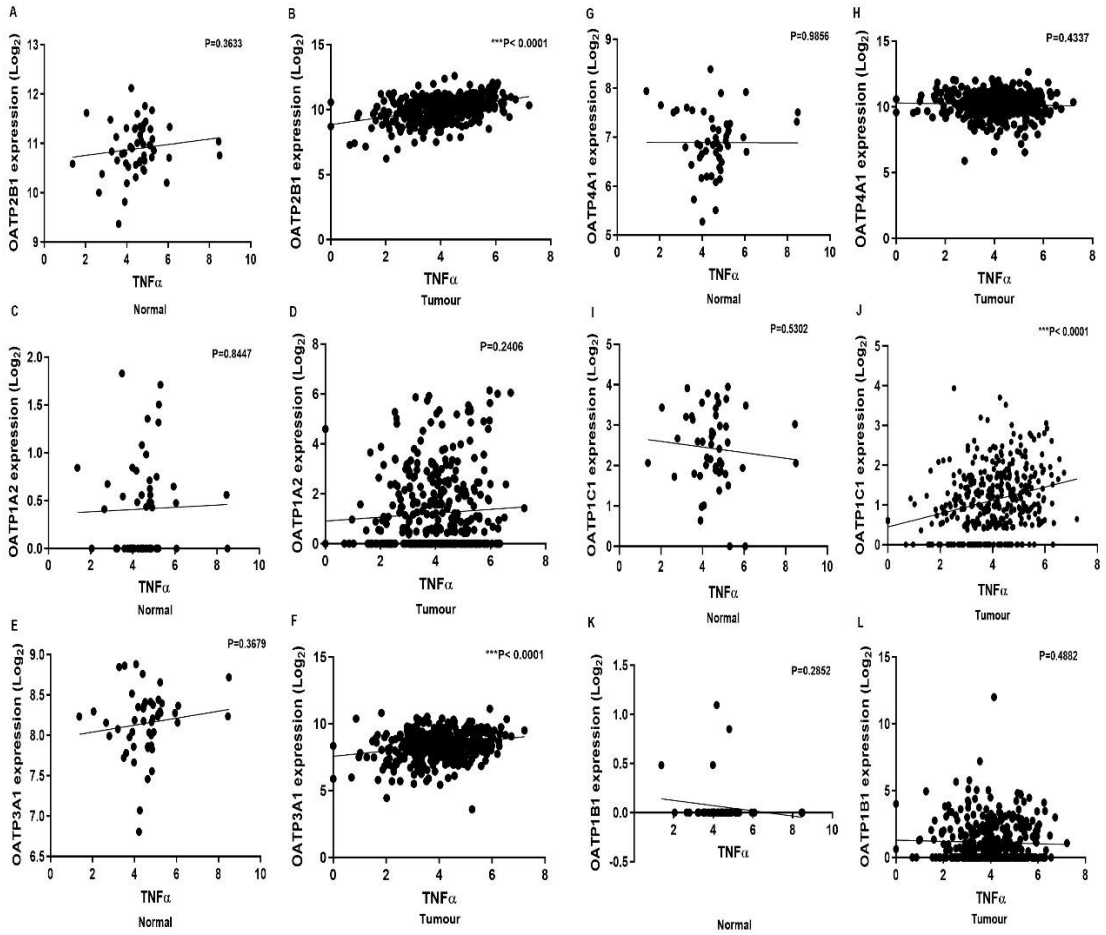


Figure 5.3: The COADREAD correlation of TNFα and OATPs expression in CRC. Correlation between TNFα in CRC tissue and OATPs expression in the COADREAD from the TCGA dataset. The correlation in normal and tumour between TNFα and OATP2B1 (A and B), OATP1A2 (C and D), OATP3A1 (E and F), OATP4A1 (G and H), OATP1C1 (I and J) and OATP1B1 (K and L). Correlation statistical analysis was done by using Prism, *** $p < 0.001$ compared to control. (n=433).

No correlation was seen between OATP2B1 and TNFα in normal tissue (P=0.36,) (Figure 5.3A) and a positive correlation with tumour CRC tissues (*** $p < 0.001$) (Figure 5.3B). The OATP1A2 have no correlation with TNFα in normal tissue (P=0.84) (Figure 5.3C) and tumour CRC tissues (P=0.24) (Figure 5.3D). However, a positive correlation was seen between OATP3A1 in tumour CRC tissues and TNFα (*** $p < 0.001$) (Figure 5.3F), but no correlation was seen between these in normal tissue (P=0.36) (Figure 5.3E). For OATP4A1, no correlation was seen with TNFα expression in normal tissue (P=0.98) (Figure 5.3G) or tumour CRC tissues (P=0.433) (Figure 5.3H). Also, a positive correlation was seen between OATP1C1 in tumour CRC tissues and TNFα (*** $p < 0.001$) (Figure 5.3J), but no correlation was seen between these in normal tissue (P=0.53) (Figure 5.3I). Finally, no correlation was seen between OATP1B1 and TNFα expression in normal tissue (P=0.28) (Figure 5.3K) or tumour CRC tissues (P=0.488) (Figure 5.3L).

5.3.4 The effect of TNFα on OATPs expression in CRC cell Lines

As the TCGA COAD data suggested the potential effects of TNFα on OATP expression, the next studies focused on examining these changes in CRC cell lines. To do this, OATP expression was measured in CRC cells after treatment with TNFα (10, 20, and 40 ng/ml) for 24-48 hours. We measured the expression of the OATP1B1, OATP1C1, OATP2B1, OATP1A2, OATPO3A1, and OATP4A1 genes in CRC cells using quantitative real-time PCR.

5.3.4.1 The effect of TNFα on OATP2B1 expression in CRC cell Lines

The mRNA expression of the OATP2B1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

HCT116 cells treated with TNFα (10 ng/ml, 20ng/ml, and 40 ng/ml) for 24-48 hours have an increase in the OATP2B1 expression after 24 hours treatment (6.37 ± 1.69, 6.07 ± 3.50 and 8.35 ± 4.45 fold increase) and have a significant increase in the OATP2B1 expression after 48 hours incubation with TNFα (10 and 20 ng/ml) *P=0.05 and with (TNFα 40 ng/ml) **P=0.01 (10.22 ± 2.69, 10.35 ± 3.82 and 12.73 ± 6.44 fold increase respectively). Also, the highest increase was observed in Colo205 cells treated with TNFα (20 ng/ml) for 24 hours and (40 ng/ml) for 48 hours (24.5 ± 31.6 and 25.17±11.31 folds respectively). There were no changes in OATP2B1 expression across Lovo and SW620 cells (Figure 5.4C and D). The survival outcome of CRC patients with higher TNFα with OATP2B1 expression has been improved (Figure 5.4E)

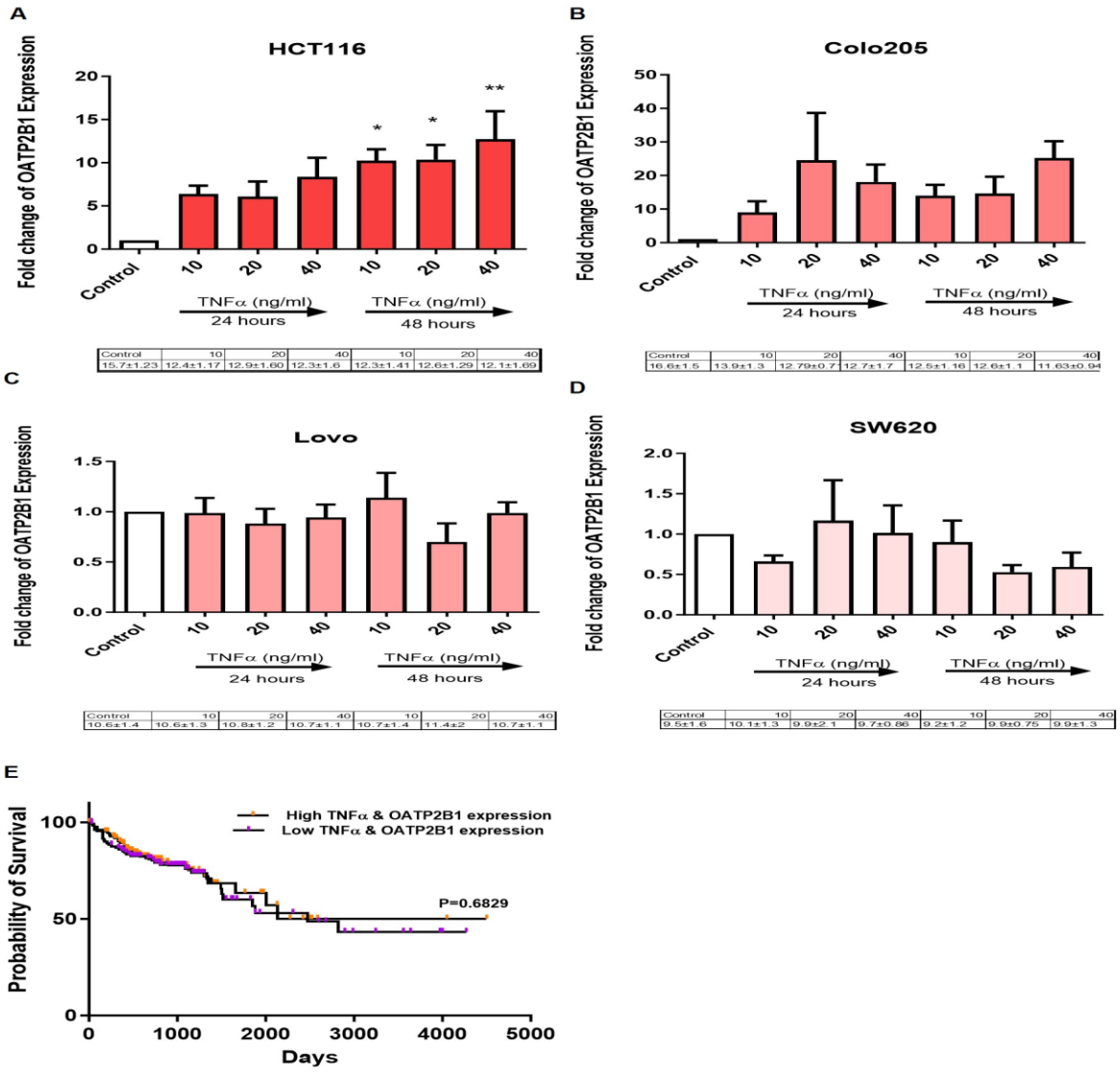


Figure 5.4: The effect of TNFα on OATP2B1 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP2B1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP2B1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. *P< 0.05 and **p < 0.01 compared to control. n= 3 independent experiments.

5.3.4.2 The effect of TNFα on OATP1A2 expression in CRC cell lines

The mRNA expression of the OATP1A2 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

HCT116 cells treated with TNFα (20 ng/ml and 40 ng/ml) for 24 hours have a very modest, non-significant increase in the OATP1A2 expression after 24-48 hours (1.34 ± 0.87 and 1.37 ± 0.94 fold increase). Also, a non-significant increase in the OATP1A2 expression was observed in Colo205 cells treated with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml) for 24-48 hours, and the highest expression was observed within 48 hours (1.85, 4.21 ± 3.46 and 5.41 ± 4.75 fold respectively). For Lovo and SW620 cells, there is a general non-significant reduction in OATP1A2 expression with TNFα treatment for both 24 hours and 48 hours (Figure 5.5C and D). The survival outcome of CRC patients with higher TNFα with OATP1A2 expression has been improved (Figure 5.5E).

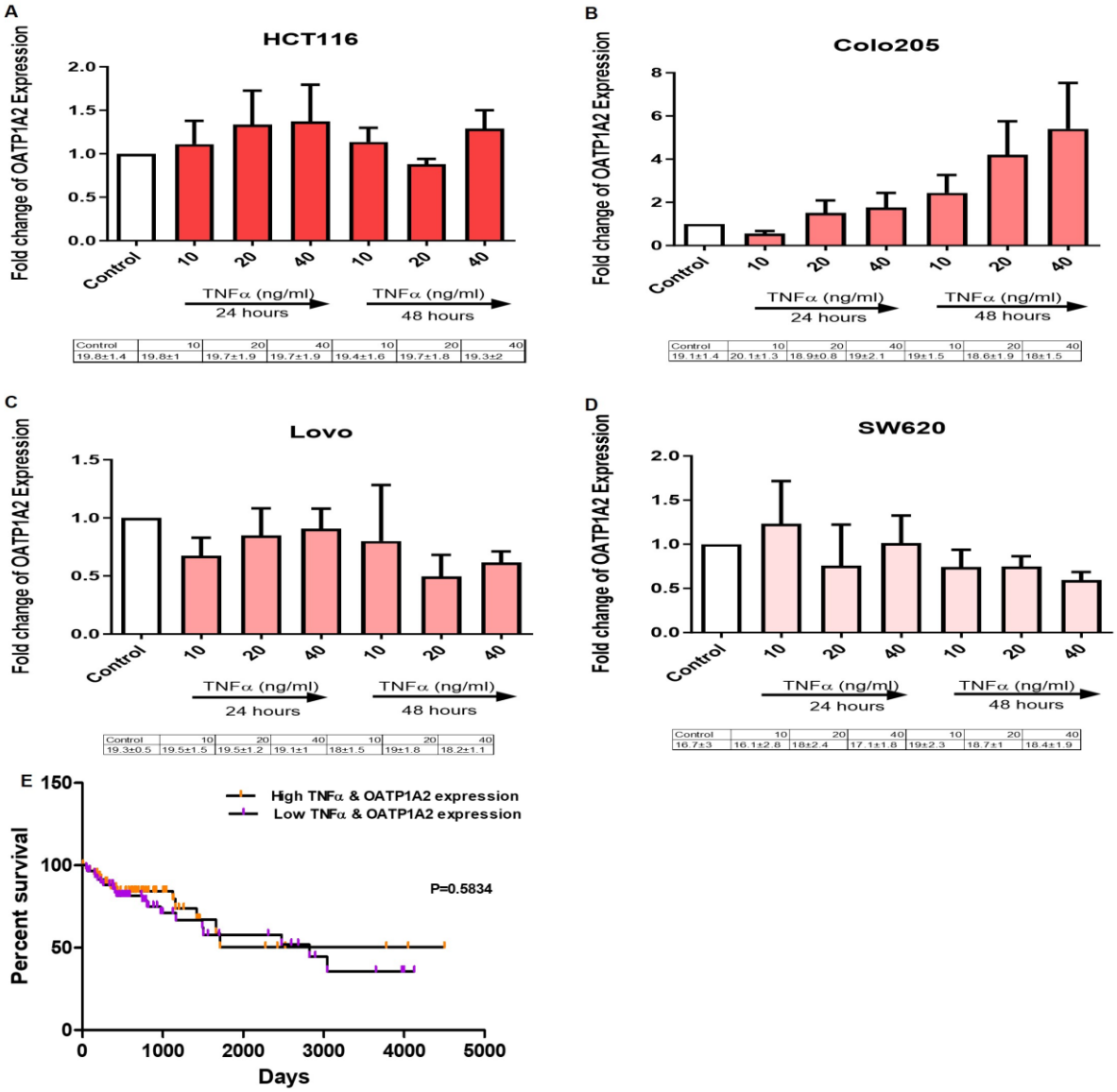


Figure 5.5: The effect of TNFα on OATP1A2 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP1A2 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP1A2(E). TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

5.3.4.3 The effect of TNFα on OATP3A1 expression in CRC cell lines

The mRNA expression of the OATP3A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

HCT116 and Colo205 cells treated with TNFα for 24 hours had, in general, a non-significant decrease in the OATP3A1 expression. However, for the HCT116 cell line after 24 hours of treatment with 20ng/ml TNFα (0.42 ± 0.17 fold), this decrease was significant (*p>0.05) compared to control. All other time points and treatments in the different cell lines did not significantly alter OATP3A1 expression in these CRC cell lines. The survival outcome of CRC patients with higher TNFα with OATP3A1 expression has been improved (Figure 5.6E).

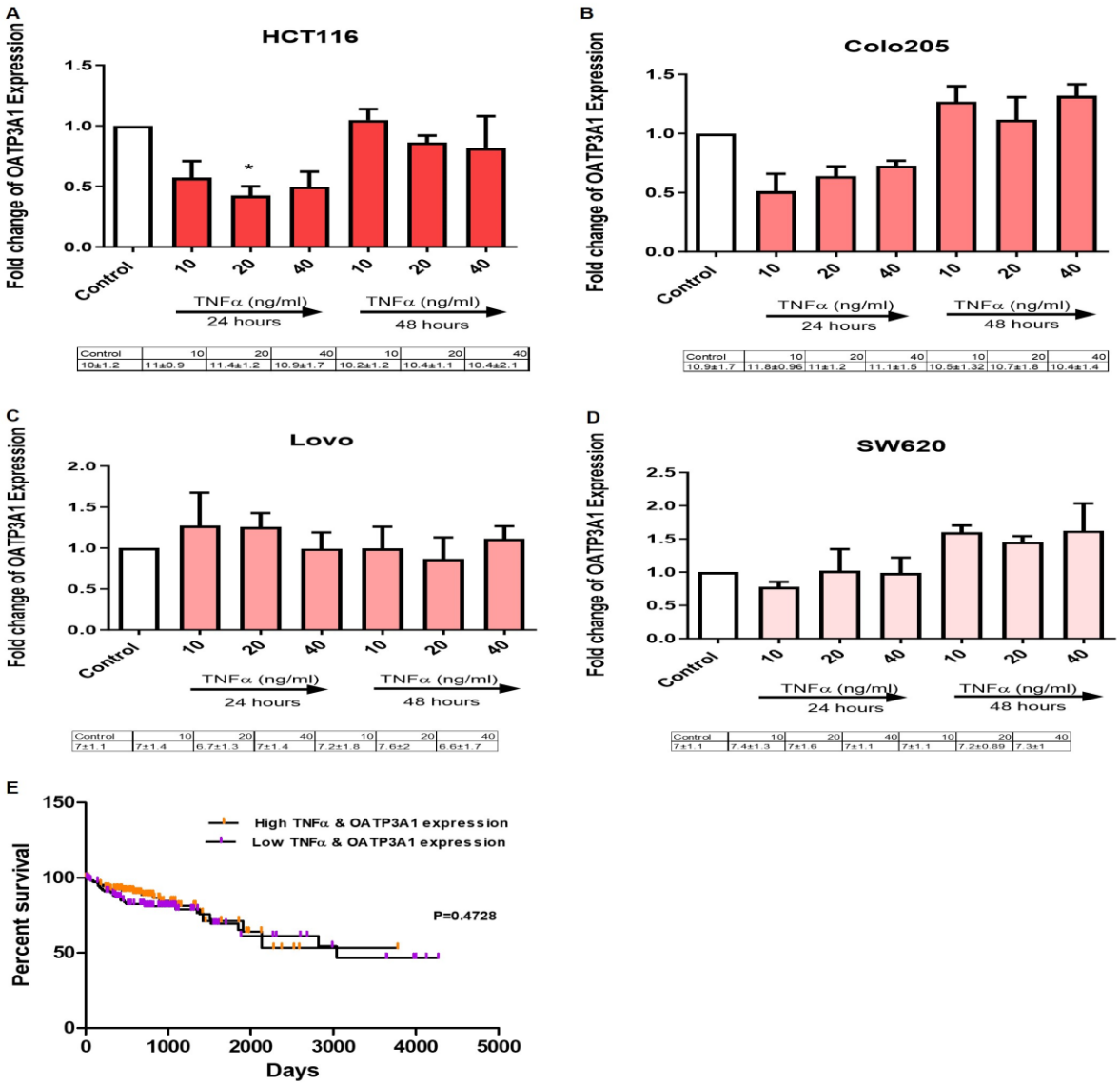


Figure 5.6: The effect of TNFα on OATP3A1 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP3A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP3A1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments. *p > 0.05 compared to control.

5.3.4.4 The effect of TNFα on OATP4A1 expression in CRC cell lines

The mRNA expression of the OATP4A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

Across all cell lines, there was no change in the expression of the OATP4A1 in cells treated with different doses of TNFα for 24-48 hours. Colo205 and LoVo cells had a non-significant increase in the OATP4A1 expression after being treated with (10ng/ml, 20ng/ml and 40ng/ml) for 48 hours in Colo205 (1.77 ± 0.82,1.33 ± 0.57 and 1.84 ± 0.42 fold) and 24 hours in LoVo (1.27 ± 0.15, 1.4 0± 0.60 and 1.53 ± 0.16 fold). Patients with low TNFα & OATP4A1 expression have high survival percent (Figure 5.7E).

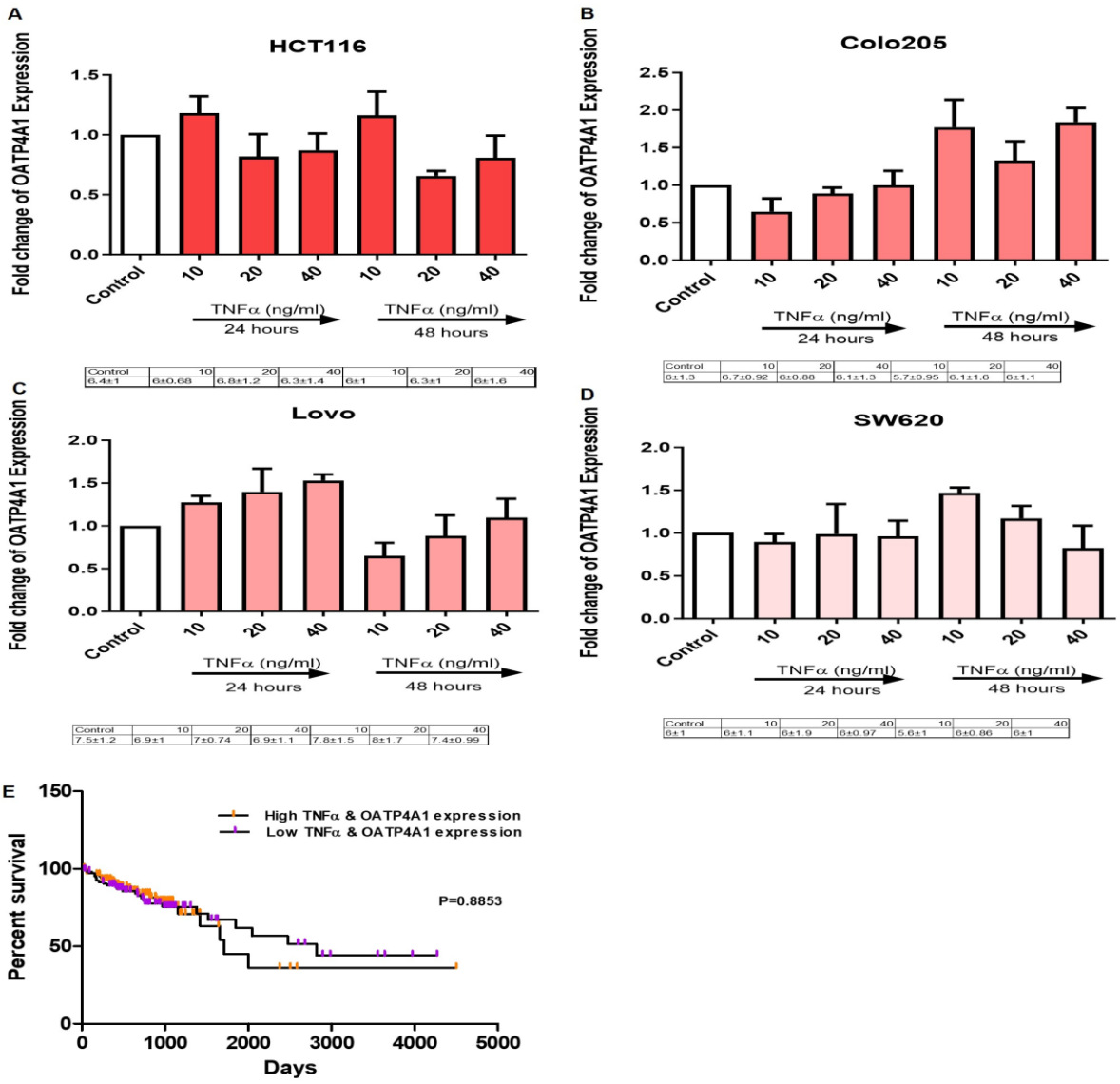


Figure 5.7: The effect of TNFα on OATP4A1 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP4A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP4A1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

5.3.4.5 The effect of TNFα on OATP1C1 Expression in CRC Cell Lines

The mRNA expression of the OATP1C1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

Across all cell lines, there was no change in the expression of the OATP1C1 in cells treated with different doses of TNFα for 24-48 hours. Colo205 cells had a non-significant increase in the OATP1C1 expression after being treated with (20ng/ml and 40ng/ml) for 48 hours (3.00 ± 2.48 and 2.75 ± 0.37 fold). The survival outcome of CRC patients with higher TNFα with OATP1C1 expression has no differences (Figure 5.8E).

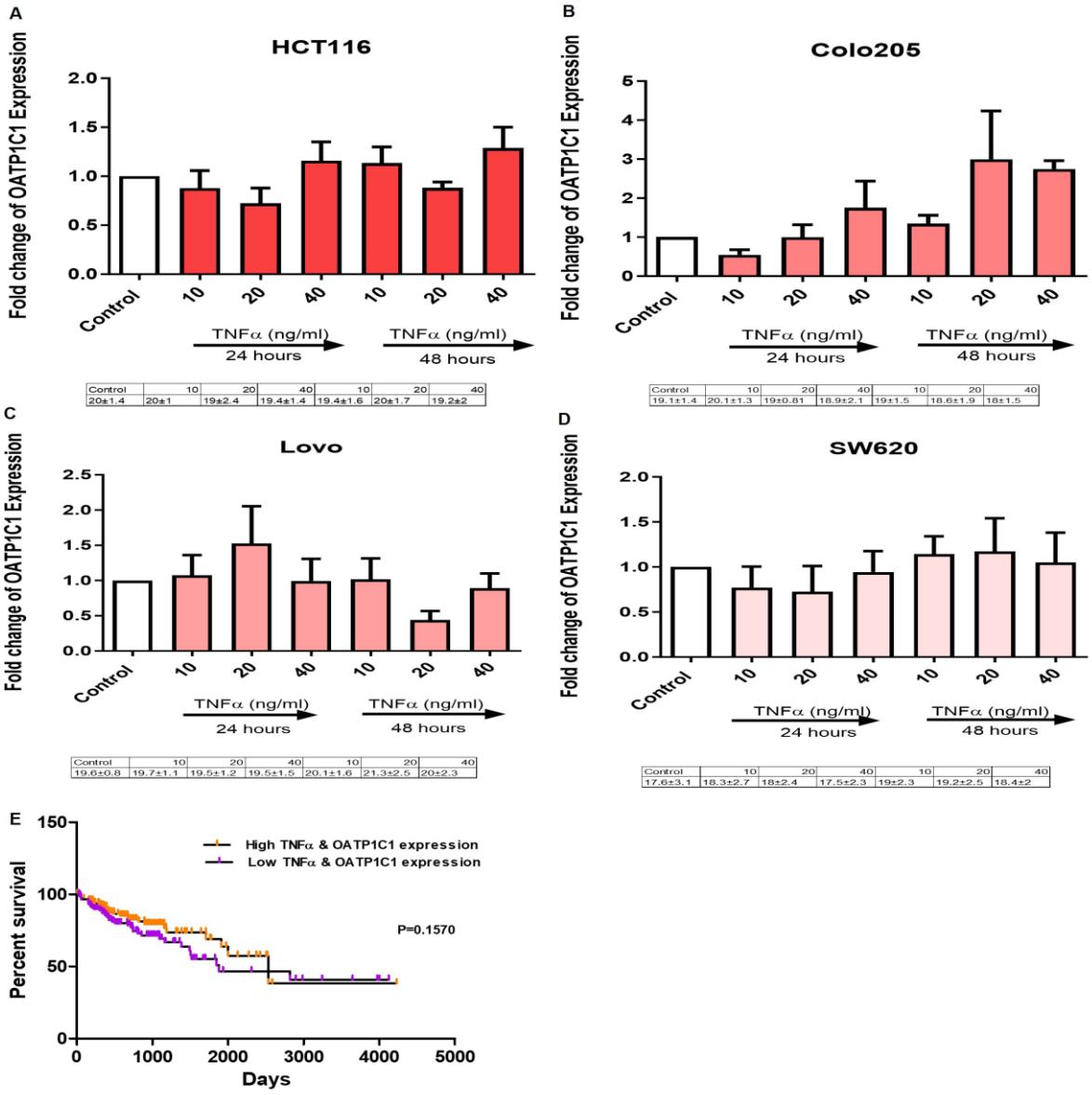


Figure 5.8: The effect of TNFα on OATP1C1 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP4A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP1C1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments.

5.3.4.6 The effect of TNFα on OATP1B1 Expression in CRC Cell Lines

The mRNA expression of the OATP1B1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), LoVo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours.

HCT116 cells have a non-significant increase in the OATP1B1 expression in cells treated with (40ng/ml) after 24 hours (2.33 ± 2 fold). Also, Colo205 have a non-significant increase in the OATP1B1 expression after treatment of TNFα (10 ng/ml, 20 ng/ml) with (5.54 ± 1.00 and 3.48 ± 1.35 fold) and a significant increase after TNFα (40 ng/ml) for 48 **P=0.01 (12.14 ± 9.67 fold). Lovo cells showed a non-significant increase in the OATP1B1 expression with TNFα (10, 20 and 40 ng/ml) incubated for 48 hours (1.79 ± 1.44, 1.16 ± 0.78 and 1.72 ± 0.88 fold). SW620 cells showed a non-significant increase in the OATP1B1 expression with TNFα (20 and 40 ng/ml) incubated for 24 hours (3.49 ± 3.6 and 2.34 ± 1.66 fold). The survival outcome of CRC patients with higher TNFα with OATP1B1 expression has been improved (Figure 5.9E).

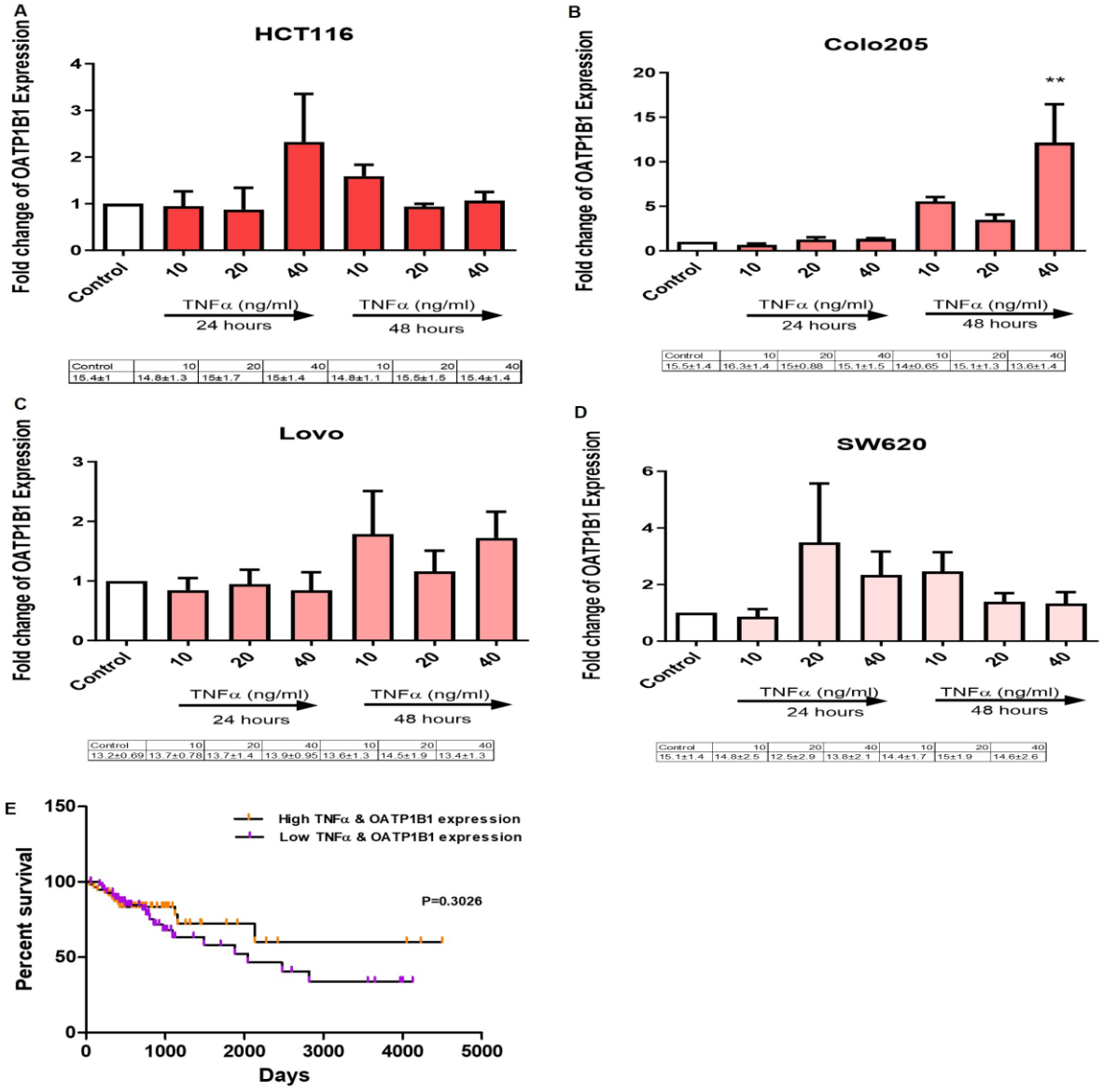


Figure 5.9: The effect of TNFα on OATP1B1 expression in HCT116, Colo205, Lovo and SW620 cell lines. The mRNA expression of the OATP4A1 and RPLPO genes was measured by qRT-PCR in HCT116 (A), Colo205 (B), Lovo (C) and SW620 (D) cells treated with TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) for 24 and 48 hours. Tabulated values represent mean ΔCt values ± SD. Statistical analysis is a one-way ANOVA with Tukey post-test. The survival curve of OATP1B1(E)**.** TCGA survival curves are based on Kaplan-Meier analysis, and statistical analysis is the log-rank test. n= 3 independent experiments. **P < 0.01 compared to control.

5.3.4.7 Survival curve and COAD correlation of TNFα and OATP1B3

A positive correlation was seen between OATP1B3 in normal tissues and TNFα (*P=0.045) (Figure 5.10A), and there was no correlation between OATP1B3 and TNFα in CRC tissue (P=0.56) (Figure 5.10B). The survival outcome of CRC patients with higher OATP1B3 expression has been improved (Figure 5.10 C). While patients with low and high TNFα & OATP1B3 expression have similar survival percent (Figure 5.10 D). The OATP1B3 expression was significantly high in Stage I, Stage III and Stage IV *P=0.05 and Stage II ***P=0.001 (Figure 5.10E). There was no OATP1B3 expression in the normal tissue of the colon, and it was only expressed in tumours and with high expression in both sexes, but the highest expression was in males under 50 and females over 50 (Figure 5.10F).

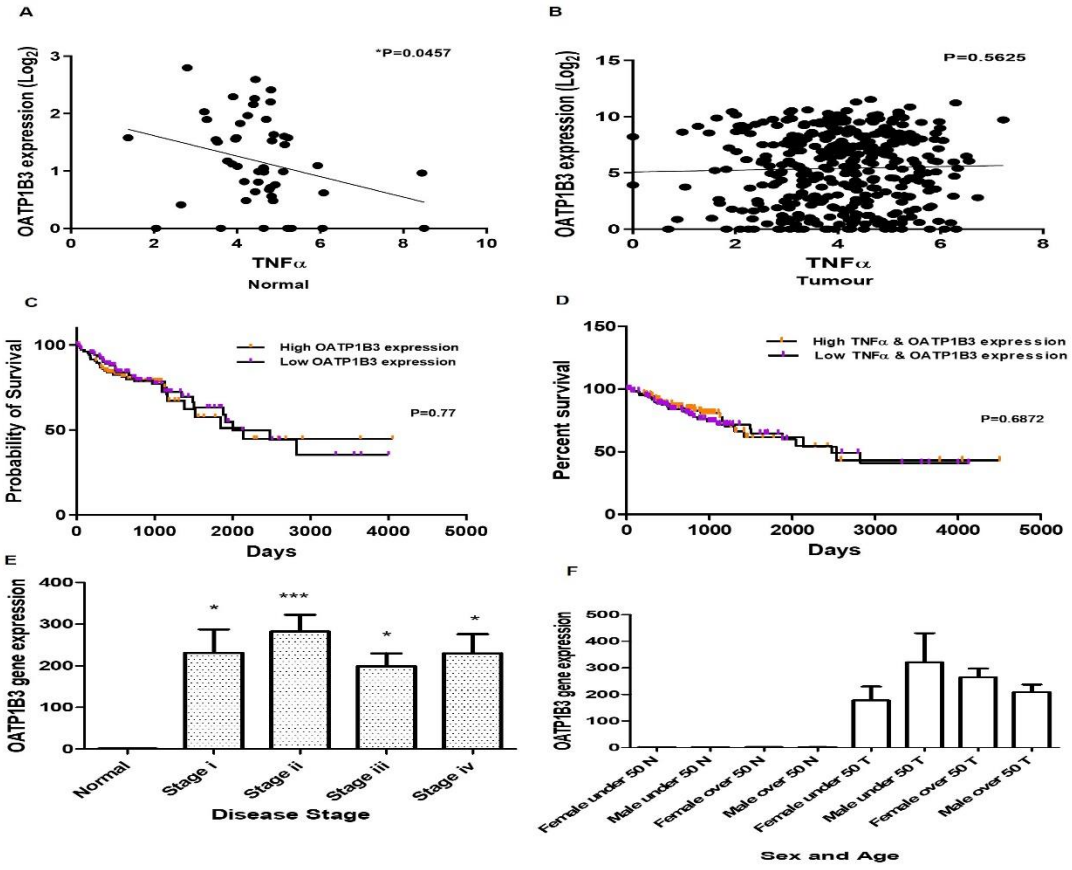


Figure 5.10: The effect of TNFα on OATP1B3 expression in CRC Cell Lines, the survival curve and COAD correlation of TNFα and OATP1B3 expression in CRC. Correlation between TNFα in CRC tissue and OATP1B3 expression in the COADREAD from the TCGA dataset. The correlation in normal and tumour between TNFα and OATP1B3 (A and B). The survival curves of high expression of OATP1B3(C) compared to low OATP1B3 expression. The survival curves of high expression of TNFα and high expression of OATP1B3 (D) compared to low TNFα and low OATP1B3 expression. Disease stages and sex differences of OATP1B3 expression in normal colon and CRC (E and F). ***$p < 0.001$ and * $p < 0.05$ compared to control. (n=433). Statistical analysis was performed using the log-rank test and one-way ANOVA with Tukey post-test.

5.3.4.8 Survival curve and COAD correlation of TNFα and OATP4C1

A positive correlation was seen between OATP4C1 in CRC tissues and TNFα (*P=0.004) (Figure 5.11B), and there was no correlation between OATP4C1 and TNFα in CRC tissue (P=0.83) (Figure 5.11A). The survival outcome of CRC patients with higher OATP4C1 expression has been significantly improved (**P=0.003) (Figure 5.11C). The OATP4C1 expression was significantly reduced in all CRC stages (***P=0.001) (Figure 5.11D). There was a reduction in OATP4C1 expression in the tumour tissue of the colon, and it was highly expressed in normal tissue and with high expression in both sexes, but the highest expression was in females over 50 (Figure 5.11 E and F).

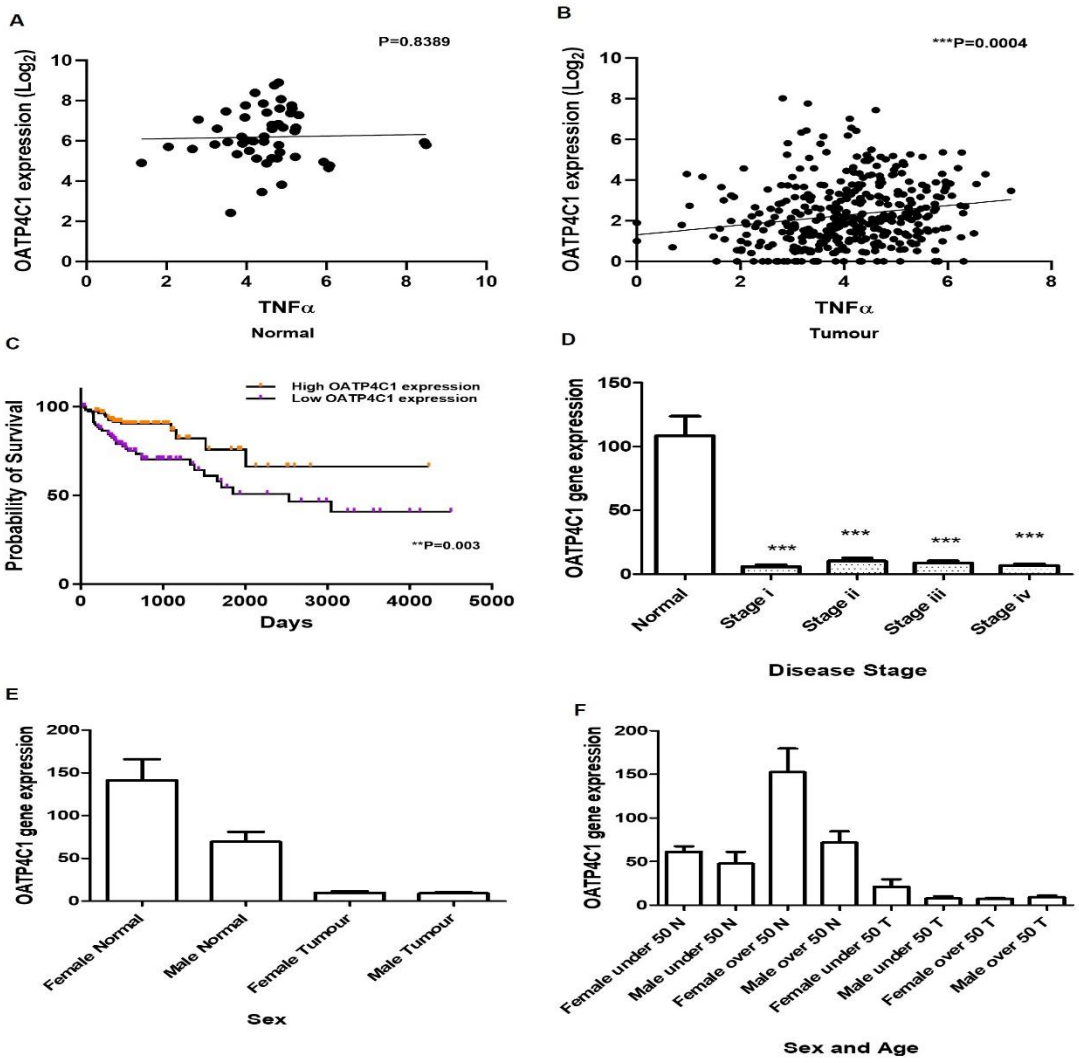


Figure 5.11: The effect of TNFα on OATP4C1 expression in CRC Cell Lines, the survival curve and COAD correlation of TNFα and OATP4C1 expression in CRC. Correlation between TNFα in CRC tissue and OATP4C1 expression in the COADREAD from the TCGA dataset. The correlation in normal and tumour between TNFα and OATP4C1 (A and B). The survival curves of high expression of OATP4C1 (C) compared to low OATP4C1 expression. Disease stages (D) and sex differences of OATP4C1 expression in normal colon and CRC (E and F). ***$p < 0.001$ and ** $p < 0.01$ compared to control. (n=433). Statistical analysis was performed using the log-rank test and one-way ANOVA with Tukey post-test.

5.3.5 Inflammation and uptake study of OATPs in CRC.

As TNFα caused significant expression changes in some OATPs, it was decided to further examine whether this alteration caused greater E1S uptake. CRC cells were treated with TNFα (40 ng/ml) for 24 hours, followed by E1S (100nM) treatment for 24 hours. HCT116 and SW620 cells have a non-significant increase in the E1S uptake compared to the control (Figure 5.12 A and D). Both the Colo205 and Lovo cells demonstrated a significant decrease in the E1S uptake compared to the control (***$P < 0.001$ and **$P < 0.01$) (Figure 5.12B and C).

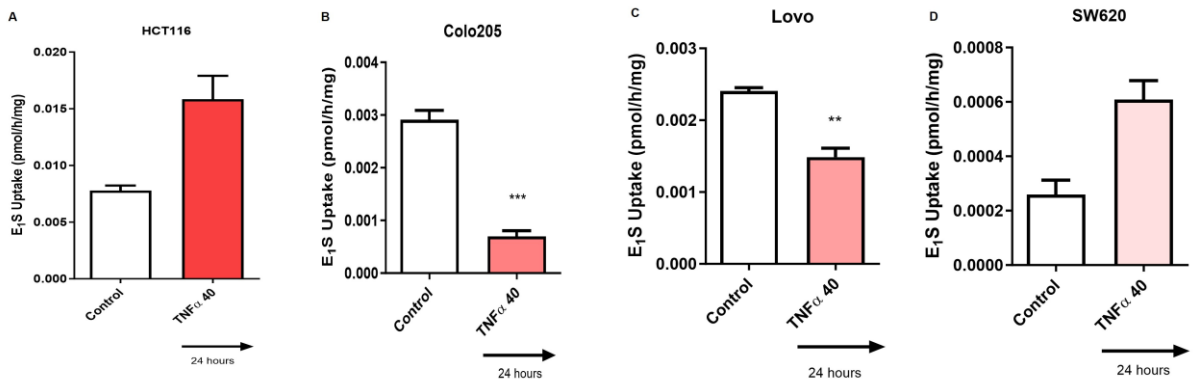


Figure 5.12: Cellular E1S uptake of OATPs. Uptake of E1S over 24 hours in four CRC cell lines, HCT116 (A), Colo205 (B), Lovo (C) and SW620 cells (D), after 24 hours of treatment with 40ng/ml. The results are expressed as mean ± standard deviation. A statistic was carried out with a t-test using GraphPad Prism.

5.4 Discussion

5.4.1 OATPs and CRC

According to my results, there was a significant increase in CRC samples in the expression of OATP1A2 (**P < 0.01), OATP1B1 (***P< 0.001), OATP1B3 (***P< 0.001), OATP3A1 (**P < 0.01) and OATP4A1 (***P< 0.001) mRNA compared to the normal epithelial samples. There was a significant reduction in OATP2B1 (***P< 0.001), OATP1C1 (***P< 0.001) and OATP4C1 (***P< 0.001) mRNA expression in CRC samples compared to normal colon tissue. Due to significant changes in OATP expression in CRC patients, it was determined whether these effects affected survival outcomes. Accordingly, TCGA COADREAD expression data and clinical survival data were analysed (Figure 5.2). These survival analyses focused on CRC and the OATP genes. Survival outcomes have improved for CRC patients expressing OATP1A2, OATP4A1, OATP1C1 and OATP1B1. Also, a high survival rate is observed in patients with low OATP2B1 and OATP3A1 expression. The results were consistent with previous studies, which reported that the OATPs expression has been shown to be altered in many cancerous tissues and cell lines compared to healthy tissues in many studies. The excessive production of these OATPs in cancer cells could elevate the cellular concentrations of hormones, such as oestrogens and androgens, which promote the growth of hormone-dependent cancer cells (Buxhofer-Ausch et al., 2013). Oestrone sulphate conversion may be affected by variations in steroid absorption by cells based on variations in OATP transport proteins or membrane permeability (Hankele et al., 2018).

The OATP1B3 is predominantly expressed at the basolateral membrane in hepatocytes. At the same time, when cancer develops, the distribution pattern is disrupted, and

OATP1B3 are upregulated in malignant tissues (colon, breast, prostate) (Svoboda et al., 2011; Chen et al., 2020). Compared to normal tissue, colorectal carcinoma expressed higher levels of OATP1B3 protein and mRNA (Lee et al., 2008). Despite not being present in normal colon tissue, OATP1B1 and OATP1B3 mRNA are substantially expressed in a variety of gastrointestinal tract malignancies, including the colon (Abe et al., 2001; Buxhofer-Ausch et al., 2013). There is an association between higher OATP1B1 expression and higher tumour grade in colon cancer specimens (Pressler et al., 2011). Also, the mRNA of OATP2B1 has been detected in colon adenocarcinomas, and the expression is higher in neoplastic colon specimens than in normal colon specimens (Tamai et al., 2000; Bleasby et al., 2006; Kleberg et al., 2012).

5.4.2 TNFα Regulates OATPs Expression

Tumour necrosis factor (TNFα), an inflammatory cytokine, was used in previous studies to treat cultured human CRC cells, and this resulted in a downregulation or up-regulation of OATPs expressions and activity. Our results of the analysis of COAD data demonstrated a significant correlation was seen between TNFα in CRC tissue and OATP2B1 (***P<0.0001), OATP3A1 (***P<0.0001), OATP1C1 (***P<0.0001) and OATP4C1 (***P=0.0004) expression, suggesting TNFα could upregulate these OATPs. Also, A survival analysis of The Cancer Genome Atlas (TCGA) data showed improved survival outcomes for patients with CRC who expressed high expression of TNFα and high expression of OATP2B1, OATP1A2, OATP3A1, OATP1B1. Survival rates are high in patients with low levels of TNFα & OATP4A1.

My findings demonstrate that TNFα (10, 20 and 40 ng/ml) treatment for 24 hours increased the expression of OATP2B1 in HCT116 and that this increase was significant

after 48 hours with TNFα (10 and 20 ng/ml) and (TNFα 40 ng/ml) *P=0.05 and ***P=0.01 and a non-significant rise in OATP2B1 expression was also seen in Colo205 following 24-48 hours of TNFα treatment. These findings were comparable with other studies that indicated increased OATP2B1 in colorectal cancer (Tamai et al., 2000; Bleasby et al., 2006; Kleberg et al., 2012). Moreover, OATP1A2 expression is non-significantly increased in HCT116 and Colo205 cells treated with TNFα after 24-48 hours. The expression of OATP3A1 in HCT116, Colo205 and SW620 cells decreased non-significantly after 24 hours of TNFα treatment with a significant decrease in the cells treated with TNFα (20 ng/ml) after 24 hours *P=0.05. Also, a non-significant increase in OATP4A1 expression was observed in HCT116 cells treated with TNFα (10ng/ml) after 24 hours, and Colo205 also showed a non-significant increase in OATP4A1 expression after 48 hours incubation with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml). The findings were similar to those of other studies which demonstrated low levels of OATP3A1 mRNA and high levels of OATP4A1 mRNA in CRC tumours (Kleberg et al., 2012; Rawłuszko-Wieczorek et al., 2015).

Across all cell lines, there was no change in the expression of the OATP1C1 in cells treated with different doses of TNFα for 24-48 hours, and only Colo205 cells had a non-significant increase in the OATP1C1 expression after being treated with (20ng/ml and 40ng/ml) for 48 hours. Finally, in HCT116 cells treated with TNFα (10ng/ml, 20ng/ml, and 40ng/ml) for 24-48 hours, OATP1B1 expression is not significantly increased in cells treated with TNFα (40ng/ml) after 24-48 hours and TNFα (10ng/ml) after 48 hours and Colo205 have a non-significant increase in the OATP1B1 expression after treatment of

TNFα (10 ng/ml, 20ng/ml) and a significant increase after TNFα (40 ng/ml) for 48 hours (**P=0.01).

HCT116 and SW620 cells treated with TNFα (40 ng/ml) for 24 hours have increased the E1S uptake. This indicates that TNFα may affect the OATPs uptake.

5.5 Conclusion

CRCs alter the physiological expression pattern of OATPs; variations in expression levels and gene variants of transporters and enzymes can affect their expression and function. As steroid hormone precursors (E1S) are taken up by cancer cells through OATPs, hormone-dependent tumour growth may also be affected. In the prognosis of CRC, OATPs and E1S could be significant factors. In this study, we investigated whether inflammation may have contributed to the increased STS activity observed in CRC. In CRC, the STS activity is increased by inflammation primarily through TNFα. Furthermore, TNFα might increase the expression of OATP2B1, OATP1A2, OATP4A1 and OATP1B1, which increases the uptake of E1S. Consequently, STS activity increases in response to TNFα because more E1S substrate enters the cell. In conclusion, TNFα can alter OATP expression, which may have implications for CRC and treatment effectiveness, and its expression in cancer should be investigated further.

CHAPTER 6. FINAL DISCUSSIONS AND FUTURE STUDIES

6.1 Discussions:

The human CRC exhibits a dysregulated oestrogen metabolism that favours oestradiol (E2) synthesis (Gilligan et al., 2017), and STS activity has been shown to be frequently elevated in CRC (Sato et al., 2009), although what regulates this effect was unknown. Oestradiol (E2) is the most abundant and potent oestrogen form in humans. In CRC, HSD17B2 and 4 are involved in converting E2 into estrone (E1), an antiproliferative compound (English et al., 1999; English et al., 2001), and HSD17B7 and 12 are involved in converting E1 to estrone (E2) (Gilligan et al., 2017). HSD17B2 was also strongly expressed in healthy colon's small intestine and surface epithelium and decreased expression in HCT116 and Colo205 (English et al., 1999; Oduwole et al.,2003; Gilligan et al. 2017), HSD17B7 is expressed more highly in CRC than in healthy colon tissue, and this is the same for HSD17B12 (Gilligan et al., 2017) but again what factors regulating this effect was unknown. Earlier findings have suggested that TNFα may partly control STS activity in prostate and breast cancer cell lines in vitro (Newman et al.,2000; Suh et al., 2011). This thesis focuses on whether pro-inflammatory cytokines, mainly TNFα, can alter oestrogen metabolism and uptake in CRC cell lines (HCT116, Colo205, Lovo and SW620). Here, and after treatment with TNFα, STS activity increased in CRC cell lines. The data presented in this thesis confirm that TNFα alters oestrogen metabolism and uptake in CRC, implicating oestrogens as proliferative factors in this malignancy.

6.1.1 TCGA datasets suggest TNFα alters key oestrogen metabolism pathways in human CRC

The TCGA dataset has been analysed to study STS, HSD17Bs and OATPs expression in COADREAD and to investigate whether STS, HSD17Bs and OATPs mRNA expression is associated with CRC. There was a reduction in STS mRNA expression in CRC patients compared to normal (Figure 3.1A). Also**,** comparing CRC samples to normal epithelial revealed a significant increase in HSD17B7 and HSD17B12 mRNA expression and a significant reduction in HSD17B2 mRNA expression was observed in CRC (Figure 4.1). This suggests that CRC favours the synthesis of oestradiol. Interestingly, patients with CRC had a significant reduction in ERα, ERβ and GPER expression compared to normal (Figure 4.15). However, as previously shown (Gilligan et al. 2017), GPER expression was higher than ER□ and ER□, suggesting this receptor is still present in CRC despite its down-regulation.

Finally, compared to other OATPs analysed, CRC patients had high levels of OATP1B3, OATP2B1, OATP3A1 and OATP4A1. Expression of OATP1A2, OATP1B1, OATP1C1 and OATP4C1 was very low. In comparison to normal epithelial cells, CRC samples showed a significant increase in OATP1A2, OATP1B1, OATP1B3, OATP3A1 and OATP4A1. As compared to normal colon tissue, OATP2B1, OATP1C1, and OATP4C1 expression were significantly reduced in CRC samples (Figure 5.1).

Also, the expression correlations for TNFα compared with STS, HSD17Bs and OATPs were originally determined using the COADREAD dataset. In normal colon tissue, TNFα expression did not correlate to STS expression (Figure 3.1C). However, in CRC, a

positive correlation was observed between STS and TNFα expression (Figure 3.1D). These results were similar when analysing another inflammatory cytokine, IL-6. There was no significant correlation of IL-6 with STS in normal colon tissue (Figure 3.1E). However, in CRC tissue, increased IL-6 expression correlated with increased STS expression (Figure 3.1F). Furthermore, a positive correlation was seen between TNFα in CRC tissue and HSD17B4 and HSD17B12 (Figure 4.3F and J). There was no correlation between ERα and ERβ with TNFα expression in normal colon tumours (Figure 4.16A and Figure 4.17A). However, a positive correlation was seen between ERα and ERβ with TNFα in tumour CRC tissues (Figure 4.16B and Figure 4.17B). This suggests that TNFα expression may regulate in ERα and ERβ expression in CRC. Finally, analysis of COADREAD data demonstrated a positive correlation between TNFα in CRC tissue and OATP2B1, OATP3A1 and OATP1C1 expression (Figure 5.3B and F and J), suggesting TNFα could play a role in upregulating these OATPs.

6.1.2 TNFα causes STS activity to increase in CRC

Fraumeni first observed the role of oestrogen in CRC in 1969, showing an increased risk of colon and breast cancer in a community of nuns; they have a regular menstrual cycle and higher lifetime exposure to oestrogen (Fraumeni et al., 1969). Our group has recently shown that oestrogen metabolism is dysregulated in CRC (Gilligan et al.,2017). Although there were no differences in the expression of STS mRNA between normal and CRC epithelial specimens, STS activity in human CRC tissue was increased compared with normal matched controls. Similarly, in CRC, various other studies have shown a relationship between oestrogen metabolism and cancer by analysing epidemiological and clinical data, HSD17Bs, aromatase, STS/ SULT1E1 activity, and *in vitro* as well as *in*

vivo genomic influences (Fiorelli et al., 2002; Barzi et al., 2013; Gilligan et al., 2017). A recent study (Gilligan et al. 2017) demonstrated that STS activity is significantly elevated in human CRC and that STS overexpression accelerates CRC proliferation *in vitro* and *in vivo.*

STS is increased in various malignant tissues, and it has been shown that the proinflammatory cytokines TNFα and IL-6 modify STS enzyme kinetics in different cancers (Mueller et al., 2015). TNFα and IL-6 have previously been shown to increase the expression and/or function of STS in breast MCF7 (Purohit et al., 1996; Reed and Purohit, 1997), and up to 95% of diagnosed CRC patients do not have genetic predispositions. Still, they share common risk factors, including IBD, age and an unhealthy Western lifestyle (Kern et al., 2019). Both obesity and age are linked to increasing CRC, and there is a known increase in circulating TNFα and IL-6 in obese patients/rats. (Kern et al., 2019) and a study by (Bruunsgaard et al.,2001) has shown increased circulating TNFα and IL-6 levels with aging. Thus, increased TNFα may increase colonic STS activity, which may have a role in the development and progression of CRC. Also, in all five groups, patients with CRC had higher serum IL-17A levels and TNFα levels than those with UC, colon polyps, and healthy volunteers. In the CRC group, TNF levels were significantly higher than those in any of the control groups, suggesting that these proinflammatory cytokines may be useful biomarkers. (Ma et al.,2022).

The data in this project supports the role of TNFα in regulating STS activity. The mechanism by which TNFα alters STS activity remains unclear, while post-translation modifications have been hypothesised, probably through STS glycosylation. However, it

could be that these inflammatory cytokines increase substrate availability by altering OATP expression and increasing sulfated substrate uptake into the cell (Newman et al., 2000). The expression of STS in human CRC is increased relative to normal colonic mucosa (Gilligan et al.,2017), and these indicate that local oestrogens (E1 and E2) play an important role in disease progression. Indeed, the combined status of high STS and low expression of SULT1E1 was significantly correlated with higher oestrogen concentrations in CRC tissue (Sato et al., 2009). Therefore, the increase in STS activity in the CRC cell line is not due to higher STS mRNA expression, and cytokines can increase STS activity by post-translation modification. Understanding how expression is regulated is crucial since malignant colon tissues are likely to have high STS activity. In order to answer this, we investigated if the cytokines, which can activate STS, could be implicated. It's also possible that these cytokines change membrane permeability, enhancing substrate availability and increasing STS.

To confirm that the STS activity is regulated by the cytokines, intact and lysed (non-intact) HCT116, Colo205, Lovo and SW620 cell types were analysed to determine the STS activity after treatment with different cytokines. Results show that other cytokines (IL-6, IFN-γ, and IL-4) did not affect STS activity in some CRC cell lines, suggesting STS activity is primarily regulated by TNFα activity. There was a significant increase in STS activity in intact CRC cells after TNFα treatment, but there were minimal changes in STS activity in cell lysates. The activity of STS was increased in HCT116, Colo205, LoVo, and SW620 cells by TNFα. HCT116 and Colo205 cells showed significant STS activity (Figures 3.2 & 3.3). In addition, LCMS results of HCT116 and Colo205 cells treated with TNFα (40ng/ml) showed an increase in E1 concentrations compared to

untreated cells after incubation with 100 nM of E1S (Figure 3.8A). Earlier findings have suggested that TNFα may partly control STS activity in prostate and breast cancer cell lines *in vitro*.

Also, to understand how inflammation affects oestrogen metabolism, we examine STS activity in cell lysates. It has been shown that both lysated HCT116 and Colo205 have increased STS activity after TNFα treatment. Lysated LoVo and SW620, however, showed an increase in their STS activity after TNFα (10 ng/ml, 20 ng/ml, 40 ng/ml) was applied for 24 or 48 hours. Gilligan et al. (2017) found that cell protein from cell lysates had a significantly higher STS activity, which suggests that E1S membrane transport may be limiting oestrogen hydrolysis.

Finally, we also examined the proliferation of CRC cells after being treated with 40ng/ml TNFα and 100nM E1S, E1 and E2 for 24 and 48 hours. HCT116 and Colo205 cells treated with TNFα and E1S proliferated significantly after 24 and 48 hours. The proliferation of HCT116 cells treated with E2 increased significantly after 24 hours, whereas the proliferation of HCT116 and Colo205 cells was non-significant after 24 and 48 hours. Foster et al. (2006) concluded that STS activity directly promotes the proliferation of oestrogen-dependent breast cancer. In colon cancer cells, E1S stimulates proliferation via a series of reactions that include uptake, desulfation by STS, conversion of E1 to E2 by HSD17Bs, binding to nuclear ER, and transcriptional regulation. Based on the results of this study, TNFα can increase STS activity by either post-translationally altering the enzyme or by increasing substrate availability.

6.1.3 TNFα alters HSD17Bs expression in colorectal cancer

In the normal colon, cancerous colon, and CRC cell lines, HSD17B1 is not expressed (Gilligan et al. 2017). However, HSD17B2 is highly expressed in the small intestine and surface epithelium in normal colon tissue. However, with the onset of CRC, there is a decrease in HSD17B2 and HSD17B4 expression (Vihko et al., 2002). HSD17B2 and HSD17B4 convert E2 to E1, which is antiproliferative in human colon cancer cell lines. (English et al., 2001). In previous studies, IL-6 and TNFα have been shown to modulate this steroidogenesis. For example, TNFα can significantly decrease the E1/E2 ratio by decreasing E1 synthesis and increasing E2 concentrations. This E1/E2 ratio alteration suggests that TNF-α alters HSD17Bs expression (Zhang et al., 2012). Also, it has been reported by Adams et al. that IL-6 has a paracrine role in the regulation of breast cancer tissue levels of E2 and IL-6 can regulate the expression of several enzymes involved in sex steroid formation and inactivation in breast cells (Adams et al., 1991; Turgeon et al.,1998) and regulate STS (Purohit et al., 1996) and P450 aromatase (Zhao et al., 1995).

The results in this thesis support these previous findings. TNFα did not significantly alter HSD17B1 expression in all CRC cell lines tested. Also, there was a non-significant decrease in HSD17B2 expression in HCT116, Colo205, and LoVo cells treated with TNFα, again supporting previous evidence of this enzyme's loss in CRC. Furthermore, there was a non-significant downregulation in HSD17B4 expression in HCT116 and Colo205 cells treated with TNFα.

Other enzymes responsible for the conversion of E1 to E2, HSD17B7 and HSD17B12, were also studied. Both mRNAs were found to be expressed in CRC cell lines. While, across all cell lines, there was a non-significant increase in HSD17B7 and HSD17B12

expression, these demonstrate that in CRC, TNFα may increase pathways that favour E2 synthesis and thus act as a potential pro-proliferative mechanism.

Data from TCGA COADREAD and previous studies (English et al., 2000; Gilligan et al., 2017) support these findings, in which HSD17B1 expression was not affected by TNFα, and HSD17B2 expression was downregulated in human CRC. In addition, LCMS results of HCT116, Colo205, LoVo and SW620 cells treated with TNFα (40ng/ml) showed a decrease in E1 concentration in comparison to untreated cells incubated with 100 nM of E2. In response to TNFα treatment, HSD17B2 and HSD17B4 activity declined, suggesting that E1 synthesis from E2 is decreased.

6.1.4 TNFα alters OATPs expression in CRC

In HCT116 and Colo205 cells treated with TNFα, this is unlikely to explain the large increase in STS activity. My study demonstrated that TNFα might increase STS activity by increasing E1S uptake into HCT116, Colo205, LoVo, and SW620 cells. For E1S uptake, OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1 and OATP4A1 are responsible (Muller et al., 2015). As a result of TNFα (10, 20 and 40 ng/ml) treatment for 24 hours in HCT116, OATP2B1 expression was significantly increased after 48 hours, and a non-significant increase in OATP2B1 expression was also observed in Colo205 after 24 to 48 hours of TNFα treatment. Other studies have also found an increase in OATP2B1 in CRC (Tamai et al., 2000; Bleasby et al., 2006; Kleberg et al., 2012). Furthermore, OATP1A2 expression is not significantly increased in HCT116 and Colo205 cells treated with TNFα after 24-48 hours. OATP3A1 expression decreased non-

significantly in HCT116, Colo205 and SW620 cells treated with TNFα but significantly in cells treated with TNFα (20 ng/ml) after 24 hours.

HCT116 cells treated with TNFα (10 ng/ml) showed a non-significant increase in OATP4A1 expression after 24 hours, and Colo205 cells showed a non-significant increase after 48 hours incubation with TNFα (10 ng/ml, 20 ng/ml, and 40 ng/ml). A similar finding was reported in other studies in which OATP3A1 mRNA levels were low, and OATP4A1 mRNA levels were high (Kleberg et al., 2012; Rawuszko-Wieczorek et al., 2015). Furthermore, OATP2B1 and OATP4A1 had the highest expression across CRC cell lines (Gilligan et al., 2017). A non-significant increase in OATP1C1 expression was only observed in Colo205 cells after treatment with (20ng/ml and 40ng/ml) for 48 hours. Finally, in HCT116 cells treated with TNFα (10ng/ml, 20ng/ml, and 40ng/ml) for 24-48 hours, OATP1B1 expression was not significantly increased in cells treated with TNFα (40ng/ml) after 24-48 hours and TNFα (10ng/ml) after 48 hours. After treatment with TNFα (10 ng/ml, 20 ng/ml), Colo205 had a non-significant increase in OATP1B1 expression and a significant increase after 48 hours when treated with TNFα (40 ng/ml). E1S uptake was increased in HCT116 and SW620 cells treated with TNFα (40 ng/ml) for 24 hours. This indicates that TNFα may affect the uptake of OATPs. Therefore, OATP2B1, OATP1A2, OATP1B1 and OATP4A1 were hypothesised to be the most likely candidates to be regulated by TNFα to increase E1S uptake in CRC.

6.2 CONCLUSION

This thesis aimed to understand how inflammation, specifically TNFα, affects oestrogen metabolism and oestrogen uptake through OATPs in CRC. I explored how inflammation

affects STS in chapters 3 and 4, the HSD17Bs enzymes in chapters 4, and the organic transporter of oestrogens in chapter 5. Consequently, I would like to highlight the major points of the previous discussions to demonstrate the links between the results and facilitating CRC research and development. Our findings are summarised in Figure 6.1.

Here, it is shown that TNFα considerably increased STS activity in CRC cells. Other cytokines tested (IL-6, IL-4, and IFN-γ) had no effect on STS activity. All other tested cytokines had no effect on the expression or activity of the HSD17Bs. The TCGA COADREAD dataset analysis also established a positive association between TNFα and STS, HSD17Bs and OATPs that supports the CRC cell line findings. These results indicate that CRC TNFα increases E2-favourable pathway synthesis and thus serves as a possible proliferative mechanism. Together with our previous findings that TNFα may increase the pathways for oestrogen uptake, this data suggests that TNFα is an important regulator of oestrogen metabolism in CRC.

By understanding how chronic inflammation leads to cancer, new drugs can be designed to counter its effects; the drugs focus on inhibiting the cyclooxygenase-2 (COX-2) enzyme.

In chronic inflammatory diseases with high cancer risk, regularly suppressing a major signalling factor, like NFκB or TNFα, could prolong the premalignant phase and inhibit tumour progression. Understanding the connection between inflammation and cancer is still a long way off. A better understanding of inflammation and cancer will lead to more effective prevention and treatments.

Also, it is possible to target oestrogen metabolism enzymes as a potential new treatment for CRC. When targeting the oestrogen pathway, for example, drugs should inhibit oestrogen-mediated proliferation and survival signalling in CRC cells. It would be ideal if these drugs demonstrated efficacy in reducing tumour size, preventing metastasis, and improving patient survival. The effectiveness of these drugs can be assessed using in vitro and in vivo models in preclinical studies. CRC cell lines could be used in in vitro studies to evaluate how drugs or inhibitors affect cell proliferation, apoptosis, and other cellular processes relevant to CRC. To evaluate whether these inhibitors are effective in reducing tumour growth and metastasis in vivo, animal models of CRC can be used, such as patient-derived xenograft (PDX) models and genetically engineered mouse models (GEMMs). Additionally, clinical trials can be conducted to determine whether the drugs are safe and effective in human CRC patients.

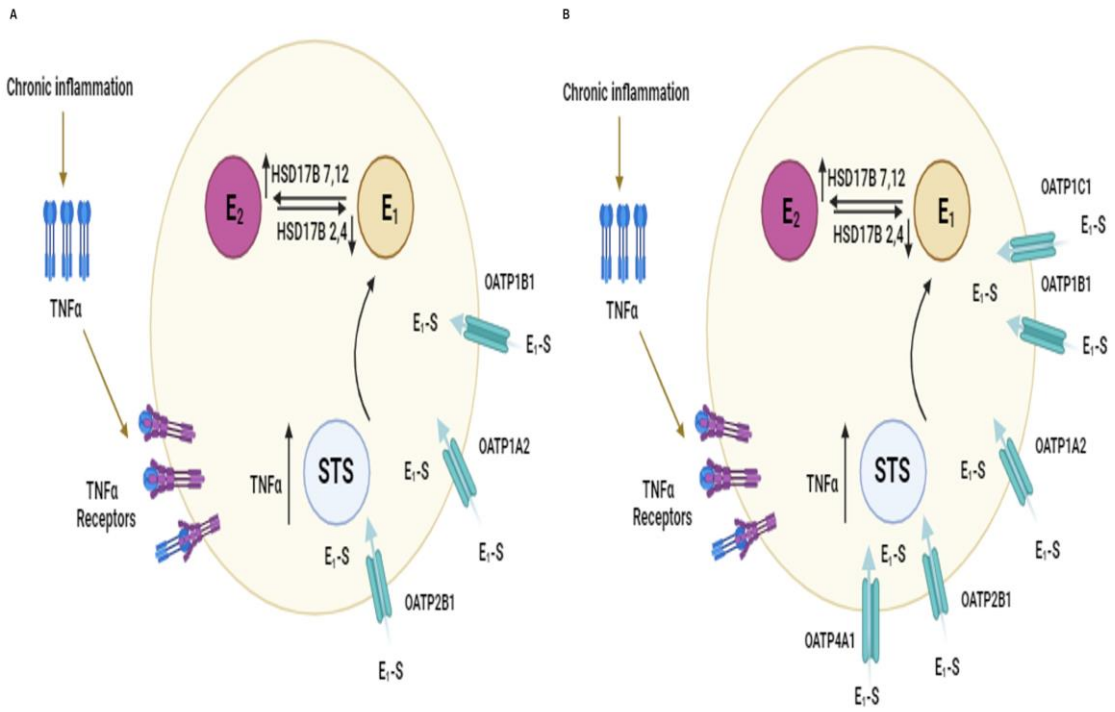


Figure 6.1: TNFα regulates steroidogenesis and STS activity in colorectal cancer cell lines. HCT116 cell (A) and Colo205 Cell (B). E1S in the circulation provide a reservoir for E1 and could be transported by organic anion transporters into tumour cells. When E1S is converted in situ into E1 in tumour cells, it then has the potential to be converted into E2 by HSD17B1 (HSD17B 7,12) to form E_2, which has the highest binding affinity for ERs. Also, the conversion of E_2 to E_1 is catalysed by oxidative (HSD17B 2,4). In tumour cells, this may be the primary route for the production of oestrogen. In HCT116 and Colo205, TNFα increased the activity of STS and HSD17B 7,12. TNFα increases the uptake of E1S via OATP2B1, OATP1A2 and OATP1B1 in both HCT116 and Colo205 cells and via OATP1C1 and OATP4A1 in Colo205. (Created with BioRender.com).

6.3 Limitations

-mRNA can vary among samples when assessing enzyme expression levels, affecting the accuracy of enzyme activity measurements and leading to fluctuations in PCR results. These fluctuations can make it difficult to interpret and analyse PCR results.

-CRC samples were not tested using western blotting. After using PCR to screen for samples expressing a particular gene, Western blotting can confirm its expression at the protein level.

-In vitro work using cancer cell lines has advantages and limitations. Even though cancer cell lines are easy to grow and useful for direct comparisons between experiments, they may not fully represent cancer tissue in vivo. Cell lines can also lose differentiation and be contaminated with mycoplasma.

6.4 Future work:

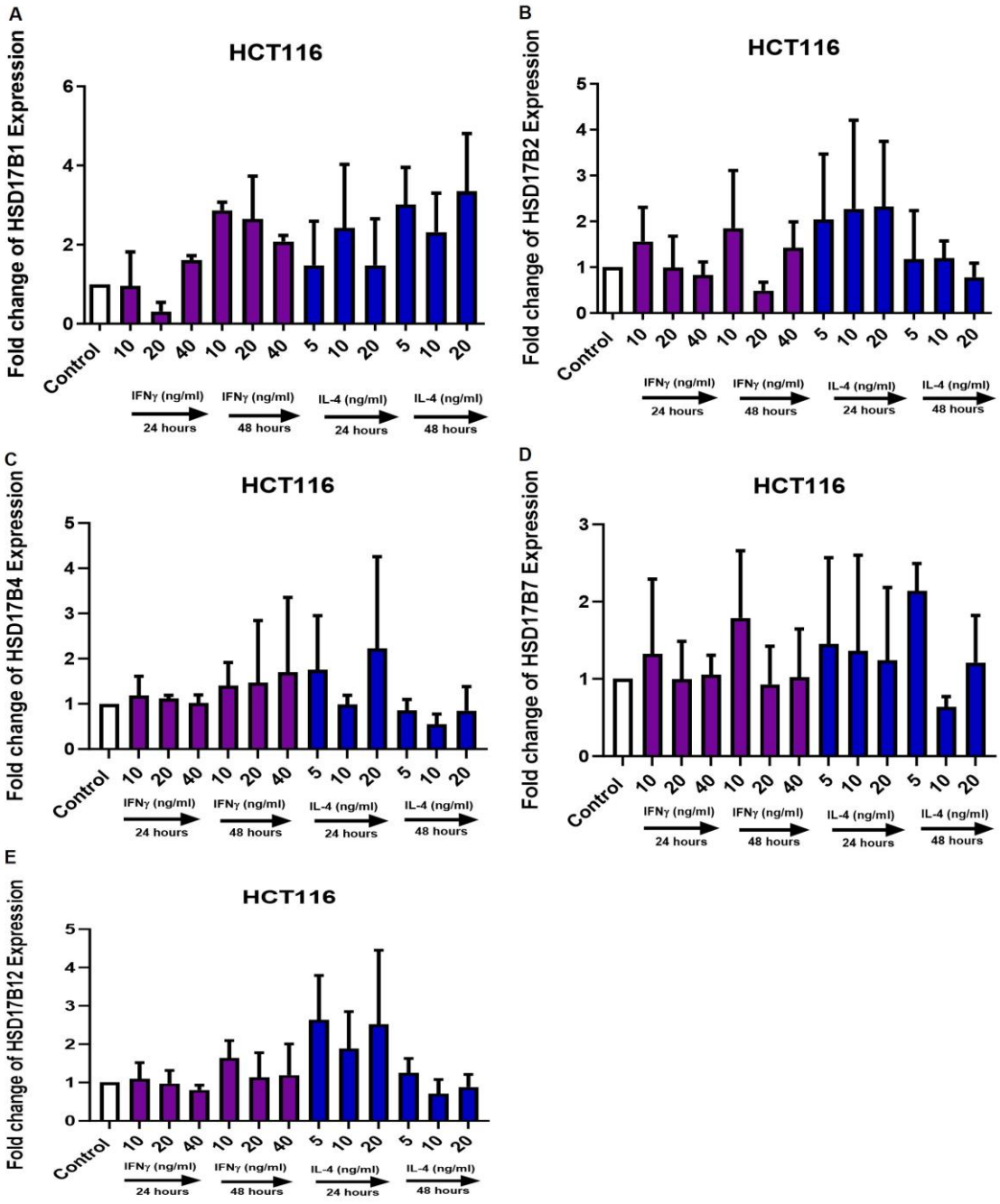
1- No studies directly linked TNFα treatment with ERα, ERβ, and GPER expression in CRC cells. Thus, it is important to measure how TNFα affects oestrogen receptor expression.
2- Performing qRT-PCR for OATP1B3, a number of studies have shown that OATP1B3 is expressed in CRCs, which differs from its expression pattern in the normal colon (Lee et al., 2008; Evangeli et al., 2017).
3- Using western blotting, to measure the protein expression in the CRC samples.

4- Non-steroidal anti-inflammatory drugs (NSAIDs) can lower oestrogen levels in postmenopausal who regularly use them, which may be associated with a decreased breast or ovarian cancer risk (Hudson et al., 2008**;** Gates et al., 2010; Oghazian et al., 2022). In several studies, nonsteroidal anti-inflammatory drugs (NSAIDs) were shown to reduce the development of CRC significantly (Chan et al., 2005; Yan et al., 2006; Bibbins-Domingo, 2016). We need to investigate whether they affect oestrogen metabolism enzymes indirectly.

APPENDICES

APPENDIX I

Figure I: **IL-4 and IFN Effect on HSD17Bs expression in HCT116 cell lines.**



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