Oestrogen Receptors in Oesophageal Cancer

A thesis submitted for the degree of PhD by

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Abstract

Introduction: Oesophageal cancer is more common in men than women. Oestrogen, which mediates its effects via oestrogen receptors (ERs), may be responsible for the gender disparity. This thesis investigates the role of ERs in oesophageal cancer development and explores potential therapeutic possibilities.

Methods: ERα and ERβ expression in oesophageal AC cell lines (OE19 and OE33) was knocked down using siRNA, and the effect of knockdown on the expression of proliferation-associated proteins (Ki67, PCNA, E-cadherin, Cyclin D) was assessed. The effect of the SERM, tamoxifen, on oesophageal cancer cell proliferation was investigated using proliferation assays and by evaluating the effect of tamoxifen on proliferation-associated proteins. Finally, a pilot study of tamoxifen in patients with oesophageal cancer was undertaken to assess feasibility of a clinical trial and to determine the short-term biological effect of tamoxifen on proliferation, assessed by a change in the immunohistochemical expression of Ki67 between paired biopsies.

Results: ERα and ERβ are expressed at the mRNA (RT-PCR) and protein level (Western Blotting) in the OE19 and OE33 cell lines. ERβ mRNA knock down was achieved in the OE33 cell line ($p=<0.0001$). However, reproducible significant ERβ protein knockdown was not demonstrated, and there was no change in the expression of proliferation-associated proteins. Treatment with tamoxifen significantly inhibited OE33 cell proliferation in a dose-dependent manner ($p=<0.0001$). Interestingly, treatment with tamoxifen decreased the expression of E-cadherin, but failed to change the expression of the remaining proliferation-associated proteins.

Eight patients (6 male with AC and 2 female with SCC) included in the pilot study completed a median on 30 days (range: 28 - 45 days) tamoxifen treatment; the mean Ki67 Labelling Index between paired biopsies increased by 0.625% (ns). Of the two women included, Ki67 expression decreased with tamoxifen treatment. A correlation was demonstrated between a reduction in Ki67 and mean ERβ expression ($r= -0.2272$, ns).

Discussion: ERβ is the dominant ER subtype expressed in oesophageal cancer cell lines and human cancer tissue. Tamoxifen inhibits the proliferation of oesophageal cancer cell lines *in-vitro*. Further studies to define the role of the ERβ subtype in oesophageal cancer and a clinical trial of tamoxifen in patients with oesophageal cancer is needed.

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Dedication

I dedicate this work to the 13 patients who enrolled into the pilot study. I will forever be grateful for their generosity and commitment to the study.

Finally, I dedicate my PhD to my parents Maria and Antonio, for their endless love and support and to my sisters Mariella and Nadia.

Table of Contents

Abstract ..... **i**

Acknowledgments ..... **ii**

Dedication ..... **iii**

Table of contents ..... **iv**

List of figures ..... **viii**

List of tables ..... **xxiii**

Declaration ..... **.xxv**

Abbreviations ..... **xxvi**

1. Chapter One Introduction **1**

1.1 Oesophageal cancer 3

1.1.1 Epidemiology 3

1.1.2 Oesophageal anatomy and histology 4

1.1.3 Risk factors 7

1.1.3.1 Oesophageal AC 7

1.1.3.1.1 Gastro-oesophageal reflux disease 7

1.1.3.1.2 Obesity 8

1.1.3.1.3 Gender 9

1.1.3.2 Oesophageal SCC 9

1.1.3.2.1 Tobacco smoking and alcohol 9

1.1.3.2.2 Dietary factors 10

1.1.3.2.3 Other risk factors 11

1.1.4 Oesophageal cancer development 11

1.2 Sex hormones 13

1.2.1 Androgens and androgen receptors 13
1.2.2 Oestrogen..... 14
1.2.2.1 Environmental oestrogen exposure 15
1.2.2.2 Oestrogens in cancer 16
1.2.2.2.1 Oestrogen in oesophageal cancer..... 16
1.2.2.2.1.1 Exogenous oestrogen..... 17
1.2.2.2.1.2 Endogenous oestrogen 19
1.2.2.2.1.3 Tamoxifen exposure 20
1.2.3 Oestrogen receptors..... 22
1.2.3.1 Oestrogen receptor structure 23
1.2.3.2 Oestrogen receptors – mechanism of action 25
1.2.3.2.1 Genomic pathway 25
1.2.3.2.2 Non-genomic pathway..... 27
1.3 Oestrogen receptors in cancer..... 28
1.3.1 Oestrogen receptors in breast cancer 28
1.3.2 Oestrogen receptors in colorectal cancer..... 31
1.3.3 Oestrogen receptors in oesophageal cancer..... 33
1.3.3.1 Oestrogen receptor expression..... 33
1.3.3.2 Effects of oestrogen receptor ligands *in-vitro*..... 36
1.3.3.3 Effect of ER receptor expression manipulation 38
1.4 Synthetic ER ligands 38
1.4.1 Tamoxifen 39
1.4.1.1 Pharmacokinetics 40
1.4.1.2 Mechanism of action 41
1.5 Aims and objectives..... 45
1.5.1 Research aim 46

1.5.2 Objectives 46

2 Chapter Two Methodology 47

2.1 Cell culture 48

2.1.1 Cell lines 48

2.1.2 Recovery of cells from liquid nitrogen 49

2.1.3 Subculture of cell lines 49

2.1.4 Cryopreservation 50

2.2 Western blotting 51

2.2.1 Cell homogenisation 51

2.2.2 Protein determination 51

2.2.3 SDS-PAGE Electrophoresis 52

2.2.4 Electroblotting 53

2.2.5 Blocking & Antibody application 53

2.2.6 Analysis (Chemiluminescent Densitometry) 54

2.2.7 Antibody stripping method for re-blotting 55

2.3 PCR quantification 56

2.3.1 Total RNA Extraction 56

2.3.2 Concentration and purity of Total RNA 57

2.3.3 Agarose gel electrophoresis 57

2.3.4 Reverse Transcription of TOTRNA 58

2.3.5 RT-PCR Controls 59

2.3.6 Real Time quantitative PCR protocol 60

2.3.7 Creation of standard curves 61

2.3.8 RT-PCR data processing using GENEX software 61

2.4 siRNA 62

2.4.1 Preparation of cell lines for siRNA transfection 63

2.4.2 siRNA transfection protocol 63
2.5 5'-bromo- 2'-deoxyuridine (BrdU) cell proliferation assay .. 64
2.5.1 Drug preparation 64
2.5.2 BrdU protocol 65
2.6 Effect of tamoxifen on proliferation-associated proteins 66
2.7 Statistical Analysis 67
3 Chapter Three Oestrogen Receptor Knockdown 68
3.1 Introduction 69
3.2 Methods 71
3.3 Results 73
3.3.1 ER expression in oesophageal cancer cell lines 73
3.3.1.1 ER mRNA expression 73
3.3.1.1.1 ESR1 mRNA expression 74
3.3.1.1.2 ESR2 mRNA expression 75
3.3.1.2 ER protein expression 76
3.3.1.2.1 ERα protein expression 76
3.3.1.2.2 ERβ protein expression 78
3.3.2 siRNA transfection 80
3.3.2.1 Positive control cell line 80
3.3.2.1.1 Optimisation of the positive control cell line 80
3.3.2.1.1.1 Cell density and DharmaFECT volume optimisation 81
3.3.2.1.1.2 Target siRNA optimisation 82
3.3.2.1.2 Simultaneous mRNA and protein knockdown .. 85
3.3.2.2 Oesophageal cancer cell lines 94

3.3.2.2.1 Optimisation of the oesophageal cancer cell lines 94

3.3.2.2.1.1 Optimisation of DharmaFECT volume 95

3.3.2.2.1.2 Target siRNA optimisation 98

3.3.2.2.2 Simultaneous mRNA and protein knockdown .101

3.3.2.2.2.1 ERβ protein expression at different time points 109

3.3.3 Changes in expression of proliferation-associated proteins 112

3.3.3.1 Positive control cell line (MCF7) 113

3.3.3.2 OE33 cell line.....115

3.4 Discussion 117

4 Chapter Four The effect of tamoxifen on oesophageal cancer cell lines 128

4.1 Introduction 129

4.2 Methods.....132

4.3 Results 132

4.3.1 Effect of different concentrations of tamoxifen on cell growth 132

4.3.2 The effect of tamoxifen on the expression of proliferation-associated proteins 134

4.3.2.1 Positive control cell line (MCF7) 135

4.3.2.2 OE33 cell line.....136

4.3.2.3 E-cadherin expression - repeat.....138

4.4 Discussion 138

5 Chapter Five A pilot study of tamoxifen in oesophageal cancer 144

5.1 Introduction 145

5.1.1 Study aims and objectives 147

5.2 Methods 148

5.2.1 Study protocol 148

5.2.1.1 Study amendments 149

5.2.2 Immunohistochemistry 149

5.2.2.1 Biomarker scoring 150

5.2.3 Statistical analysis 151

5.3 Results 153

5.3.1 Patient identification and screening 153

5.3.2 Patient recruitment 154

5.3.2.1 Patient characteristics 156

5.3.3 Immunohistochemistry results 156

5.3.3.1 Ki67 expression 156

5.3.3.2 ERα expression 160

5.3.3.3 ERβ expression 161

5.3.3.3.1 Change in ERβ expression in patients who demonstrated a reduction in Ki67 expression with treatment 163

5.3.3.4 Correlation between change in Ki67 and mean ERβ expression 163

5.3.3.5 Correlation between clinicopathological characteristics and biomarker scoring 164

5.3.3.5.1 Initial Ki67 score and death 164

5.3.3.5.2 Ki67 change according to sex and histological subtype 165

5.3.3.5.3 ERβ staining score and sex of the patient 165

5.3.3.5.4 ERβ score and death 166

5.3.4 Summary table.....166

5.4 Discussion.....166

6 Chapter Six Discussion.....181

7 References.....189

8 Appendix.....236

8.1 Appendix 1.....236

8.2 Appendix 2.....246

8.3 Appendix 3.....255

Declaration

I declare that the research presented in this thesis is my own, unless stated in the text. The research was undertaken at the Faculty of Medicine and Health Sciences, the Medical School, Royal Derby Hospital, University of Nottingham and the Royal Derby Hospital from October 2014 to October 2016.

Antonella De Rosa, 1st April 2018

List of abbreviations

AC – Adenocarcinoma
AF – Activation function
AP1 - Activating protein 1
APC - Adenomatosis Polyposis Coli
AR – Androgen receptor
BCA - Bicinchoninic acid
BME - β-mercaptoethanol
BM – Barrett's metaplasia
BrdU – 5'-bromo-2'-deoxyuridine proliferation assay
BSA – Bovine serum albumin
Ca - Calcium
Cdk1 - Cyclin-dependent kinase 1
cDNA - Complementary DNA
CI - Confidence interval
Cq - PCR quantification cycle
CRISPR - Clustered regularly interspaced short palindromic repeats
DBD - DNA-binding domain
DMSO – Dimethly sulfoxide
DNA – Deoxyribonucleic acid
DPN - (2,3-bis (4-hydroxyphenyl) propionitrile
EC_{50} - Half maximal effective concentration
EDTA - Ethylenediaminetetraacetic acid
EGFR - Epidermal growth factor receptor
EMR - Endoscopic mucosal resection
ERE – Oestrogen response element
ERs – Oestrogen receptors
ERα – Oestrogen receptor α
ERβ – Oestrogen receptor β
ESR1 – Oestrogen receptor 1 gene
ESR2 – Oestrogen receptor 2 gene
FCS – Fetal calf serum
FDA - Food and Drug Administration
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GORD – Gastro-oesophageal reflux
HBSS – Hank's Balanced Salt Solution
H12 - Helix 12
H&E - Haematoxylin and eosin stain
HRT - Hormone replacement therapy
kDa - Kilodaltons
LBD - Ligand-binding domain
LN_2 – Liquid Nitrogen
mA – Milli amps
MCC - Mutated in Colorectal Cancer

MCF7 – Human breast cancer cell line
MDT - Multidisciplinary team
Mg – Magnesium
MIQE - Minimum Information for Publication of Quantitative Real-Time
MPP - 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride
mRNA – messenger RNA
PCR Experiments
NC – Negative control
NS – Not significant
NTC - No template control
OE19 – Human male oesophageal adenocarcinoma cell line
OE33 – Human female oesophageal adenocarcinoma cell line
PBS - Phosphate Buffered Saline
PC – Positive control
PCNA – Proliferating cell nuclear antigen
PCR – Polymerase chain reaction
PGK1 - Phosphoglycerate kinase 1
pH – Potential of hydrogen
PPT - 1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole
PHTPP - 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol
PR - Progesterone receptor
PRF – RPMI – Phenol red free RPMI
Rb – Retinoblastoma
REC – Research Ethics Committee
RISC - Ribonuclear protein complex
RNA - Ribonucleic acid
rpm – Revolutions per minute
RPMI – Roswell Park Memorial Institute 1640
RT – Reverse transcription
RT-PCR – Real time quantitative polymerase chain reaction
SCC – Squamous cell carcinoma
SD - Standard deviation
SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERM - Selective Oestrogen Receptor Modulators
sFCS – Stripped FCS
shRNA - Small hairpin RNA
siRNA - Small interfering RNA
SP1 - Specificity protein 1
SRC – Steroid receptor coactivator
TBE - Tris Borate EDTA
TBS - Tris Buffered Saline

TBST - Tris Buffered Saline + 0.1% Tween
TGF-β - Transforming Growth Factor-β
TGX – Tris-Glycine extended
TOTRNA – Total RNA
TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling
U - Untreated
UK - United Kingdom
WB – Western blotting
WHI - Women's Health Initiative
5-FU - 5-fluorouracil
5' –UTRs - 5' -untranslated regions
US – United States

1. Chapter One

Introduction

Scientific cancer research over recent decades has made remarkable progress in the understanding of cancer at a molecular level, translating to improved patient care and outcomes. With continued ageing and growth of the world's population, the global burden of cancer will continue to increase and will bring further complexity to the management of this heterogeneous and adaptive disease.

Oesophageal cancer is a deadly disease with a poor prognosis; only one-third of patients are amenable to curative resection at the time of diagnosis and treatment options available for the remaining two-thirds of patients are limited. Interestingly, there is a male predominance for oesophageal cancer, for reasons that remain unexplained. Numerous authors have proposed that oestrogen, acting via oestrogen receptors (ERs) has a protective effect in women, and is respon sible for the gender bias. Research investigating the role of ERs in oesophageal cancer development will further our understanding of this disease and may uncover the potential for additional treatment approaches. Thus this thesis explores the role of ER in oesophageal cancer development and investigates the potential of ER ligands in the treatment of this disease. Before the role of ERs in oesophageal cancer development is discussed, oesophageal cancer epidemiology and known risk factors will be described.

1.1 Oesophageal cancer

1.1.1 Epidemiology

Oesophageal cancer is highly aggressive and carries a dismal prognosis [1]. The 10-year survival for patients diagnosed with oesophageal cancer during 2010-2011 in England and Wales was 12% [2], a figure reflected in other Western populations [3]. It is the eighth most common cancer in the world [4] and the incidence varies according to the histological subtype, race, gender, and geographical location. For example, the incidence of squamous cell carcinoma (SCC) is higher in black men than in white men [5-7], and the incidence of adenocarcinoma (AC) is substantially higher in males than females, and in whites compared with blacks [7].

Globally, the predominant histological type is SCC, prevalent in high-risk areas contained within the "Asian oesophageal cancer belt", extending from northeast China westward through Central Asia to Northern Iran [4, 7, 8]. From the 1970s onwards there was a dramatic increase in the incidence of oesophageal AC in the Western world, predominantly among Caucasian men [9-12]. The United Kingdom (UK) has the highest incidence of oesophageal AC and the highest mortality [7]; the reason for this remains unexplained and is not thought to be attributed to the prevalence of risk factors alone and is discussed further in section 1.1.3. The increase in incidence of oesophageal cancer in Caucasian men in the UK has now plateaued, and has indeed started to fall [2] (Figure 1). The rates of SCC on the other hand are relatively stable or decreasing [7, 9].

Worldwide, there is a male predominance for oesophageal cancer (all histological types) [9, 10, 12-14] and in the UK, in 2014 it was the 9th most common cancer in males (3% of all male malignancies), and the 14th most common in females (2%) [2].

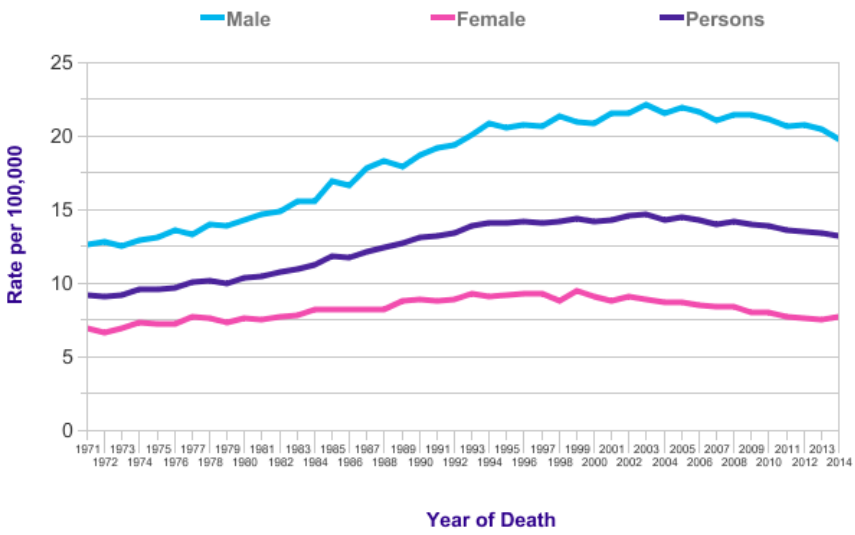


Figure 1. European Age-Standardised Incidence Rates of Oesophageal cancer by sex, UK, 1971-2014. (Source: Cancer Research UK, website accessed on 21/01/17).

1.1.2 Oesophageal anatomy and histology

The oesophagus is a muscular tube connecting the pharynx to the stomach. It travels from the neck through the posterior mediastinum and through the diaphragm to end at the gastro-oesophageal junction, where it joins the stomach. Anatomically it is divided into 3 sub-divisions; the cervical, thoracic and abdominal oesophagus, and for descriptive purposes, it is referred to as the upper, middle, and lower third (Figure 2) [15].

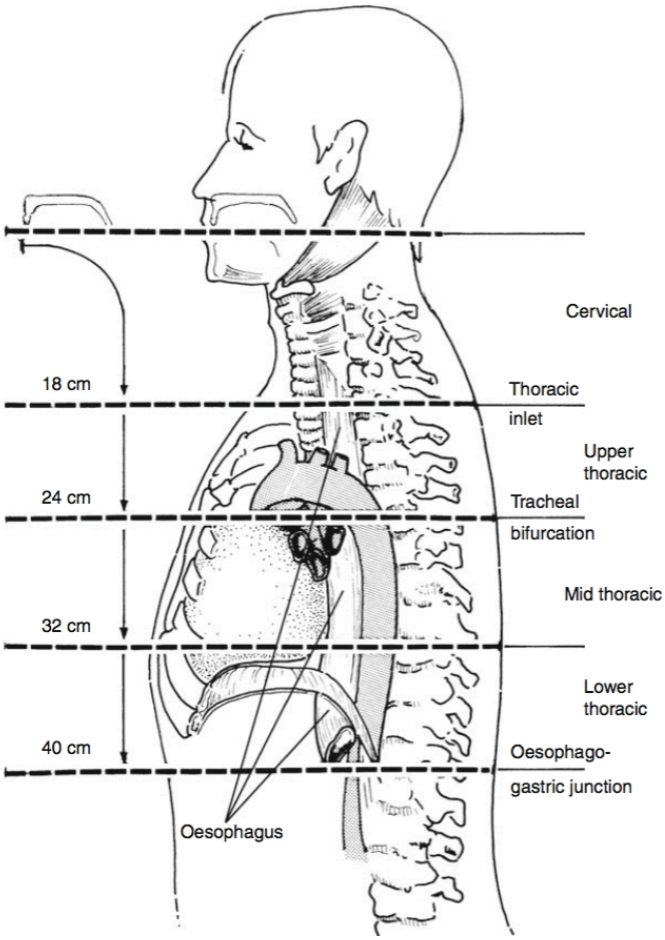


Figure 2. Anatomical sub-divisions of the oesophagus. (Used with permission from Allen [15])

The oesophageal mucosa consists of stratified squamous epithelium, continuous with the lining of the oropharynx. The histological junction between the oesophagus and stomach is marked by the "Z-line", which represents the irregular boundary between pale stratified squamous epithelium of the oesophagus and glandular, simple columnar epithelium of the stomach (Figure 3) [16]. Of the two main histological subtypes of oesophageal cancer, SCC arises from the squamous epithelium lining the oesophagus and AC arises from intestinal metaplastic cells that replace the squamous epithelium in

the lower oesophagus with columnar epithelium of the stomach [17, 18].

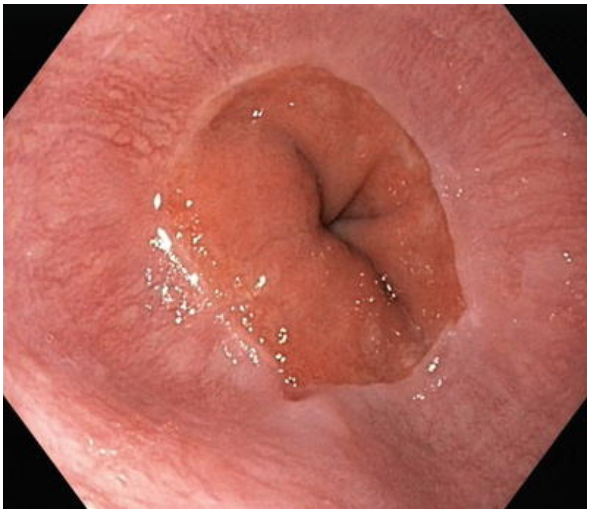


Figure 3. A view of the "Z-line" at endoscopy. (Used with permission from Eliakim [19])

The submucosa consists of loose connective tissue containing venous, lymphatic and neural plexuses. The muscularis externa is composed of internal circular and external longitudinal muscle. The upper third is composed of striated voluntary muscle, while the distal third is composed of smooth muscle, with the middle third containing a mixture of both muscle types. The oesophagus lacks a true adventitial layer and instead is surrounded by loose, elastic, areolar connective tissue that permits expansion during swallowing, with the disadvantage that it allows easier dissemination of oesophageal cancer [16].

1.1.3 Risk factors

1.1.3.1 Oesophageal AC

1.1.3.1.1 Gastro-oesophageal reflux disease

Of all established risk factors for oesophageal AC, Gastro-oesophageal reflux disease (GORD) and its manifestation, Barrett's Metaplasia (BM) [20-22] are the strongest known to date. BM describes the metaplastic replacement of squamous epithelium located in the lower oesophagus with intestinal type columnar epithelium; an adaptation that arises because intestinal columnar epithelium is more resistant to low pH levels of the stomach than squamous epithelium. BM is characterised histologically by the presence of specialised intestinal metaplasia [17, 18]. It affects approximately 1% of the UK population and is a premalignant lesion with the potential to progress to dysplasia and eventually oesophageal AC, with an estimated incidence rate of 1 for every 100 patients per year [18]. There is insufficient evidence to suggest the difference in incidence of GORD or BM between both genders, is responsible for the enigmatic sex difference in oesophageal AC [23, 24]. Limited studies have shown that the risk of acquiring BM is higher in men [25, 26]. Some authors have suggested men are more likely to develop erosive reflux disease, compared with women [25, 27]. Others have demonstrated the prevalence and severity of reflux symptoms peak and then decline at age 50-70 years in men, whereas for women it increases with age [27, 28], suggesting the female sex hormones may have a protective role.

1.1.3.1.2 Obesity

Obesity is a recognised risk factor for the development of AC [29-34]. The presence of abdominal obesity (intra-abdominal fat including visceral fat) is more important than total obesity [35] as the increased intra-abdominal pressure predisposes to GORD [14, 29, 32, 36-39]. Adiposity alone, however, independent of the predisposition to GORD, appears to play a role in the development of oesophageal AC [40, 41]. Adipose tissue is a complex, metabolic, endocrine organ, and contributes to the metabolism of glucocorticoids and sex steroids. Adipose tissue also secretes several hormones and growth factors such as adiponectin, insulin-like growth factor and leptin [42], which stimulate tumour growth in a variety of tissues, including oesophageal cancer cells [43, 44]. The contribution of obesity in the gender bias in oesophageal AC is unclear. Numerous studies have reported the difference in the prevalence of obesity between the sexes is not sufficient to account for the disparity [35, 45]. Whereas others have reported abdominal obesity [21, 29] and visceral fat [46] are more abundant in men than women with oesophageal AC, possibly being responsible for the gender bias. Moreover, numerous authors have reported the association of obesity as a risk factor for the development of oesophageal AC is stronger among men than in women [39, 40]; findings refuted another study which found that obese women were not less vulnerable to the risk of developing oesophageal AC than men [47].

It has been hypothesised that the higher levels of oestradiol in females increase sensitivity to leptin preventing the accumulation of visceral fat [48] and that mutations of ERs or an imbalance of the ER subtypes are strongly linked to abnormal fat distribution and metabolic disease [49].

1.1.3.1.3 Gender

As discussed in section 1.1, both subtypes of oesophageal cancer are more common in men and the male to female ratio is up to 9:1 and 3:1 for oesophageal AC and SCC, respectively [9, 11, 13, 14, 50, 51]. The male predominance is particularly marked in oesophageal AC, which occurs approximately 20 years earlier in men compared to women [11, 52]. The reason for this sex disparity in oesophageal AC is unknown and is not explained by disparity in the prevalence of risk factors between both sexes [38, 47]. Numerous authors have hypothesised that the female sex hormone oestrogen, mediated by ERs, may have a protective role [11, 13, 14, 24, 53-55] and will be discussed in further detail in section 1.2.2.2.1.

1.1.3.2 Oesophageal SCC

1.1.3.2.1 Tobacco smoking and alcohol

Tobacco smoking and alcohol are strong established risk factors for oesophageal SCC. It is estimated that 90% of oesophageal SCCs are attributable to tobacco and alcohol in developed countries and they work synergistically [56, 57]; both alcohol consumption and tobacco

smoking increase the risk of oesophageal SCC in a dose and time dependent manner [8, 58]. Ethanol-induced carcinogenesis is a result of the carcinogenic effect of acetaldehyde, a by-product of ethanol metabolism [58]. Mutations of the enzymes responsible for the elimination of acetaldehyde (aldehyde dehydrogenase-2 and alcohol dehydrogenase-2) predispose to the development of oesophageal SCC and the mutation is prevalent in East Asian populations [57, 59]. Tobacco smoking increases the risk of oesophageal SCC due to the effect of over 60 known carcinogens, the most important being polycyclic aromatic hydrocarbons and N-nitrosamines, that induce mutations in oncogenes and tumour-suppressor genes such as p53 [58]. Tobacco smoking also increases the risk of oesophageal AC but the association is not as strong, resulting in only a two-fold increase in risk [56, 57]. The male predominance observed in SCC of the oesophagus has been attributed almost entirely to sex differences in the exposure to alcohol and tobacco smoking [60], but it is not enough to account for the gender bias in oesophageal AC [13].

1.1.3.2.2 Dietary factors

The development of oesophageal SCC is associated with the consumption of a variety of food types prevalent in regions contained within the "Asian oesophageal cancer belt", such as extremely hot beverages, fermented (pickled) vegetables, smoked meats and fish containing N-nitroso compounds (nitrates, nitrites, and nitrosamines) and maize contaminated by mycotoxins (fumosinins) [61]. Nutritional deficiencies [62, 63] and a low intake of

fresh fruit and vegetables [8] have also been associated with SCC. The consumption of meats cooked at high temperatures or barbecued/ grilled meats are also strongly associated with the development of oesophageal SCC, thought to be due to the presence of carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons, respectively [64, 65]. Different dietary habits between the genders may theoretically contribute to the gender bias in oesophageal cancer, although to date, no dietary factor has been identified.

1.1.3.2.3 Other risk factors

A higher risk of oesophageal SCC is also associated with low socioeconomic status [8] and poor oral health [63]. Other diseases such as the autosomal dominant genetic disease, Tylosis, conditions that cause mucosal damage such as Achalasia, Plummer-Vincent syndrome, oesophageal webs, and radiation-induced strictures are associated with an increased risk of developing oesophageal SCC [56].

1.1.4 Oesophageal cancer development

Tumourigenesis is a multistep process of genetic alterations that result in the transformation of normal cells into malignant cells [66]. The successive accumulation of genetic changes leads to the evolution of cell clones that are independent of cell death regulatory pathways, with the ability to divide indefinitely [67]. It is thought that cancer results from numerous altered cellular processes termed, the hallmarks of cancer, and includes; sustained proliferation, lack of

growth suppression and apoptosis, immortality, angiogenesis, invasion and metastasis, reprogramming of cellular metabolism, evasion of immune destruction and tumour-promoting inflammatory responses [68, 69]. The path the cell takes to become malignant is highly variable and the particular sequence in which mutations in oncogenes and tumour suppressor genes occur vary widely even among tumours of the same type. Unlike colon cancer, where the key mutations involved in the multi-step adenoma-adenocarcinoma pathway are known, the sequence of mutations involved in the development oesophageal cancer is not well defined. Nevertheless, mutations in common oncogenes and tumour suppressor genes have been identified. In oesophageal AC, over expression of the epidermal growth factor receptor (EGFR) [70, 71], the c-erbB2 protein [72, 73] and loss of Transforming Growth Factor-β (TGF-β) [74] expression, have been detected. In addition, loss or alterations of the following tumour suppressor genes have been reported: Mutated in Colorectal Cancer (MCC) [75], Adenomatosis Polyposis Coli (APC) [76] and Retinoblastoma (Rb) [77]. Finally, mutations in the genes encoding the tumour suppressors p53, P16, and Cyclin D1, a regulator of the G1/S checkpoint of the cell cycle, have been detected in both oesophageal AC and SCC [58, 59, 61, 78, 79].

On a histopathological level, oesophageal AC, arises in gastric-like mucosa with intestinal metaplasia, the majority being associated with conventional BM [80], which then undergoes stages of transformation from low to high grade dysplasia and finally AC. Most

cases of SCC develop as a result of carcinogen exposure (tobacco, alcohol) that act as chemical mutagens, increasing the basal mutation rate and resulting in the mutation of genes involved in the transition of a normal cell to a tumour cell [69], described histopathologically as hyperplasia, dysplasia (mild to severe), and invasive carcinoma [79].

1.2 Sex hormones

As described above, differences in the exposure to known risk factors for oesophageal cancer alone are not enough to account for the gender bias [25, 38, 81]. It has been suggested that the sex hormones testosterone and oestrogen, which mediate their effects via the androgen receptors (ARs) and ERs respectively, may be responsible [82, 83 13, 53, 55].

1.2.1 Androgens and androgen receptors

An androgen is defined as a steroid hormone that plays a role in the development and maintenance of male characteristics. Androgens are synthesised by the testes, ovaries and adrenal glands and act by binding to the AR, a member of the superfamily of nuclear receptors that act as transcription factors[84]. Testosterone is the major androgen in males and the role of testosterone in prostate cancer is well-described[85]. Its role in oesophageal cancer development however, is less substantial. *In-vitro* studies have demonstrated testosterone enhances the growth of oesophageal SCC cell lines[83, 86,

[87] and SCC cell line xenografts [88], but no such effect on growth has been reported in oesophageal AC cell lines. Authors of a retrospective database study of >38,000 men with prostate cancer hypothesied that the use of anti-androgen therapy in the treatment of prostate cancer was responsible for the significantly reduced risk of oesophageal AC development [82]. The number of patients who actually received anti-androgen therapy, however was not available. Moreover, a study by Awan *et al* [89] demonstrated the median serum testosterone level in 25 oesophageal cancer patients was significantly higher than in aged matched controls, although subsequent studies have failed to demonstrate a correlation between serum testosterone levels and clinic-pathological features [90]. Likewise, AR expression has been reported in oesophageal cancer specimens although at proportionally low levels compared with ER expression [89, 91 92 93] and is discussed in further detail in section 1.3.3.1. The contribution of androgens and ARs in oesophageal cancer development is still undefined and warrants further investigation. The work presented in this thesis, however, will focus on the role of ERs.

1.2.2 Oestrogen

Oestrogen is a steroid hormone produced from testosterone by the aromatase enzyme cytochrome P450 [94]. The primary site of oestrogen production in premenopausal women is the ovaries and the adrenal cortex, and in postmenopausal women, the extra-gonadal tissues such as adipose tissue, vascular endothelium,

breast, bone and skin [95]. In men, oestrogen in produced in the testes and extra-gonadal tissues. The most potent oestrogen produced is 17β-oestradiol which is a stronger ER agonist than its metabolites oestrone and oestriol, although they bind to the ER with a higher affinity [96]. Oestrogens are primarily responsible for reproductive and sexual function, in addition to playing a role in the cardiovascular, immune, and musculoskeletal skeletal systems [49, 97-99]. Moreover, oestrogens are involved in critical cellular signalling pathways influencing cell proliferation and differentiation, and through these mechanisms, are involved the development of numerous cancers [100].

1.2.2.1 Environmental oestrogen exposure

Oestrogen-like molecules, hidden in unlikely food sources are an important source of exogenous oestrogen. For example, soybeans contain the isoflavone genistein, which has weak anti-oestrogenic properties. Soyfood consumption is therefore reported to have a protective effect in breast, colon and prostate cancers [101]. The effect of increasing environmental estrogens present in water and other natural solids such as water is of growing global concern [102]. The main source of environmental oestrogens is from animal manure and by effluent from sewage treatment plants [102]. Causal links with environmental oestrogen exposure with breast [103] and prostate cancer [104] have been reported. There are no reports of oesophageal cancer incidence and environmental oestrogen exposure, although an association is plausible.

1.2.2.2 Oestrogens in cancer

The role of oestrogen in mammary gland morphogenesis and carcinogenesis is well-established [105-107]. The single most important risk factor for developing breast cancer is being female, and factors that increase oestrogen exposure in women such early menarche, late menopause, and treatment with oestrogen or hormone replacement therapy (HRT), increase the risk of developing breast cancer, whereas pregnancy, decreases the risk [106, 108-110].

The role of oestrogen in the development of other cancers is less well defined. In colorectal cancer, women are less likely to develop the disease than in men hence, it has been postulated that oestrogen has a protective effect [111]. Indeed, the use of HRT in post-menopausal women has been shown to lower risk of developing colorectal cancer in numerous observational and prospective cohort studies [108, 112, 113]. Furthermore, the Women's Health Initiative (WHI) randomised trial found that the combined oestrogen plus progesterone HRT pill significantly decreased colorectal cancer incidence compared with placebo [114, 115].

1.2.2.2.1 Oestrogen in oesophageal cancer

In oesophageal AC, epidemiological studies have demonstrated that the male: female ratio peaks in the fifth decade of life, suggesting oestrogen has a protective effect that declines following the

menopause [51, 116]. Additional studies have investigated the effect of both exogenous and endogenous oestrogen exposure on the risk of developing oesophageal cancer.

1.2.2.2.1.1 Exogenous oestrogen

In a large prospective cohort study of 201,506 post-menopausal women in the United States (US), 56 developed SCC and 25 developed AC of the oesophagus [117]. The use of HRT significantly reduced the risk of oesophageal SCC and also reduced the risk of oesophageal AC, although this was not significant. This association was more evident with oestrogen and progesterone containing HRT and women who had not undergone a hysterectomy. Moreover, older age at menopause was inversely associated with oesophageal SCC. There was no association with age at first birth, oral contraceptive use, parity, hysterectomy, or bilateral oophorectomy [117]. Another study from the US included women enrolled in the WHI clinical trials plus data from an observational study [118]. Of the 161,080 included participants, 23 developed oesophageal AC and 34 oesophageal SCC. In concordance with the findings reported by Freedman *et al* [117], the risk of oesophageal SCC was lower among women taking the oestrogen and progesterone HRT, but not oestrogen only containing HRT. There was no association between HRT use and the risk of developing AC. Although breastfeeding was associated with a non-significant reduction in the risk of AC, no other reproductive or hormonal factors were significantly associated with the risk of either oesophageal SCC or AC [118].

A later prospective nested case–control study using data available in the UK General Practice Research Database from 1999 to 2005, also found that women prescribed HRT had a significant reduction in the risk of oesophageal cancer [119]. There were no significant differences in cancer risk by HRT type or duration of use. The authors combined these results with published data in a meta-analysis and found that the risk of developing oesophageal cancer was significantly reduced in women who had ever used HRT vs those that had never used HRT. The study, however, failed to distinguish between the histological subtypes [119]. Interestingly, an earlier case-control study also using data from the UK General Practice Research Database from 1994 to 2001, failed to find an association between HRT use and the risk of oesophageal cancer in 299 women [120]. The study methodology was different however, with the later study manually reviewing the case notes of every patient included.

As well as HRT for the treatment of post-menopausal symptoms in women, exogenous oestrogens are used in the management of prostate cancer in men. Lagergren *et al*. [13] reported results of a retrospective cohort study of over 100,000 Swedish men with a diagnosis of prostate cancer and failed to show an association between prostate cancer and the development of oesophageal AC. A later retrospective cohort study of 38,627 men in the UK, however, did demonstrate that a diagnosis of prostate cancer reduced the risk of developing oesophageal AC [82]. The limitation of both of these

studies is that information on how many of the patients had actually received oestrogen treatment was not available.

1.2.2.2.1.2 Endogenous oestrogen

Serum oestradiol levels fall rapidly at the time of the menopause [121] and studies examining the association between oesophageal cancer with endogenous reproductive factors may provide further insight into the role of oestrogen in oesophageal cancer. For example, in a large UK cohort study of 1.3 million women (the Million Women Study), post-menopausal women were at higher risk of developing oesophageal cancer than pre- or peri-menopausal women and an earlier age at menopause increased the risk. In addition, they reported an increase in risk of SCC, but not AC, in nulliparous compared with parous women [122], similar to findings from a pooled case–control study of 114 women with SCC in Italy and Switzerland [123]. A case-control study of Swedish women, however, failed to show an association between childbearing and the risk of oesophageal AC [14] and neither did a previously mentioned study by Freedman *et al*. of women from in US [117]. A later nested-case control study from the Swedish Multi-Generation Register found the risk of oesophageal AC and SCC was reduced in parous women compared to non-parous women, which is contradictory to the hypothesis that oestrogen has a protective effect in women. This finding was also present in the male cohort, which was included as a control, suggesting other confounding factors other than sex hormones [124].

A later pooled case-control study from the UK, Australia and US, of 218 women with oesophageal AC found breastfeeding was associated with a dose-dependent reduced risk of cancer [125], similar to the findings of a regional UK case–control study [126] and a previously mentioned study of women in the United States [118]. In addition, they reported cancer risk was higher in nulliparous women compared with multiparous women although this was not significant [125]. They also found no association with age at menarche or menopause [125], in concordance with findings in women in China [127], and a previously mentioned study in the US [118].

1.2.2.2.1.3 Tamoxifen exposure

As described above, the role of endogenous oestrogen and synthetic oestrogen in the form of HRT is still inconclusive. To further investigate the potential role of ERs, the effect of tamoxifen, used in the treatment of breast cancer (see section 1.4), on the incidence of oesophageal cancer has been reported. In a Danish population-based cohort of 864 postmenopausal women who received adjuvant tamoxifen treatment for 48 weeks, there was no association with the development of oesophageal cancer after median follow-up of 7.9 years [128]. Likewise, studies of women receiving tamoxifen in the US [129], Sweden [130] and Japan [131] did not find an association between adjuvant tamoxifen treatment and oesophageal cancer incidence.

In a nationwide population-based cohort study of 138,885 Swedish postmenopausal women, patients diagnosed with breast cancer before 1978 (before the introduction of tamoxifen) were considered unexposed and patients diagnosed after this date as potentially exposed to tamoxifen treatment [132]. In the potentially exposed cohort, 9 patients developed oesophageal AC resulting in a 60% non-significant increase and there was no association with the unexposed cohort. For oesophageal SCC, in the cohort unexposed to tamoxifen, there was an overall increase in the risk of oesophageal SCC, but no change in risk in the cohort exposed to tamoxifen. The main limitation of this study is the lack of information about the tamoxifen treatment in the individual patients and hence, is at risk of exposure misclassification [132].

Although the findings from these population-based studies suggest tamoxifen treatment does not prevent oesophageal cancer, implying tamoxifen would be ineffective as a chemopreventative agent [128] [129] [130] [131], this does not suggest that tamoxifen will be ineffective in the treatment of established oesophageal cancer, a hypothesis that will be investigated in this thesis.

The published evidence on oesophageal cancer incidence in relation to oestrogen exposure is inconsistent and may be explained by different study designs and study populations. The major limitation, however, is the low incidence of oesophageal cancer among women,

which results in statistically underpowered studies, even in pooled data sets.

1.2.3 Oestrogen receptors

Oestrogens mediate their effect mainly through two intracellular receptors: ERα and ERβ [133]. ERα and ERβ are not isoforms of each other, but are the products of individual genes (ESR1 and ESR2), located on the 6th and 14th chromosome (6q25.1 and 14q23.2), respectively [134].

ERα was cloned from MCF7 human breast cancer cells in 1985 [135-137] and consists of 595 amino acids. The ERα gene has multiple promoters; transcriptional initiation at multiple promoters, as well as alternative splicing generates numerous mRNA variants with unique 5' -untranslated regions [138, 139], giving rise to numerous protein isoforms. The most abundant isoform is 66 kilodaltons (kDa) in size, although many other isoforms with different functions and tissue specific expression have been characterised [140-142]. A 36kDa variant for example, has been identified as a poor prognostic marker in breast cancer, via the initiation of membrane-initiated non-genomic signalling pathways [143]. ERα is found in the mammary gland, female (uterus, ovary) and male (testes, epididymis prostate), reproductive organs, bone and liver [144, 145].

ERβ was cloned in 1996 from rat prostate [146-148] and consists of 530 amino acids. The ERβ protein is produced from 8 exons, in addition

to 2 untranslated exons that can be spliced to exon 7 [149]. ERβ mRNA transcripts may be subject to alternative 3' splicing, exon deletions, or alternative usage of untranslated exons in the 5'region [149, 150] which gives rise to at least 5 protein isoforms [151-154], with different functions and tissue distributions [155]. ERβ has a wider tissue distribution than ERα, being present in the colon, lung, brain, the male reproductive organs [153, 156] and of equal amounts to ERα in the ovary [145, 156, 157].

1.2.3.1 Oestrogen receptor structure

The ER is a nuclear receptor, belonging to a family of transcription factors, that bind steroid hormones such as thyroid hormone, Vitamin D and retinoic acid. The nuclear steroid receptors share a common structure of defined functional domains. The DNA-binding domain (DBD) is centrally located and highly conserved. It interacts with the hormone response elements on DNA through two zinc-finger motifs, controlling the activation of genes. There is 96% sequence homology of the DBD between ERα and ERβ (Figure 4); the ER subtypes therefore interact with identical oestrogen response elements (EREs) and exhibit a similar binding affinity for an array of EREs [158]. The ligand-binding domain (LBD) consists of 12 α-helices, highly conserved in structure but only moderately conserved in sequence, between different nuclear receptors. As well as ligand binding via the deep hydrophobic cavity, it binds co-regulatory proteins and contributes to receptor dimerization and nuclear translocation [159]. Only 54% of the LBD is homologous between the

ER subtypes, which may explain why the same ligands act as antagonists or agonists, translating into different biological effects [160]. The N-terminal region (A/B domain) is highly variable between nuclear receptors and has only 18% amino acid homology between the two ER subtypes. Transcriptional activation is mediated by two distinct activation functions (AF-1 and AF-2) that recruit coregulatory proteins to the DNA-bound receptor [133]. AF-1 is located in the A/B domain and is involved with ligand-independent activation of transcription, AF-2 is located in the LBD which recruits transcription factors only in the presence of bound ligand. As previously mentioned, there is only 18% amino acid homology of the A/B domain and hence, of AF-1 between the two ER subtypes, which translates a functional difference: the AF-1 in ERα is active in stimulating gene expression and can function autonomously, whereas the activity of the AF-1 domain in ERβ is negligible [144]. Of the remaining domains, the F domain (C-terminal region) has a modulatory role [161, 162] and the D domain acts as a flexible hinge between the C and E domains and contains the nuclear localisation signal.

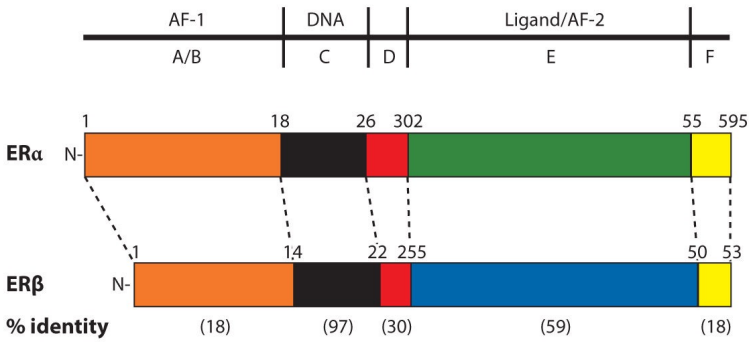


Figure 4. A diagram showing the relationship of ERα and ERβ. (Used with permission from Huang et al. 2010[159]). A/B–F designate the segments of the receptor polypeptides and structural domains. The specific amino-acid sequences are demonstrated above by numbers. The functional domains: DNA binding

domain, ligand binding domain and transactivation functional domains (AF-1, AF-2) are shown. The homology of the amino acid sequences is indicated as a % (in brackets).

1.2.3.2 Oestrogen receptors – mechanism of action

The effect of ER activation on cellular function occurs via two main pathways: the genomic and non-genomic pathway.

1.2.3.2.1 Genomic pathway

The classical pathway of ER action involves ligand binding to the hydrophobic pocket embedded deep within the LBD [159], inducing conformational and allosteric changes on the receptor surface, at sites of receptor - coregulator and dimer-dimer interactions [163]. The ligand-binding pocket consists of a central layer of helices lined by hydrophobic residues [164] (Figure 5). The pocket shape and conformation is highly inducible depending on the bound ligand, and the helical segment, Helix 12 (H12), undergoes dramatic shifts in position dependent on the bound ligand. Oestrogen binding stabilises a conformation of LBD where H12 folds back on top of the ligand-binding pocket, positioning a sequence of amino acids that support the formation of a binding surface for coactivators. Antagonists such as tamoxifen, induce an alternate conformation in the LBD where H12 is displaced away from its agonist conformation (Figure 5). Upon ligand binding to the ER, heat-shock proteins (e.g. hsp90, hsp56) and corepressors (e.g. SMRT, NCoR) dissociate from the complex and the ER is phosphorylated by a variety of src kinases [165]. The ligand-bound, phosphorylated ER then undergoes dimerisation and binds via the DBD to the ERE, a 13bp palindromic

sequence located upstream of the transcription initiation site of oestrogen-regulated genes. The ligand-receptor complex then interacts with basal transcription factors to make a pre-initiation transcription complex [166]. Coregulatory proteins interact with this core transcription unit to either initiate or represses gene transcription, altering mRNA levels, translating to the production of associated proteins and a physiological response [95, 110, 133, 166]. Instead of binding directly to the ERE, the ligand bound ER dimers can regulate gene transcription by acting as a coactivator for other DNA-binding transcription factors like activating protein 1 (AP1) [167] or specificity protein 1 (SP1) [168] and influence transcription of genes devoid of EREs (Figure 6). These different transcription activation mechanisms highlight the complex role of coregulators and response elements in eliciting specificity in gene output.

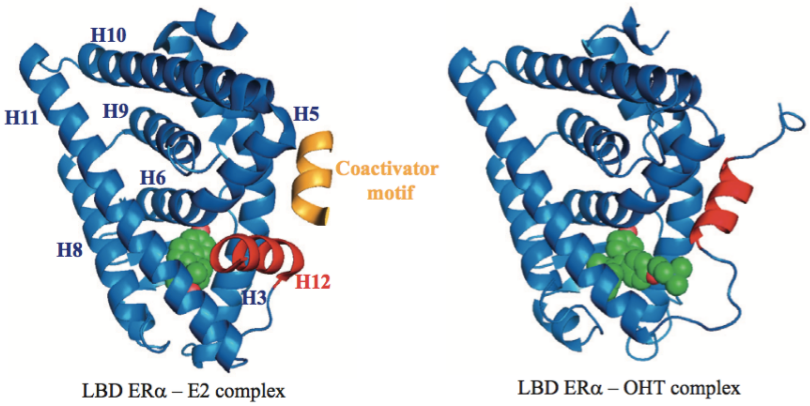


Figure 5. A diagram showing the 12-helical canonical fold of the ER LBD (Adapted from Traboulsi et al. 2017[169] with permission). Binding of the ER agonist oestrogen (E2) results in a conformation in the LBD, particularly at helix H12 (H12), which supports binding of co-activators. Antagonists such as 4-hydroxytamoxifen (OHT), through the bulky side-chain, induce conformations in the LBD where H12 is displaced away from its agonist conformation. ER: blue; Helix 12: red; ligand: green; α helical TIF2 coactivator motif: gold.

1.2.3.2.2 Non-genomic pathway

ER activation may also occur independent of ligand and result in non-genomic effects that occur within minutes, compared to the transcriptional events described above that take several hours [110, 170]. These rapid effects are thought to be mediated through extra-nuclear ERs located at the plasma membrane, or other plasma membrane-associated oestrogen-binding proteins like EGFR, insulin-like growth factors and HER2 receptors [133, 171, 172]. These rapid non-genomic effects result in a variety of cellular responses such as modulation of potassium currents, nitric oxide levels, calcium levels, and the activation of an array of intracellular kinase cascades such as mitogen-activated protein kinase, phosphoinositide 3-kinase, and protein kinase C [110, 171]. The non-genomic pathway is either ER-dependent or ER-independent and interacts with the genomic pathway through transcription factors such as AP1 and SP1 [110, 163, 170] (Figure 6).

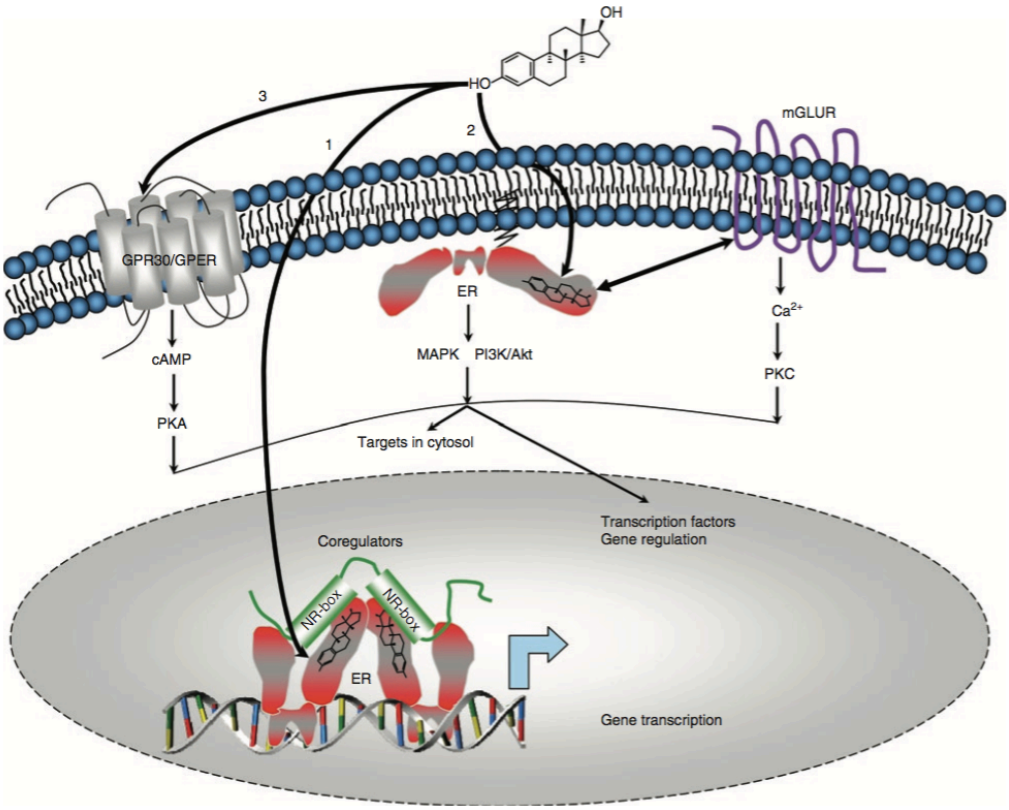


Figure 6. A schematic demonstrating the various oestrogen and ER signaling pathways (Adapted from Nilsson 2010[173] with permission). In the genomic pathway (1), the ligand-bound ER acts as a nuclear transcription factor by binding to the EREs on DNA, modulating target gene transcription. Pathway 2 involves ER present within the cell membrane or cytoplasm, which interact with other membrane-associated receptors. In pathway 3, oestrogen binds to other membrane-associated receptors resulting in signal transduction via different cytoplasmic kinases, independent of the ER.

1.3 Oestrogen receptors in cancer

1.3.1 Oestrogen receptors in breast cancer

The role of ERs in carcinogenesis is most comprehensively reported in breast cancer, where ERα is associated with proliferation, and ERβ with growth suppression [95, 163, 174]. Numerous *in-vitro* studies have been conducted involving manipulation of ER receptor subtype expression by knockdown, up-regulation or using subtype-specific agonists or antagonists.

In the mouse mammary epithelial cell line HC-11, silencing of ERβ expression using small interfering RNA (siRNA) resulted in a proliferative cell growth response to oestradiol, and silencing of ERα led to apoptosis [175]. Moreover, selective ERα activation using a selective ERα agonist, PPT (1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole) increased cell proliferation, whereas activation of ERβ using a selective ERβ agonist, DPN (2,3-bis (4-hydroxyphenyl) propionitrile) inhibited proliferation and reduced cell number by 25-30% [175]. In DPN treated cells, the expression of proteins associated with proliferation such as cyclin D1, PCNA, and Ki67 were reduced compared to control and demonstrated increased apoptosis [175].

The role of ERβ on the cell cycle was investigated in a study of T47D breast cancer cells, where induction of ERβ (tetracycline-regulated) reduced proliferation and altered the expression of cell cycle components associated with proliferation such as cyclin E, Cdk2 and Cdc25A (a regulator of Cdk2) [176]. In MCF7 cells, high ERβ expression induced G2 cell cycle arrest by inactivating cyclin-dependent kinase 1 (CDK1, critical for G2/M progression), inhibition of the cyclin B1 gene (activator of CDK1), and by stimulating expression of GADD45A and BTG2 (inhibitors of CDK1)[177]. In a further study in T47D and MCF7 cells, high ERβ expression (tetracycline-regulated) inhibited cell proliferation by decreasing Akt signalling (regulates cell motility, proliferation, and apoptosis), down-regulating proto-oncogene c-ErbB-2 /receptor tyrosine kinase erbB-3 (HER2/HER3) receptor dimer, and resulting in decreased activation of the PI3K–

AKT phosphorylation signalling pathway. In addition, ERβ up-regulated the levels of PTEN, which is an inhibitor of AKT signalling and a tumour suppressor [178].

The role of the ER subtypes on gene expression in breast cancer cell lines has also been investigated. In MCF7 cells, DNA microarray analysis showed that ERβ induction (using adenoviral gene delivery) resulted in up-regulation of genes involved in negative regulation of proliferation [179]. In ERα expressing cells, oestradiol up-regulated the expression of genes that promote cell cycle progression and knockdown of ERα (using siRNA) attenuated this response [179]. In an effort to elucidate the gene regulatory function of ERα and ERβ, Williams *et al*. 2008 [180] performed a full microarray transcriptome analysis of the ERα expressing T47D cell line. Of the 35,000 genes and transcripts analysed, 1434 genes (4.1%) were regulated in the presence of oestradiol, and the expression of ERβ using a lentivirus vector, supressed ~1000 ERα regulated genes, as well as altered the expression of 152 non-ERα regulated genes. In line with previous reports, the induced ERβ expression opposed the ERα up-regulation of cell proliferation genes. In a cell proliferation assay, the induction of ERβ inhibited the increase in cell number observed in ERα expressing cells in response to oestradiol [180].

Numerous xenograft models have demonstrated that oestradiol increases proliferation and tumour growth when ERα positive breast cancer cells are implanted into mice [181, 182]. In an attempt to

investigate the role of the ERβ subtype, Hartman *et al.* 2006 [183] transfected T47D cells with a tetracycline-regulated ERβ expression plasmid which were injected into severe combined immunodeficient mice. ERβ resulted in a reduction in tumour growth, a reduction in the number of intratumoural blood vessels and a reduction in expression of the pro-angiogenic factors, vascular endothelial growth factor and platelet-derived growth factor β [183].

1.3.2 Oestrogen receptors in colorectal cancer

A role for oestrogen acting via ERs, in the development of colorectal cancer, has been suggested based on the observation that the incidence and mortality of colorectal cancer in women is lower than in men [111], and the risk of developing colorectal cancer is lower in women who have taken HRT [112, 115, 184, 185]. Both ERα and ERβ have been identified in colonic tissue [186, 187]. ERβ however, is the prominent subtype, expressed as numerous isoforms [188, 189], and is thought to protect against carcinogenesis. Loss of ERβ expression in malignant colonic tissue parallels loss of cell differentiation and grade of disease [187, 190-193] and secondarily, is associated with a worse prognosis [194]

In-vitro experiments have shown that colon cancer cell growth is affected by ER ligands, but with inconsistent results [195-200]. In two separate studies using non-malignant mouse colonocytes (YAMC) and the ERβ expressing colon cancer cell lines (COLO205), oestradiol reduced cell growth and increased apoptosis [198, 201]. In

DLD-1 colon cancer cells, oestradiol increased ERβ mRNA via p38/MAPK activation [202]. In a study of colon cancer cells expressing ERβ but not ERα, tamoxifen caused dose related growth inhibition with down-regulation of surviving, an anti-apoptosis protein [203]. In an attempt to determine the individual role of ERβ, numerous studies have up-regulated ERβ in colon cancer cell lines. Up-regulation of ERβ in LoVo cells induced apoptosis and inhibited proliferation by increasing p53 signalling in an oestrogen-dependent manner [204], and in SW480 colon cancer cells ERβ up-regulation resulted in inhibition of proliferation and G1 phase cell cycle arrest, by transcriptional inhibition of proliferation-regulating genes such as c-Myc (an activator of numerous G1-S-phase genes) [183]. Further work by the same group showed that induction of ERβ expression into different colorectal cancer cell lines (SW480, HT29, and HCT-116) lead to oestrogen independent, down-regulation of a wide array of genes involved in tumorigenesis [205] and induction of ERβ into SW480 cells resulted in a significant change to the miRNA pool (24 down-regulated and 3 were up-regulated), with subsequent changes to gene expression and functional effects [206]. In a study of colon cancer cells which express only ERβ (NCT8), up-regulation of ERβ using a plasmid vector, inhibited cell proliferation and resulted in a decrease in cyclin E and an increase in p21CIP1 (cdk inhibitor), causing a G1 cell cycle arrest [207].

The effect of ERβ in colonic cancer development has also been demonstrated *in-vivo*; when ERβ expressing cells were transplanted

into immunodeficient mice, tumour volume was 70% less than controls lacking ERβ [208]. Wild-type mice treated with oestradiol developed significantly fewer pre-neoplastic lesions than ERβ knockout mice [198], and in another study, the incidence of aberrant crypt foci was significantly higher in ERβ knockout mice compared to wild-type mice, following treatment with azoxymethane, a chemical carcinogen [209]. Another research group demonstrated ERβ and APC (a tumour suppressor and key regulator of colon tumourigenesis) knockout mice developed larger colon tumours compared with APC knockout mice alone [210, 211]. Furthermore, administration of DON (a selective ERβ agonists), significantly reduced the number of small intestinal polyps in APC knockout mice. In addition, colonocytes from mice treated with the ERβ agonist DPN, demonstrated higher levels of p27 (a cell-cycle inhibitor), increased TGF-β1, TGF-β3, and Smad2 phosphorylation transcripts (a TGF-β pathway cytoplasmic effector), suggesting the effects of ERβ activation are mediated by the TGF-β signalling pathway [212].

1.3.3 Oestrogen receptors in oesophageal cancer

1.3.3.1 Oestrogen receptor expression

The presence of ERs in oesophageal tissue was initially reported in a study of 48 oesophageal carcinoma specimens; Wang *et al*. demonstrated the expression of ERα and progesterone receptor (PR) in 44% (21/48) and 21% (10/48) of specimens respectively, and failed to detect either receptor in normal oesophageal tissue[93].

Later, in a study of Barrett's oesophagus and AC tissue samples (n = 20), ER staining was present in 16 specimens, whereas AR staining was positive in only two of the 20 specimens [92]. The results of these two studies implied the importance of ER over PR or ARs. Discrimination between the ER subtypes provided further information on their role in oesophageal cancer development. Kalayarasan *et al.*[213] studied the expression of ERα, ERβ and PR in tumour tissue and adjacent normal mucosa of 45 oesophageal cancer specimens (SCC = 30 cases, AC = 15 cases). They failed to identify ERα or PR in any of the specimens. ERβ expression however, was strongly positive in all cancer specimens (AC and SCC), and the intensity of staining was significantly higher than in normal adjacent mucosa [213]. In a study by Nozoe *et al*. [214] of 73 SCC specimens, ERα was identified in the cytoplasm, and ERβ in the nucleus. ERα expression was found to be inversely correlated to ERβ expression, and a positive expression of ERα plus negative expression of ERβ was associated with a worse prognosis [214]. In a similar study, Akgun *et al*. [215] investigated the expression of ERβ in BM and associated AC in 31 oesophagectomy specimens. Nuclear ERβ was present in >50% of cells in all 23 AC specimens (100%), 10 out of 11 (91%) BM specimens with high-grade-dysplasia, 8 out of 11 (83%) with low-grade dysplasia, and in 10 of 15 (66.6%) of BM negative for dysplasia [215]. In an attempt to determine which ERβ isoforms were expressed, the same research group examined 33 oesophagectomy specimens, which were stained for the ERβ isoforms: ER-β1, ER-β2, ER-Bβ3 and ER-β5 [216]. They reported all

ERβ isoforms were overexpressed in oesophageal AC compared to BM negative for dysplasia, suggesting a significant biological role for ERβ in the evolution of oesophageal cancer. Interestingly, in all cases of BM negative for dysplasia, all isoforms showed only nuclear expression, whereas cytoplasmic expression was seen in dysplasia and AC specimens[216]. Likewise, Zuguchi *et al.* [217] found increased nuclear ERβ reactivity in SCC oesophagectomy specimens (88/90 specimens) compared to matched normal mucosa, and increased ERβ expression correlated unfavourably to the clinical outcome. ERα was expressed, but to a lesser extent, in 38/90 specimens [217]. Similar findings were reported in a more recent study of 89 oesophageal SCC specimens; Dong *et al*. [218] reported ERβ expression was significantly higher in SCC compared to normal mucosa (p= 0.0243), the inverse was true of ERα expression, which was significantly lower in SCC than that in normal mucosa (p=0.0445). They further correlated ER expression with clinical features and found an inverse correlation between ERα expression and depth of tumour invasion (p=0.0426) and ERα positive patients (21/89) had a better outcome than ERα negative patients (p=0.0280), whereas patients with high ERβ expression (44/89) had significantly worse survival (p=0.0366, Kaplan-Meier survival analysis) [218]. Due *et al*. [219] were the first to demonstrate a high proportion of ERα positive specimens in oesophageal AC resection specimens (8/10), which they attributed to the use of biotin-free polymeric visualization system, which is more sensitive and specific

ERα characterisation in breast cancer [220]. They also reported ERβ positivity in 7/10 specimens [219].

1.3.3.2 Effects of oestrogen receptor ligands *in-vitro*

Research using ER ligands in oesophageal cancer began in the late 1980s. Matsuoka *et al.* [86] reported growth of SCC cancer cell lines (KSE-1), which expressed ER and testosterone receptors, was inhibited by oestradiol and enhanced by testosterone [86]. Later work by Utsumi *et al.*[221] supported this finding *in-vivo* with nude mice transplanted with ER positive SCC. Tumour growth was significantly greater in male than female mice. Tumour growth was enhanced in oophorectomised female mice which was then subsequently suppressed following oestradiol treatment [221]. A later study by the same research group reported oestradiol significantly inhibited growth of the ER-positive SCC (ES-25C) but not the ER-negative SCC cell line (ES-8C) [222]. A similar study, by Ueo *et al.* [87] of ER positive (KSE-1) and ER negative (KSE-2) oesophageal SCC cell lines, demonstrated that administration of oestradiol significantly inhibited the growth of KSE-1 but not KSE-2 both *in-vitro* and *in-vivo* when transplanted into nude mice [87]. Most recently, Zhang *et al*. [223] investigated the effect of oestradiol on oesophageal SCC cells (EC109) that expressed both ERα and ERβ. Oestrodiol reduced oesophageal SCC cell proliferation, which was reversed by the use of an oestrogen antagonist (ICI1 82,780) [223]. They also demonstrated oestradiol induced intracellular Ca^{2+} release and extracellular Ca^{2+} entry into EC109 cells, and hypothesized that oestrogen exerts its

antiproliferatve effect on oesophageal SCC cells through estrogen receptor-Ca^{2+} signaling pathways [223].

Sukocheva *et al*. [55] were the first group to publish results on the effect of ER ligands on oesophageal AC (OE33 and OE19) and Barrett's oesophagus cell lines (ChTRT and QhTRT, GihTRT). The presence of ERα and ERβ was demonstrated using western blotting. Treatment with oestradiol, and the Selective Oestrogen Receptor Modulators (SERM), tamoxifen and raloxifene, resulted in growth arrest. Treatment with ER ligands also reduced cell number in the Barrett's oesophagus cell lines, which expressed ERs (ChTRT and QhTRT), but not in the cell line devoid of ERs (GihTRT). The authors proposed the ER ligands induced apoptosis and cell cycle arrest as they detected an increase in the number of cells in apoptosis, a decrease in the expression of Ki67, and an increase in the expression of the cell adherence protein E-cadherin [55]. The same research group took the work further by investigating the effect of tamoxifen alone and in combination with cisplatin and 5-fluorouracil (5-FU), the mainstay chemotherapeutic agent used in oesophageal cancer, on OE19 and OE33 cell lines [219]. They demonstrated that 4-hydroxytamoxifen resulted in increased cell death compared to cisplatin or 5-FU (using the MTS assay) and that the addition of 4-hydroxytamoxifen to both cisplatin and 5-FU alone or in combination, resulted in a significant increase in cell death in both cell lines [219].

1.3.3.3 Effect of ER receptor expression manipulation

Manipulation of ER expression provides further information on the role of ERs in oesophageal cancer. ER up-regulation has been attempted previously in oesophageal SCC (ECGI-10) using expression vectors [217]. In ERα up-regulated cells, treatment with an ERα specific agonist significantly decreased the number of cells. Both oestradiol and ERβ specific agonist treatments, however, significantly increased the proliferation of ERβ expressing cells. Moreover, cell proliferation of ERβ expressing cells with oestradiol was significantly suppressed following treatment with a pure ER antagonist (ICI 182780) [217]. Manipulation of ER expression, such as knockdown using RNA-based or genomic techniques will further increase our understanding of the role of ER and ER ligands in oesophageal cancer development and treatment.

1.4 Synthetic ER ligands

Synthetic ER ligands compete with endogenous oestrogen for ER binding and are classified by their molecular structure into either steroidal or non-steroidal [144, 169]. A limited number of highly selective pure ER agonists or antagonists exist, however, the majority of ER ligands have both mixed agonist and antagonistic effects, dependant on the ER subtype expression and coregulators present in different target tissues and are thus, more accurately termed SERMs [224]. There are two SERMs currently licenced for clinical use: tamoxifen, a first-generation triphenylethylene derivative used in the treatment of breast cancer and raloxifene, a second-generation benzothiopene

derivative, used for the treatment and prevention of osteoporosis [225].

1.4.1 Tamoxifen

Tamoxifen is a non-steroidal SERM with antagonist activity in the breast and partial agonist activity in uterus and bone [226]. It has been the gold standard endocrine treatment for oestrogen receptor positive breast cancer for over 30 years, following approval by the US Food and Drug Administration (FDA) in 1977 [227]. It was initially licensed for use in metastatic disease, followed by use in the adjuvant setting, and in 1999, it became the first drug to be approved by the US FDA to prevent breast cancer following the publication of evidence demonstrating it reduces the incidence of disease in healthy women [228]. A meta-analyses of 20 trials showed tamoxifen treatment in breast cancer was associated with a reduction in disease recurrence by 39%, and mortality by 30% [229]. In the adjuvant setting, treatment is for 5 years [230, 231], which may be increased to 10 years for even further clinical benefit according to evidence reported in two recent phase III trials [232, 233]. In addition to treatment of breast cancer, tamoxifen is licenced for use in the UK for anovulatory infertility [234] and high-dose tamoxifen has been investigated in clinical trials of patients with prostate cancer [235, 236], renal cell carcinoma [237-240], glioma [241-243] gastro-intestinal adenocarcinoma [244], malignant melanoma [245] and non-small cell lung cancer [246].

1.4.1.1 Pharmacokinetics

Tamoxifen is administered orally at doses of 20mg or 80mg/ day for the treatment of breast cancer or anovulatory infertility, respectively [234], and is absorbed rapidly by the gastrointestinal tract, with a peak plasma concentration at approximately 3–4 hours [247]. There is a high degree of inter-individual variability in steady-state levels of tamoxifen and its metabolites [242, 248-255] and studies describing the pharmacokinetics of standard dose tamoxifen (10-30mg/ day), report steady state serum levels of 100-333ng/ml [255-259]. Steady state levels are usually achieved within 3-4 weeks with 20mg/ day [247], but higher dosing schedules allow steady-state values to be reached in up to 1 week [257]. Tamoxifen is highly protein bound (>95%), with the majority bound to albumin, and it is distributed at high concentrations in liver, lung, pancreas, brain, ovaries and breast tissue. Tamoxifen undergoes extensive phase I metabolism in the liver by microsomal cytochrome P450 enzymes into at least 22 metabolites [255]. The two main metabolites N-desmethyltamoxifen (resulting from N-demethylation) and 4-hydroxytamoxifen (resulting from 4-hydroxylation), can be further hydroxylated and demethylated, respectively, to endoxifen [255] (Figure 7). N-desmethyltamoxifen has a low binding affinity for the ER but a long biological half-life and 4-hydroxytamoxifen and endoxifen are up to 100 times more potent than tamoxifen with a short biological half-life [225, 255]. The elimination half-life of tamoxifen is 5-7 days and it is primarily excreted in the bile and eliminated in the faeces, with urinary excretion being less predominant.

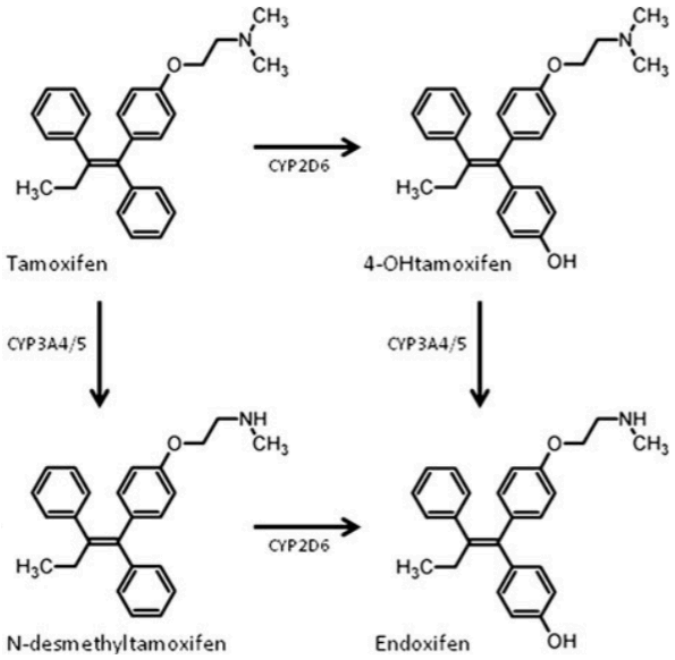


Figure 7. A schematic demonstrating the biotransformation of tamoxifen to its main metabolites by the cytochrome P450 (CYP) enzymes (Adapted from Jager 2014[255] with permission).

1.4.1.2 Mechanism of action

The simple explanation for the mechanism of action of SERMs is that they competitively displace oestrogen from the ER, eliminating the downstream, generally stimulatory effects of oestrogen. The tissue specific pharmacology of SERMs, however, is complex and is a consequence of interaction with numerous diverse cellular pathways (Figure 8).

The molecular structure of the ligand is the first variable to influence SERM action, as the external confirmation of the ER-ligand complex determines interaction with coregulatory proteins [225]. Moreover, small changes in ligand structure can result in major changes in the ER-ligand conformation and subsequent biological character of the

ER. Tamoxifen possesses a bulky alkylaminoethoxyphenyl side chain (Figure 7) that projects out of the ligand-binding cavity and via steric clashes involving the dimethylamino group, prevents the positioning of H12 over the ligand-binding cavity [164]. As previously discussed (section 1.2.3.2.1, Figure 5) the conformation of H12, folding back on top of the ligand-binding cavity, is critical for creating a binding surface for coactivators and transcriptional activation [169, 260].

Secondly, the tissue-specific repertoire and concentration of coregulatory proteins and their ability to bind to the unique shape of the ER-SERM complex offers another explanation for the target tissue specificity of SERM action [164]. Specific coregulators have been identified that recognise SERM-bound ER but not oestrogen-bound ER or vice-versa [261]. In a study using uterine cell lines, tamoxifen increased the recruitment of the coactivator SRC-1, and raloxifene, which lacks oestrogen agonist activity in the endometrium, had no effect [262]. Moreover, both tamoxifen and raloxifene, which behave as antagonists in the breast, increased the recruitment of corepressors, thereby inhibiting the growth of mammary cells [262].

The tissue distribution and ratio of ERα and ERβ with their various isoforms and splice variants adds an additional dimension to the tissue-specific effects of SERMs as they activate different, often opposing target genes, which has been discussed in detail elsewhere (section 1.3). The functional differences between the ER subtypes is

largely due to differences in the AF-1 domain, which is critical for the interaction with other coregulatory proteins controlling gene transcription [263].

Tamoxifen also has antitumor properties that are independent of the classical ER signalling pathway. As described in section 1.2.3.2.1, the ER-oestrogen complex can bind to promoters devoid of ERE through interactions with other DNA-bound transcription factors such as AP1 [167] and SP1 [168]. Likewise, the tamoxifen-ER complex is able to activate gene transcription via pathways that negate ERE binding [262]. The ER-SERM complex has also been shown to modulate transcription of other genes by acting as a coactivator for AP1 induced transcription [264, 265]; in a study using MCF7 cells, oestradiol increased, while tamoxifen decreased growth factor-induced AP1 activity [264]. Tamoxifen has also been shown to recruit coactivators such as SRC-1 and SRC-3, which enhance the transcriptional activity of other nuclear receptors [266]. Another distinct signalling pathway activated by the SERM raloxifene, but not endogenous oestrogen, is via the recently discovered polypurine DNA sequences in the promoters of certain genes, designated raloxifene response elements [267].

There are additional, non-genomic pathways by which tamoxifen can modulate the individual cells in a target tissue, independent of the ER, such as decreasing the level of cyclic nucleotides [268], inhibiting protein kinase C [269], inhibiting cAMP phosphodiesterase

activation [270], modulating mitochondrial bioenergetics [271] and signalling of cell-membrane receptors such as insulin-like growth factor and epidermal growth factor receptors [272]. For example, insulin-like growth factors are increased in the presence of oestrogen but decreased in the presence of tamoxifen [273]. Tamoxifen has also been shown to have an effect on antioxidant defence mechanisms by stimulating antioxidant detoxifying enzymes such as quinone reductase, an activity that is reversed by oestradiol [160].

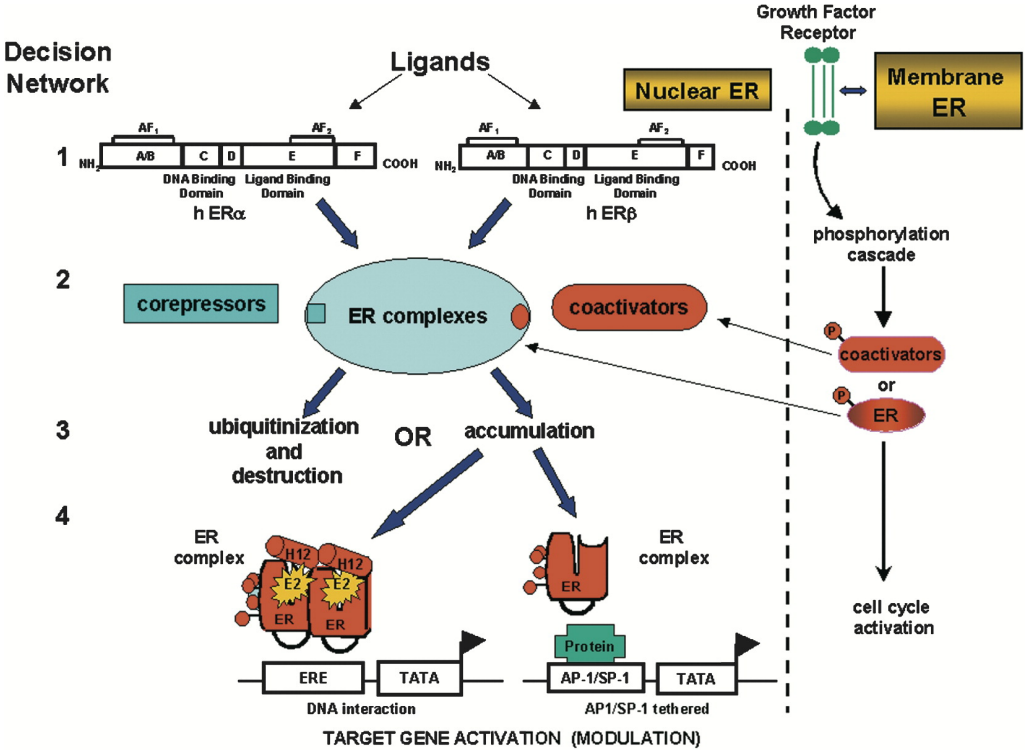


Figure 8. SERM signal transduction schematic (adapted from Jordan 2004[274] with permission). The SERM may bind to a cytoplasmic oestrogen receptor (ER) α or β or a membrane-associated ER (1). SERM binding to the ligand binding domain allows binding of coregulatros (2). The ER complex or coregulators may be destroyed or accumulate to become oestrogen-like complexes (3). The ER complex may interact with the gene transcription machinery by binding to an oestrogen response element (ERE) or via interaction with proteins such as AP1 or SP1 (4).

The intriguing biology of the ER-ligand complex in different target cells is thus determined by: the chemical structure of the ligand and its physical interaction with the ligand-binding pocket; the nature of the gene promoter responsive unit; the repertoire of coregulatory proteins and associated transcription factors; and the ratio of ERα and ERβ the cell [133, 144, 163]. The actual biology, however, is likely to be even greater as new research reveals multiple layers of complexity thereby amplifying the molecular mechanisms of action.

1.5 Aims and objectives

The existing literature on the role of ERα and ERβ in oesophageal cancer, using receptor subtype specific agonists and antagonists is conflicting. An additional approach would be to knockdown subtype receptor expression using siRNA to delineate the role of the subtypes and also to provide insight into the mechanism via which ER ligands such as tamoxifen work, i.e. ER or non-ER mediated. There are limited studies reporting the effect of tamoxifen on oesophageal cancer cell growth and given that tamoxifen is a safe, inexpensive and readily available drug, its anti-cancer properties in oesophageal cancer warrants further investigation.

1.5.1 Research aim

The aim of this research is to determine the role of ERα, ERβ and tamoxifen on oesophageal cancer growth *in-vitro* and *in-vivo.*

1.5.2 Objectives

1. To define the individual roles of ERα and ERβ in oesophageal cancer cell lines, using siRNA to induce gene knockdown (Chapter 3).
2. To investigate the effect of tamoxifen on oesophageal cancer cell lines using proliferation assays (Chapter 4).
3. To explore the mechanism of action of tamoxifen on oesophageal cancer cell lines by observing changes in the expression of markers of proliferation and identify suitable biomarkers that can be used to measure the biological effect of tamoxifen *in-vivo* (Chapter 4).
4. To investigate the therapeutic role of tamoxifen *in-vivo*, in a pilot study of patients with oesophageal cancer (Chapter 5).

2 Chapter Two

Methodology

Laboratory work was undertaken in accordance with the University of Nottingham health and safety regulations. When necessary, personal protective equipment was used and waste disposed of as clinical waste. A class II laminar flow cell culture hood and aseptic technique was used for cell culture and siRNA preparation and transfection. The laboratory temperature maintained at 22-24°C and is considered as room temperature for all experiments.

2.1 Cell culture

2.1.1 Cell lines

Two oesophageal adenocarcinoma cell lines (OE19 and OE33) were used along with the breast cancer cell line, MCF7, serving as a positive control (Table 1). All cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium, supplemented with 10% foetal calf serum (FCS), penicillin (10,000 U/ml), and streptomycin (100µg/ml). RPMI was selected as the media of choice for both cell lines as proliferation studies identified it as the optimum growth media (Figure A.2.8 & A2.9). Cell lines were grown to 70-80% confluence in a T75 or T175 ($75cm^3$ or $175cm^3$, respectively) vented flask and maintained in 5% CO_2 at 37°C in an air humidified incubator.

Table 1. Cell line tissue of origin and supplier

Cell line	Tissue of origin	Supplier	Passage number
OE19	Human male adenocarcinoma of oesophagogastric junction	Sigma-Aldrich, Poole, UK	P33
OE33	Human female oesophageal adenocarcinoma developed in Barrett`s metaplasia	Sigma-Aldrich, Poole, UK	P16
MCF7	Human female breast cancer cell line	From Dr C. Tufarelli (Identity confirmed by STR profiling)	P+142

2.1.2 Recovery of cells from liquid nitrogen

Cancer cell lines were stored in liquid nitrogen (LN_2) at -180°C. When required for culturing, an ampoule was removed and sprayed with 70% ethanol prior to opening the cap in a Class II safety cabinet to release LN_2 gases. The cap was re-tightened and the ampoule transferred to a 37°C water bath where it was immersed until thawed. The cell suspension was then transferred to a T75 vented flask containing warm culture medium before being placed in humidified air incubator at 37°C in 5% CO_2.

2.1.3 Subculture of cell lines

The culture medium was changed every 2-3 days and the cells were split once they were 70-80% confluent, according to the following protocol (volumes used correspond to a T75 flask). The cell media was removed by suction and the flask washed with 15mls of Hank's Balanced Salt Solution (HBSS, Ca and Mg free). The HBSS was then aspirated and cell were trypsinised by adding 4-6mls of 0.025% trypsin/EDTA and the trypsinisation process observed under a microscope at room temperature. Once the cells had detached (usually within seconds), 4-6mls of FCS were added to stop the

action of trypsin and the cell suspension then transferred to a Falcon centrifuge tube. The flask was washed again with 5mls HBSS to remove any remaining cells and transferred to the Falcon tube, which was centrifuged (1000rpm, at room temperature) for 5 minutes. The supernatant was then aspirated and the pellet washed with HBSS (15mls) and centrifuged again (1000rpm, at room temperature) for 5 minutes. Finally, HBSS was aspirated and the cells were re-suspended in the corresponding 5-6mls of medium depending on the cell line (OE33 and OE19 cells were seeded in a 1:6 dilution and MCF7 cells in a 1:5 dilution). 1ml of cell suspension was then pipetted into a new T75 flask containing 14 ml of warm culture medium and placed in humidified air incubator at 37°C in 5% CO_2 for further proliferation.

2.1.4 Cryopreservation

Between experiments, the cell lines were stored in the vapour phase of LN_2, by suspending the cell pellet obtained by the trypsinisation processed described above in 10% DMSO (600µl culture medium, 300µl FBS and 100µl DMSO) at a density of 4 $x10^6$cells/ml. The cells were transferred to a cryo-vial and kept on ice for 15 minutes, then transferred to a "Mr Frosty" cell freezer overnight at -80°C, before being stored in LN_2.

2.2 Western blotting

2.2.1 Cell homogenisation

Cell line homogenisation for Western blotting (WB) was carried out in homogenization buffer (sucrose 300mM; Tris base, pH. 7.4, 25mM; monothioglycerol 10mM; EDTA 1mM; Igepal 2.5%) to which, protease inhibitors (1 Pierce Protease and phosphatase Inhibitor Mini tablets, EDTA free, to 10mls of homogenization buffer) were added just prior to use. 350µl of homogenisation buffer was added to one well of a 6-well plate and the cells disrupted using a P1000 pipette tip. The lysate was then transferred to a 1.5ml eppendorf tube and placed on ice. Using a 1ml syringe with a 21 gauge green needle, the lysate was aspirated 25 times and placed in an ultrasonic bath for 15 minutes. The lysate was again aspirated with the same syringe 25 times. The lysate was the centrifuged at 10600 rpm for 1 hour at 4°C. The resulting supernatant and the pellet were separated and stored at -80°C with the supernatant used in WB.

2.2.2 Protein determination

Estimation of total protein concentration of the homogenised cell lysate was determined using the Bicinchoninic acid (BCA) reaction method. A stock solution of BSA (1mg/ml) was made up in 154mM (0.9%) sodium chloride and a standard curve constructed between zero and 1mg/ml of protein. 10µl of each standard were loaded into a well of a 96-well plate in triplicate, along with diluted samples of unknown concentrations, also in triplicate. The BCA working reagent

(0.1ml 4% copper sulphate and 4.9mls stock bicinchonic acid reagent, 200µl) was added to each well and incubated at 37°C for 30 minutes with shaking. The absorbance of all the standards and unknowns were read at 570nm in a plate reader (Wallac 1420 Victor; Perkin-Elmer, Waltham, USA).

2.2.3 SDS-PAGE Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the process used to separate proteins according to size and mass-to-charge ratio. 10% or 4-15% commercial polyacrylamide precast gels were used depending on the molecular weight of the protein (37-65kDa: Bio-Rad 10% Mini-PROTEAN® TGX™ Gel, 4561036; > 65kDa: Bio-Rad 4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gel, 4568086). The protein samples were diluted to achieve a final concentration of 15µg / 13µl using Laemmli buffer plus β-mercaptoethanol (BME; 950µl Laemmli buffer and 50µl BME) and denatured at 85°C for 5 minutes just prior to loading. The gel was inserted into the running apparatus and running buffer used to fill the inner tank to ¾ full. Running buffer was prepared by diluting premixed electrophoresis buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH8.3, Bio-Rad®) with distilled water at a ratio of 1:10. 13µl of protein sample was loaded per well and 7µl of Precision Plus™ Protein Kaleidoscope™ Marker (Bio-Rad®) was used to identify the protein according to molecular weight. The gel was run at 40mA (constant amps) for 60 minutes using a Bio-Rad® powerpack.

2.2.4 Electroblotting

The separated proteins were then transferred during electroblotting, which uses an electric current and transfer buffer solution to drive proteins from the gel to a membrane. A supported nitrocellulose membrane (Bio-Rad®) was used, and to ensure an even transfer of proteins, the gel and membrane were sandwiched between four pieces of blotting paper and four sponges that had been pre-soaked in blotting buffer at 4°C. The gel was run with an ice block at 100V (Constant), 350mA for 1 hour (2 hours for proteins >100kDa in size), to maintain the temperature and dissipate heat. The optimal transfer time was determined following a series of optimisation experiments, during which the quality of electrophoresis and protein transfer was assessed using Commassie Brilliant Blue R-250 staining solution (Bio-Rad®) to visualise the amount of protein remaining on the gel and the Ponceau Red solution (0.1% Ponceau (w/v) in 5% acetic acid (v/v), Sigma, St Louis, USA) to visualise the protein transferred to the membrane. For each subsequent experiment, once the transfer time was complete, Ponceau Red solution was applied to the membrane to ensure even protein transfer. The Ponceau Red stain was removed by washing in TBST (0.1% Tween-20 (Sigma) in Tris-Buffered Saline). TBS solution consists of 12.11g of Tris base and 146.1g NaCl in 500mls of distilled water (pH7.4).

2.2.5 Blocking & Antibody application

The nitrocellulose blot was blocked in 10% Marvel original (dried skimmed milk dissolved in TBST) for 2 hours at room temperature,

before being incubated in primary antibody solution diluted in 3% Marvel over night at 4°C with shaking (Table 2). The next day, the blots were washed six times with TBST for ten minutes each with shaking. The blot was then incubated in the secondary antibody (Table 3) diluted in 3% Marvel for 2 hours at room temperature, with shaking. The blot was then rinsed again with TBST 6 times, for 10 minutes with shaking.

Table 2. Primary antibodies used in WB

Antibody name	Catalogue number (source)	Antibody type	Dilution	Predicted size (kDa)
ERα	32063 (abcam)	Rabbit monoclonal	1:500	67
ERα	108398 (abcam)	Rabbit monoclonal	1:2000	66
ERβ	3577 (abcam)	Rabbit polyclonal	1:1500	59
ERβ	133467 (abcam)	Rabbit monoclonal	1: 1000	59
ERβ	3576 (abcam)	Rabbit polyclonal	1: 1000	59
Ki67	92742 (abcam)	Rabbit monoclonal	1:1000	359
E-cadherin	610181 (BD Transduction Laboratories)	Mouse monoclonal	1:2500	120
Cyclin D	134175 (abcam)	Rabbit monoclonal	1:10000	34
PCNA	92552 (abcam)	Rabbit monoclonal	1:1000	29
β actin	8227 (abcam)	Rabbit polyclonal	1:5000	42
GAPDH	2118 (New England Biolabs)	Rabbit monoclonal	1:20,000	37

Table 3. Secondary antibodies used in WB

Antibody name and type	Catalogue number (source)	Dilution
Anti-Rabbit IgG polyclonal Alkaline Phosphatase linked	A3937 (Sigma)	1: 30,000
Anti-Mouse IgG polyclonal Alkaline Phosphatase linked	A3562 (Sigma)	1: 30,000
Anti-Rabbit polyclonal HRP-linked	P0448 (Dako)	1:2000

2.2.6 Analysis (Chemiluminescent Densitometry)

The nitrocellulose blot was washed 3 times with TBS prior to the application of substrate enhancer mixture evenly to the membrane (50µl substrate and 2ml of enhancer mixed just prior to use,

Immun-Star, Alkaline Phosphatase substrate pack, Bio-Rad, 1705012). After 10 minutes, the excess solution was removed and the blot placed in a plastic sleeve. For assessing molecular weight, Precision Plus bands were marked with a black marker on the plastic sleeve. A Chemidoc® MP system (Bio-Rad) was used to capture images. The molecular weight markers were imaged using white light, and chemiluminescence light was used to detect the signal produced by the secondary antibody. The protein bands were then quantified using the Bio-Rad Image Lab software (Version 5.2.1, Hercules, CA); the volume analysis tool was used to measure the protein band and the mean background was then subtracted from the mean value. For semiquantification, densitometry of the bands was normalised to the loading control as described in 2.2.7.

2.2.7 Antibody stripping method for re-blotting

After probing with the primary antibody, the blots were stripped and re-probed with β actin as a loading control. The blot was washed with TBST three times and then incubated with ReBlot plus mild solution (Millipore, Watford, diluted 1:10 with distilled water) for 12 minutes with gentle shaking. The blot was then washed in TBST before blocking in Marvel and the application of the loading control antibody as described in 2.2.5.

2.3 PCR quantification

2.3.1 Total RNA Extraction

Total RNA extraction was performed at room temperature from cells cultured in 6-well plates (for cell number see 2.4.1). Firstly, the culture medium was removed and the cells washed with 200µL Phosphate Buffered Saline (PBS). The PBS was the then aspirated and 350µl of denaturing guanidine-thiocyanate–containing buffer RLT (10µl β-ME per 1ml buffer RLT) was added per well and the cells disrupted using a P1000 pipette tip. The lysate was then added to a QIAshredder spin column (QIAGEN, Manchester, UK), which homogenizes the cells by centrifugation at full speed. Pure Ethanol (350µl) was then mixed with the homogenized lysate and added to an RNeasy Mini spin column (QIAGEN, Manchester, UK), which was centrifuged at 12,000rpm for 15 seconds. Buffer RW1 (700µl) was then added to the spin column and centrifuged at 12,000rpm for 15 seconds. A further two washing steps were performed by adding buffer RPE (500µl) to the spin column and centrifuging at 12,000rpm for 15 seconds and 2 minutes respectively. The spin column was then placed in a new 2ml collection tube and centrifuged at full speed for 1 minute. Total RNA (TOTRNA) was eluted into a 1.5ml eppendorf tube by adding 50µl RNA free water and centrifuging at 12,000 rpm for 1 minute. TOTRNA was stored at -80°C.

2.3.2 Concentration and purity of Total RNA

A Nanodrop® spectrophotometer was used to measure the concentration and purity of the extracted RNA. Total extracted RNA (1.6µl) was quantified by measuring the optical density at A260nm and A289nm. Purity of the total RNA was determined by measuring the A260/A280 ratio; a ratio of 2.0 indicates high total RNA purity (Figure 9).

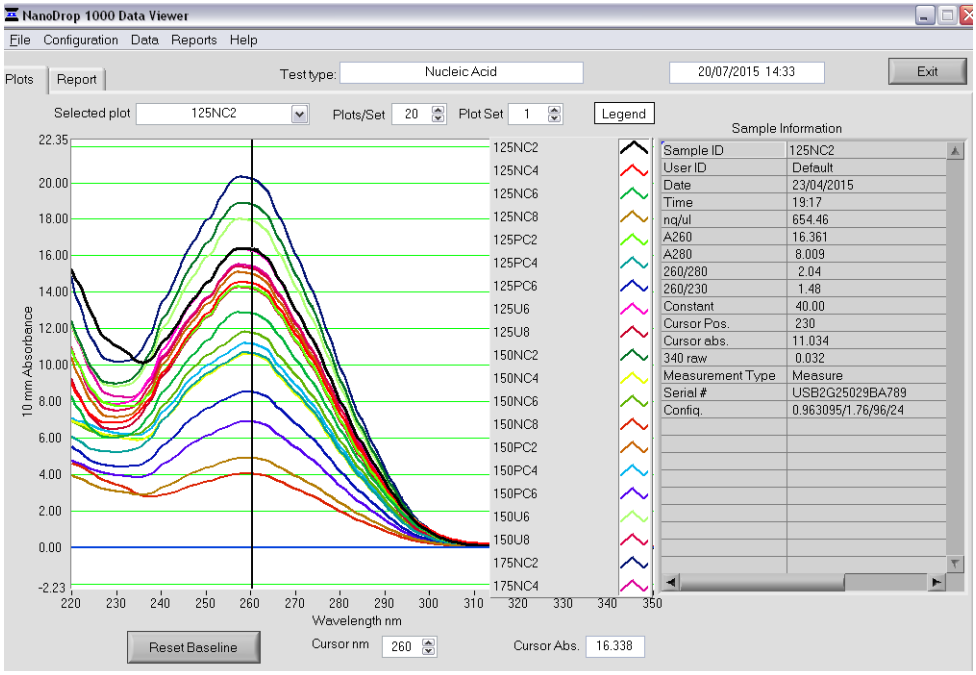


Figure 9. Graphical representation of a nanodrop spectometer reading, displaying the concentration and purity of MCF7 RNA of the siRNA optimisation samples.

2.3.3 Agarose gel electrophoresis

RNA integrity was assessed using agarose gel electrophoresis. A 1% agarose gel was prepared by adding 0.5g of agarose to 50ml of 1x Tris Borate EDTA (TBE) (10x TBE diluted in distilled water 10:1) in a conical flask. The agarose was dissolved by heating in a microwave and then cooled to 60°C. 1µl of ethidium bromide was added to the agarose solution to enable visualisation of TOTRNA and

mixed thoroughly. It was then poured into a gel tray with combs and left for approximately 30 minutes to set. The combs were then removed and the tray containing the gel was submerged in 1x TBE within the gel tank. 5µl of TOTRNA was mixed with 2µl loading buffer and loaded into the wells. The gel was run at 90V (constant voltage) for 30 minutes. The RNA was visualised using a Chemidoc® transilluminator system (BioRad, Quantity One Software Version 4.6.9). High quality RNA extraction was indicated by the presence of two tight undistorted ribosomal RNA bands (28s – 5kb, 18s – 2kb) with 28S displaying a higher intensity than 18S (Figure 10).

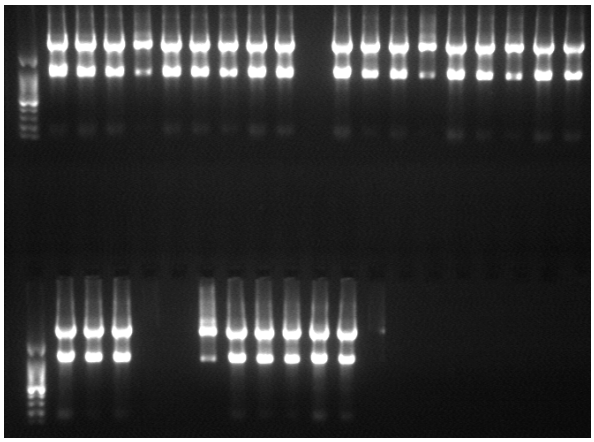


Figure 10. Agarose gel electrophoresis of MCF7 RNA of the siRNA optimization samples. Following visualisation on a Chemidoc® transilluminator system, two discrete bands at 28s and 18s demonstrated high RNA integrity.

2.3.4 Reverse Transcription of TOTRNA

All RT and RT-PCR experiments were performed on a designated bench using designated pipettes and filtered tips to minimise the risk of contamination. The bench and equipment were cleaned with 70% ethanol and RNase® ZAP prior to preparing a running reaction. All reaction components were kept on ice during preparation and a

cooling block was used during loading of samples onto the RT-PCR plate.

Reverse Transcription (RT) reaction is the process whereby single-stranded RNA is reverse transcribed into complementary DNA (cDNA), which is then used for real-time quantitative PCR (RT-PCR). Total RNA was converted into cDNA using High-capacity cDNA reverse transcription kit from Applied Biosystems according to the manufacturer's protocol (Foster City, CA, USA). Total RNA was diluted to achieve a final concentration of 180ng/µl for OE33 and OE19 and 30ng/µl for MCF7, using RNA free water. 20µl of diluted Total RNA was added to 4µl random primers and 6.4µl RNA free water (mix 1) and then incubated at 70°C for 10 minutes to denature the RNA secondary structure. 15.2µl of mix 1 was then added to 2µl of 10xRT buffer, 0.8µl dNTP mix, 1µl RNase inhibitor and 1µl Multiscribe reverse transcriptase to achieve a total volume of 20µl for each RT reaction (+RT). A negative control (-RT) was prepared by adding 15.2µl of mix 1 to 2µl of 10x RT buffer, 0.8µl dNTP mix, and 2µl RNA free water. A –RT control was used in parallel with each sample to identify any genomic DNA contamination.

2.3.5 RT-PCR Controls

For every PCR experiment each RT reaction (+RT) was loaded in triplicate and in parallel with a –RT to identify any genomic DNA contamination. On each plate was a no template control (NTC) for

each assay in which cDNA was replaced with nuclease free water to identify contamination of the PCR components. If more than one PCR plate was used, an inter-plate calibrator of MCF7 cDNA was used for each assay to allow for normalisation of any variation between plates due to instrument factors. RT was carried out by incubation of +RT and –RT in a thermocycler (GeneAmp® PCR System 9700, from Applied Biosystems (Foster City, CA, USA)). Conditions of each RT reaction consisted of 10 minutes at 25°C followed by 2 hours at 37°C and 5 minutes at 85°C. The cDNA was stored at -20°C until required for RT-PCR.

2.3.6 Real Time quantitative PCR protocol

Each PCR reaction contained 2µl of cDNA, 0.7µl of 20x TaqMan® assay (ESR1: Hs00174860_ml; ESR2: Hs01100353_ml; GAPDH: Hs02758991_g1; β Actin: Hs01060665_g1; PGK1: Hs00943178_g1), 10µl of master mix (2x TaqMan® Universal PCR Master mix from Applied Biosystems, Foster City, CA, USA), and 7.3µl of nuclease free water (total volume of 20µl). Each reaction was loaded in triplicate on PCR plates and –RT was used in parallel. In addition, a NTC, in which template (cDNA) was replaced with nuclease free water to identify possible contamination of the PCR components. An inter-plate calibrator of cDNA from MCF7 cells was used for each assay on each plate, to allow compensation for systematic variation between plates due to instrument factors. PCR amplifications were performed using the Chromo4TM real time cycler (Bio-Rad Laboratories LTD, Hemel Hempstead, UK) and the

output data were analysed on Opticon Monitor 3.3.32. Conditions of each PCR reaction consisted of 10 minutes at 95°C followed by 40 cycles of 15 second at 95°C and 1 minute at 60°C. The references genes (GAPDH, β actin, PGK1) had been previously identified as the most stable reference gene candidates for oesophageal cancer by a colleague in the same laboratory, using two separate reference gene stability analysis software packages: geNorm, and NormFinder (Genex standard version 5, MultiD Analyses AB).

2.3.7 Creation of standard curves

Standard curves were constructed to calculate the efficiency values for the respective assays and to determine the optimal concentrations for detecting the transcript. A standard source of MCF7 cDNA was used for all TaqMan assays and the standard curves were constructed with a cDNA starting concentration of 60ng/µl or 90ng/µl, using five dilutions (1:10) in duplicate.

2.3.8 RT-PCR data processing using GENEX software

Quantification was calculated using GenEX software according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [275]. Raw PCR quantification cycle (Cq) data were tabulated in an Excel datasheet and imported into GenEx software. The data was the pre-processed using data editor by normalising for variables in the following order: (1) inter-plate calibration; (2) assay efficiency correction (calculated from the

standard curve for each gene using MCF7 cDNA); (3) normalisation of the target genes (ESR1 and ESR2) to the geometric mean of the 3 reference genes (GAPDH, B-Actin, PGK1); (4) relative quantity using the maximum reference level. The pre-processed data was then saved in an Excel datasheet to allow further descriptive and statistical analysis.

2.4 siRNA

siRNA is a synthetic RNA duplex that targets a specific mRNA for degradation, inducing short-term silencing (2-4 days) of protein coding genes [276]. The ESR1 and ESR2 siRNA used, consisted of ON-TARGET plus SMARTpool siRNA and was delivered into the cell using a cationic lipid transfection reagent, DharmaFECT 2. The transfection reagent and all siRNAs were purchased from Dharmacon, USA. All siRNA transfection experiments were performed using positive control (ON-TARGETplus GAPDH Control Pool), negative control (ON-TARGETplus Non-targeting Pool) and untreated cells. The positive control siRNA targets GAPDH and was used to measure the efficiency of siRNA transfection. Negative control siRNA is non-targeting and has no significant sequence homology to human genes and was used to identify sequence-specific silencing from non-specific effects of the experimental conditions. An untreated control was used as a baseline reference for gene expression levels. Detection of target gene knockdown was confirmed by a reduction in mRNA expression using RT-PCR at 48 hours and protein expression using WB at 72 hours.

2.4.1 Preparation of cell lines for siRNA transfection

Cell lines were seeded into 6-well plates for siRNA transfection. The cells were split and counted using automated digital cell counter (Bio-Rad Laboratories, CA, USA). OE33 and MCF7 cells were seeded at 150,000 and 135,000 cells/ well respectively, and were incubated for 24 hours at 37°C in 5% CO_2.

2.4.2 siRNA transfection protocol

The volumes in this siRNA transfection protocol are for 1 well of a 6-well plate and all preparation was performed at room temperature. Firstly, 5μM of suspended siRNA solution was diluted from 100μM stock solution using 1× siRNA buffer at a ratio of 1:20. In two separate tubes, 10μl of 5μM siRNA (tube 1) and 4μl transfection reagent (tube 2) were diluted with serum-free medium to a total volume of 200μl each per well. The contents of each tube were mixed by pipetting and incubated for 5 minutes. The contents of tube 1 and tube 2 were then combined to give a total volume of 400μl; the contents were mixed by pipetting and incubated for 20 minutes. 1600μl of antibiotic-free complete medium (containing 10% FCS) was added to the mix for a final volume of 2000μl per well and a final concentration of 25nM. The culture medium from the wells containing the cultured cells was replaced with 2000μl of the transfection medium. Cells were then incubated at 37°C in 5% CO_2 for 24 hours after which time, the medium was replaced with complete medium (containing 10% FCS and antibiotics). Cells were

harvested for mRNA analysis after a total of 48 hours and for protein analysis after 72 hours.

2.5 5'-bromo- 2'-deoxyuridine (BrdU) cell proliferation assay

To measure the effect of ER knockdown and tamoxifen treatment on cell proliferation the BrdU cell proliferation assay kit (Roche-Applied-Science) was used. The BrdU assay detects BrdU incorporation (in place of thymine) into cellular newly synthesised cellular DNA during cell proliferation using an anti-BrdU antibody. Phenol red free RPMI (PRF-RPMI, Sigma-Aldrich, Poole, UK) was used for all proliferation studies to eliminate the oestrogenic effect of phenol red, and stripped FCS (sFCS) was used to avoid any interference by steroids in the serum.

2.5.1 Drug preparation

Tamoxifen (≠T5648, Sigma-Aldrich, Poole, UK) was supplied as a lyophilised solid and the stock solution was prepared by dissolution in DMSO according to the manufacturer's instructions. It was stored in the dark at –20°C and when required, was diluted using PRF-RPMI and sFCS to 0.01% DMSO. In order to eliminate any DMSO effects, all dilutions of drugs contained a 0.01% DMSO final concentration.

Etoposide is a potent, cytotoxic anti-cancer drug and was used as a positive control during the optimisation experiments. It was prepared by dissolution in DMSO according to the manufacturer's instructions. A concentration of 1000nM was selected as this had been previously identified as the optimum concentration to suppress OE33 and MCF7 proliferation in experiments in the same laboratory [90].

2.5.2 BrdU protocol

Details of BrdU optimisation is available in Appendix 2. Cells were seeded at a density of 2,000 cells/ 100µl per well and cultured in flat-bottomed 96-well plates at 37°C in 5% CO_2. 100µl/ well of the different tamoxifen dilutions (10,000nM, 1,000nM, 100nM, 10nM and 1nM) were added in triplicate, 24 hours post cell seeding to the treated cells. The BrdU assay was performed according to the manufacturer's protocol as follows: 10µl BrdU labelling reagent (1:100) was added to each well and cells were incubated for a further 2 hours at 37°C in a 5% CO_2, to allow the pyridine analogue in BrdU to incorporate in place of thymidine into the DNA of proliferating cells. The labelling medium was then removed by tapping and suction. Cells were fixed and DNA denatured by adding 200µl/ well of FixDenate for 30 minutes at room temperature. The FixDenate was then removed by flicking off and tapping. 100µl/ well of anti-BrdU-Peroxidase antibody was added to the fixed cells (prepared by adding 100µl Anti-BrdU-POD to 10ml antibody dilution solution) and incubated for a further 90 minutes at room

temperature. The antibody conjugate was removed by decanting and rinsing three times with 300µl/ well of PBS. 100µl/ well of substrate solution was then added and incubated for 30 minutes at room temperature. The reaction product was quantified by measuring the absorbance using a scanning multi-well spectrophotometer using a 370nm filter with a reference wavelength of 492nm (Thermo Multiskan Spectrum, Thermo electron company, Vantaa, Finland).

The following controls were used in each plate: a background control, which contained cells in the absence of BrdU giving information on the unspecific binding of the anti-BrdU-POD conjugate to the cells (which was <0.1 absorbance at 2,000 cells/ well); a blank control, containing cell culture medium without cells to measure background luminescence associated with the cell culture system (which was subtracted from all other values); and an untreated/ negative control of cells cultured in 0.01% DMSO.

2.6 Effect of tamoxifen on proliferation-associated proteins

To determine the effect of tamoxifen on the expression of proliferation-associated proteins, the conditions present for the BrdU assay (in 1 well of a 96-well plate) were up-scaled to a T25 flask to ensure enough protein would be harvested for the WB experiments. Cells were seeded at a density of 156,000 cells/ T25 flask and allowed to attach for 24 hours at 37°C in 5% CO_2. The media was changed at 24 hours to media containing 100nM tamoxifen and

replaced every 48hrs. Protein was harvested for WB at day 3, 4 and 5 of treatment as described in section 2.2.1.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Cell line data was normally distributed and therefore expressed as mean ± standard deviation (SD). Comparative analyses were performed using t-tests (unpaired, two-tailed) or the one-way ANOVA test with post-hoc analysis using the Tukey's or Dunnett's multiple comparisons tests. All significant results are reported as the exact p values. Non-significant values are not indicated in the figures.

3 Chapter Three

Oestrogen Receptor Knockdown

3.1 Introduction

The individual role of ERα and ERβ in breast cancer is well defined; ERα is associated with proliferation and ERβ attenuates the action of ERα, resulting in growth suppression [95, 163, 174]. Studies on other cancer cell lines, such as prostate [277, 278] and colon cancer [188, 208, 211] also suggest that the individual ER subtypes exert distinct biological functions. The presence of ERs in oesophageal cancer tissue [92, 93, 213-217] and oesophageal cancer cell lines [55, 86, 222] has been reported by numerous authors (See introduction, section 1.3.3). The precise role of ER subtypes in oesophageal cancer is unknown and previous studies have attempted to determine their role using subtype specific ER agonists and antagonists [55, 86, 87, 217, 222]. Work by the same research group demonstrated a significant dose-dependent inhibition of proliferation in oesophageal cancer cell lines (OE33 and OE19) using highly selective ERα (MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) and ERβ, (PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol) antagonists ($p = <0.05$). In the OE19 cell line, a highly selective ERα agonist (PPT) ($p= <0.01$) and ERβ specific agonist (DPN) ($p = < 0.0001$) both significantly inhibited cell proliferation compared with untreated cells [279]. Additional work, however, is still needed to help define the role of the ER subtypes.

The aim of this chapter is threefold: (1) to confirm the expression of the ER subtypes in the OE19 and OE33 cell lines; (2) to knockdown

the expression of the ER subtypes using siRNA; and (3) to define the role of the ER subtypes in oesophageal cancer by observing the effect of ER subtype knockdown on the expression of proliferation-associated proteins and on cell proliferation, using a proliferation assay.

The expression of the ER subtypes will be knocked down using siRNA which is a synthetic RNA duplex consisting of an antisense and a sense strand. Once transfected into cells, the sense strand is loaded onto a ribonuclear protein complex (RNA silencing complex or RISC), which then binds to the corresponding mRNA sequence, targeting it for cleavage and degradation, inducing short-term silencing (2-4 days) of the downstream protein coding genes [280]. Knockdown of ERα and ERβ individually and in combination, will provide insight into their role in oesophageal cancer cell growth. The degree of ER receptor knockdown will be determined by measuring the relative mRNA and protein knockdown, using RT-PCR and WB, respectively.

Changes in the expression of Ki67 [281, 282], E-cadherin [283, 284], Cyclin D [285, 286] and proliferating cell nuclear antigen (PCNA), [287-289] assessed by WB, are regularly used as markers of proliferation in *in-vitro* work on cancer cell lines. Ki67 is a monoclonal antibody, which binds with a proliferation-associated antigen, present in the nuclei of cells during the active phases of the cell cycle, but absent in resting cells [290]. The MIB-1 monoclonal raised against recombinant parts of

the Ki-67 antigen [291] is widely used in histopathology and *in-vitro* studies to measure the growth fraction in human tumours or cell lines, respectively [292, 293]. E-cadherin is a cell adhesion molecule present on the surface of epithelial cells [294], the dysfunction of which triggers invasion and metastasis [295]. As well as work on cancer cell lines [283, 284] it also has clinical applications, such as distinguishing lobular carcinoma from ductal carcinoma of the breast, which are E-cadherin negative and positive, respectively [296]. Cyclin D is a cell cycle protein and through its interaction with cyclin-dependent protein kinases, promotes cell cycle progression. Hence, increased expression of Cyclin D results in loss of normal cell cycle control and directly results in some of the hallmarks of cancer [297]. Cyclin D plays a role in the development of numerous cancers, including breast cancer [298] and it is a recognized biomarker for prognosis in oesophageal cancer [299, 300]. PCNA is a DNA sliding clamp and plays a key role in DNA replication and repair by binding with numerous partner proteins [301]. It is expressed during G1 and S-phases of the cell cycle and is a recognised marker of proliferation [302]; high expression of PCNA has been shown to correlate with poor prognosis in a variety of solid tumours [303-305].

3.2 Methods

Cell culture, WB, RT-PCR, siRNA transfection protocols and details of statistical analyses are available in Chapter 2.

Positive control (PC) siRNA, which targets GAPDH, was used for each experiment as a measure of transfection efficiency. A negative control (NC, non-targeting) siRNA was also prepared for every experiment and was used to normalise expression of the target (mRNA or protein), to ensure any effects associated with the experimental procedure were accounted for. The effect of the DharmaFECT transfection reagent and trypsinisation of siRNA treated cells, on the expression of the target mRNA was investigated in a series of optimisation experiments, which confirmed there was no statistically significant difference in target mRNA expression between cells grown with or without the DharmaFECT transfection reagent and cells that has undergone trypsinisation or not (Appendix A1.9 & A1.10).

Percentage knockdown following siRNA treatment was then calculated by normalising the target expression with the NC expression using the following formula:
% knockdown = 1 - (target expression/ NC expression) x 100.

Percentage down- or up-regulation of proliferation associated proteins, was also calculated using the same formula.

Unless otherwise specified, ESR1 and ESR2 are used to denote mRNA expression and ERα and ERβ are used to denote protein expression. For protein expression, the individual protein blots are displayed directly below the corresponding bar graph.

3.3 Results

3.3.1 ER expression in oesophageal cancer cell lines

The oesophageal cancer cell lines, OE33 and OE19, used in the following experiments originate from oesophageal adenocarcinoma in the female and male, respectively. The expression of the ER subtypes in the cell lines and the ability to reliably detect ER mRNA and protein was confirmed on at least 3 different mRNA or protein samples harvested during different experiments. The MCF7, breast cancer cell line, which expresses both ER subtypes was used as the positive control cell line for all experiments [306, 307].

3.3.1.1 ER mRNA expression

Details of the ESR1 and ESR2 PCR primers used are available in Chapter 2. The PCR primers and Taqman probes were selected as they target the maximum number of gene transcripts and have been previously validated by a colleague in the same laboratory on the MCF7, OE19 and OE33 cell lines [90].

3.3.1.1.1 ESR1 mRNA expression

The mean ESR1 mRNA expression in MCF7, OE19 and OE33 cell lines was 24.2 (SD±1.0), 4.9 (SD±2.4) and 7.1 (SD±0.6), respectively. There was a significant difference in ESR1 mRNA amongst the three different cell lines (p=<0.0001). Post-hoc analysis demonstrated a significant difference in ESR1 mRNA expression in MCF7 vs. OE19 (p=<0.0001) and MCF7 vs. OE33 cells (p=<0.0001). There was no significant difference in OE19 vs. OE33 expression (Figure 11).

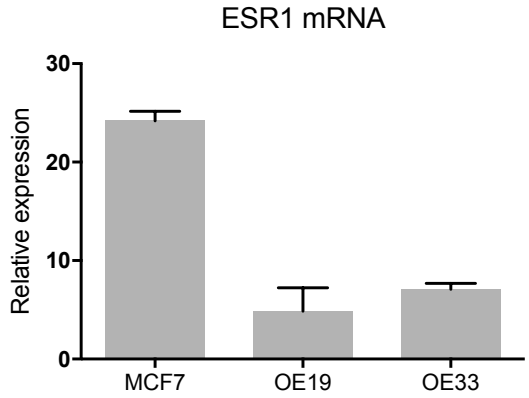


Figure 11. ESR1 mRNA expression in MCF7 (n=24), OE19 (n=18) and OE33 (n=27) cell lines. The mean expression in MCF7, OE19 and OE33 cell lines was 24.2 (SD±1.0), 4.9 (SD±2.4) and 7.1 (SD±0.6), respectively, and was significantly different between groups (p=<0.0001, One-way ANOVA). Post-hoc analysis using Tukey's multiple comparisons test demonstrated a significant difference in ESR1 mRNA expression in MCF7 vs. OE19 (p=<0.0001) and MCF7 vs. OE33 cells (p=<0.0001). Results are expressed as mean±SD.

3.3.1.1.2 ESR2 mRNA expression

The mean ESR2 mRNA expression in MCF7, OE19 and OE33 cell lines was 3.7 (SD±0.9), 0.7 (SD±0.3), and 4.2 (SD±0.3), respectively. There was a significant difference in ESR2 mRNA expression amongst the three different cell lines (p=<0.0001). Post-hoc analysis demonstrated a significant difference in ESR2 mRNA expression in MCF7 vs. OE19 (p=<0.0001) and OE19 vs. OE33 cells (p=<0.0001). The difference in ESR2 mRNA expression in MCF7 vs. OE33 was also significant (p=0.047, Figure 12).

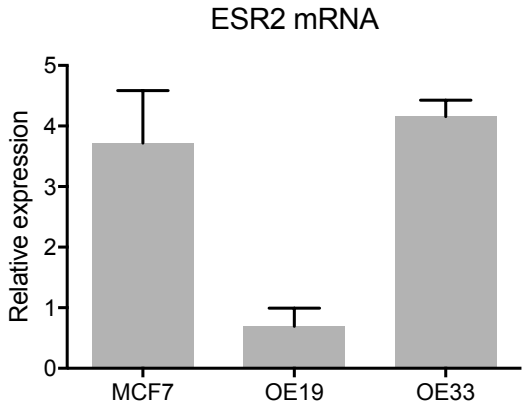


Figure 12. ESR2 mRNA expression in MCF7 (n=24), OE19 (n=22) and OE33 (n=18) cell lines. The mean expression in MCF7, OE19 and OE33 cell lines was 3.7 (SD±0.9), 0.7 (SD±0.3) and 4.2 (SD±0.3) respectively, and was significantly different between groups p=<0.0001, one-way ANOVA). Post-hoc analysis using Tukey's multiple comparisons test demonstrated a significant difference in ESR2 mRNA expression in MCF7 vs. OE19 (p=<0.0001), OE19 vs. OE33 cells (p=<0.0001) and MCF7 vs. OE33 cell lines (p=0.047). Results are expressed as mean±SD.

3.3.1.2 ER protein expression

The ER antibodies used in the experiments below are detailed in Chapter 2 and were selected following a review of the product information available from the manufacturer (Abcam).

3.3.1.2.1 ERα protein expression

The first ERα antibody used (ab32063) resulted in a poor WB with numerous protein bands (Figure A1.1, A), when tested using the protocol conditions provided by the manufacturer. The antibody was optimised by: (1) loading increasing amounts of protein for the OE19 and OE33 cell lines; (2) increasing the concentration of the primary antibody; (3) using different concentrations of secondary antibody; (4) using a different type of secondary antibody (horseradish peroxidase); and (5), using a protein-free blocking buffer (Figure A1.1, B-E). Despite attempts at optimisation, it was still unclear as to which band was the ERα protein signal. The blocking peptide specific for the ERα antibody (ab203371) was then used, but failed to discriminate between the numerous protein bands in the MCF7 cell line, as it blocked out all of the bands (Figure A1.1, F).

A second ERα antibody (ab108398) was trialled which identified a strong signal with minimal background across all cell lines at the predicted size of 66kDa (Figure A1.2), and was therefore used for further downstream experiments.

ERα protein expression was demonstrated across the three cell lines; the mean ERα protein density in MCF7, OE19 and OE33 cell lines was 1.2 (SD±0.2), 0.1 (SD±0.01), and 0.3 (SD±0.2), respectively. There was a significant difference in ERα protein density amongst the three different cell lines (p=<0.0001). Post-hoc analysis demonstrated a significant difference in ERα protein expression in MCF7 vs. OE19 (p=<0.0001) and MCF7 vs. OE33 cells (p=0.001). There was no significant difference in OE19 vs. OE33 expression (Figure 13).

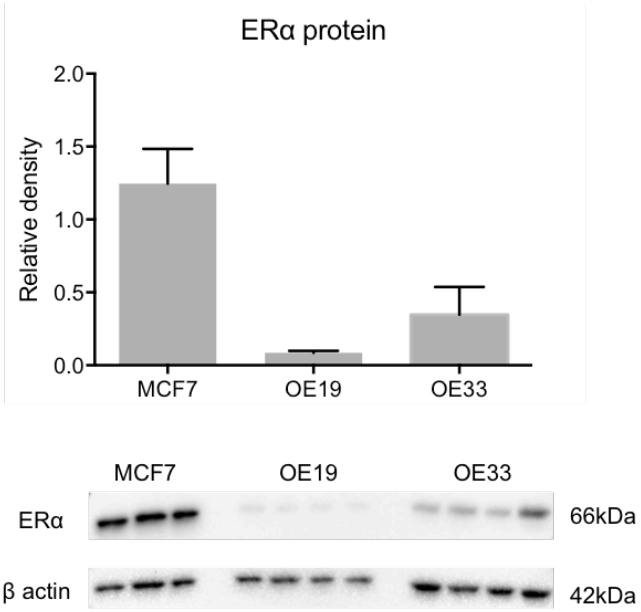


Figure 13. ERα protein expression in MCF7, OE19 and OE33 cell lines. The mean expression in MCF7, OE19 and OE33 cell lines was 1.2 (SD±0.2), 0.1 (SD±0.01), and 0.3 (SD±0.2), respectively, and was significantly different between cell lines, p=<0.0001 (One-way ANOVA). Post-hoc analysis using Tukey's multiple comparisons test demonstrated a significant difference in ERα protein expression in MCF7 vs. OE19 (p=<0.0001) and MCF7 vs. OE33 cells (p=0.001). Results are expressed as mean±SD.

3.3.1.2.2 ERβ protein expression

The first ERβ antibody used (ab3577) produced a clean WB with a protein band at the predicted size of approximately 59kDa. The protein band demonstrated on initial WB (Figure A1.3, A) was further validated using the blocking peptide specific for the ERβ antibody (ab3564, Figure A1.3, B). Failure to demonstrate knockdown of the ERβ protein following treatment with siRNA in further downstream experiments (Table 4 and Table 5), prompted the use of two other ERβ antibodies: ab3576 and ab133467 (Figure A1.4). ERβ antibody, ab133467, which detects a protein band of approximately 59kDa, produced a strong specific signal with minimal background at the predicted size and was therefore used for downstream experiments.

ERβ protein expression was demonstrated across the three cell lines; the mean ERβ protein density in MCF7, OE19 and OE33 cell lines was 0.4 (SD±0.2), 0.1 (SD±0.03), and 1.0 (SD±0.2), respectively. There was a significant difference in ERβ protein density amongst the three different cell lines ($p=0.0009$). Post-hoc analysis demonstrated a significant difference in ERβ protein expression in MCF7 vs. OE33 ($p=0.0145$) and OE19 vs. OE33 cells ($p=0.0008$). There was no significant difference in MCF7 vs. OE19 expression (Figure 14).

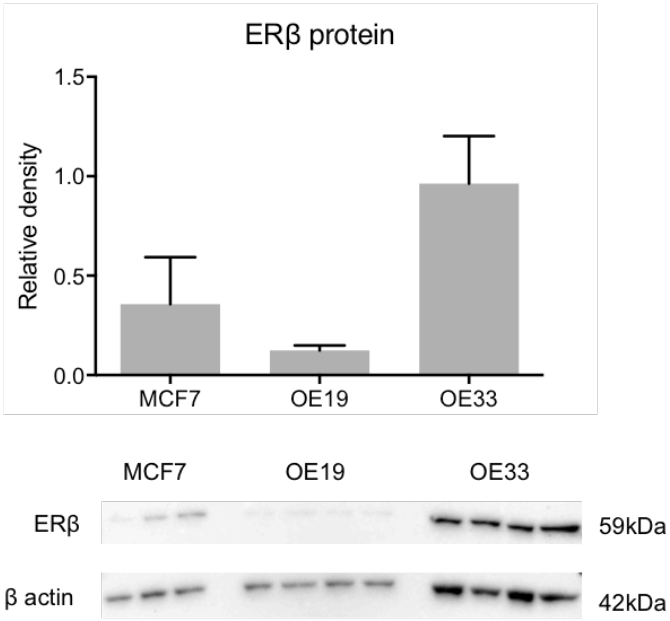


Figure 14. ERβ protein expression in MCF7, OE19 and OE33 cell lines. The mean expression in MCF7, OE19 and OE33 cell lines was 0.4 (SD±0.2), 0.1 (SD±0.03), and 1.0 (SD±0.2), respectively, and was significantly different between cell lines, p=<0.0009 (One-way ANOVA). Post-hoc analysis using Tukey's multiple comparisons test demonstrated a significant difference in ERβ protein expression in MCF7 vs. OE33 (p=0.0145) and OE19 vs. OE33 cells (p=0.0008). Results are expressed as mean±SD.

3.3.2 siRNA transfection

3.3.2.1 Positive control cell line

3.3.2.1.1 Optimisation of the positive control cell line

The MCF7 breast cancer cell line was used as the positive control cell line for the siRNA transfection experiments because there are numerous reports in the literature of successful ER knockdown in MCF7 cells using siRNA [308], and the siRNA manufacturer (Dharmacon), provided information on optimal transfection conditions for the MCF7 cell line.

The following conditions for the initial siRNA transfection were therefore based on optimal conditions reported [308] and the manufacturer's protocol: cell density of 200,000 cells/ well, DharmaFECT volume of 4µl per well, and a target siRNA concentration of 25nM. All samples were performed in duplicate, on two separate 6-well plates, one for mRNA analysis and one for protein analysis. Percentage knockdown was calculated relative to NC expression for all analyses, unless otherwise specified. The initial experiment in the MCF7 cell line using the optimal conditions described above, however, failed to demonstrate efficient transfection (Figure A1.5).

3.3.2.1.1.1 Cell density and DharmaFECT volume optimisation

As the initial experiment failed to demonstrate adequate knockdown of the PC as a marker of transfection efficiency, the protocol was optimised. As per the manufacturer's instructions, the MCF7 cells were optimised using three different cell densities (125,000 – 175,000 cells/ well) and four different DharmaFECT volumes within the recommended range (1 - 10µl), to achieve the highest transfection efficiency with minimal effect on cell viability. PC and NC siRNA were used at a final concentration of 25nM. Cell morphology was assessed at 24hours using a light microscope and cells transfected using 6 - 8µl DharmaFECT showed a marked change in morphology; the cells were contracted and lifting off the base of the well, irregular in shape with condensed chromatin in the nuclei. DharmaFECT agents are known to cause toxicity, although DharmaFECT 2 used in this study, is the least toxic [309].

The manufacturer suggested aiming for a GAPDH mRNA knockdown of ~70% using the PC siRNA which was achieved for numerous experimental conditions. Statistical analysis demonstrated a highly significant difference between all 3 DharmaFECT volumes relative to the NC for the corresponding cell number ($p=<0.0001$), and there was no significant difference in the knockdown achieved between the DharmaFECT volumes within the cell number groups (Figure 15). A cell density of 135,000 cells/ well was selected using a DharmaFECT volume of 4µl/ well, as these conditions gave optimal

transfection with minimal change in cell morphology, ensuring a high enough yield of mRNA and protein to assess knockdown.

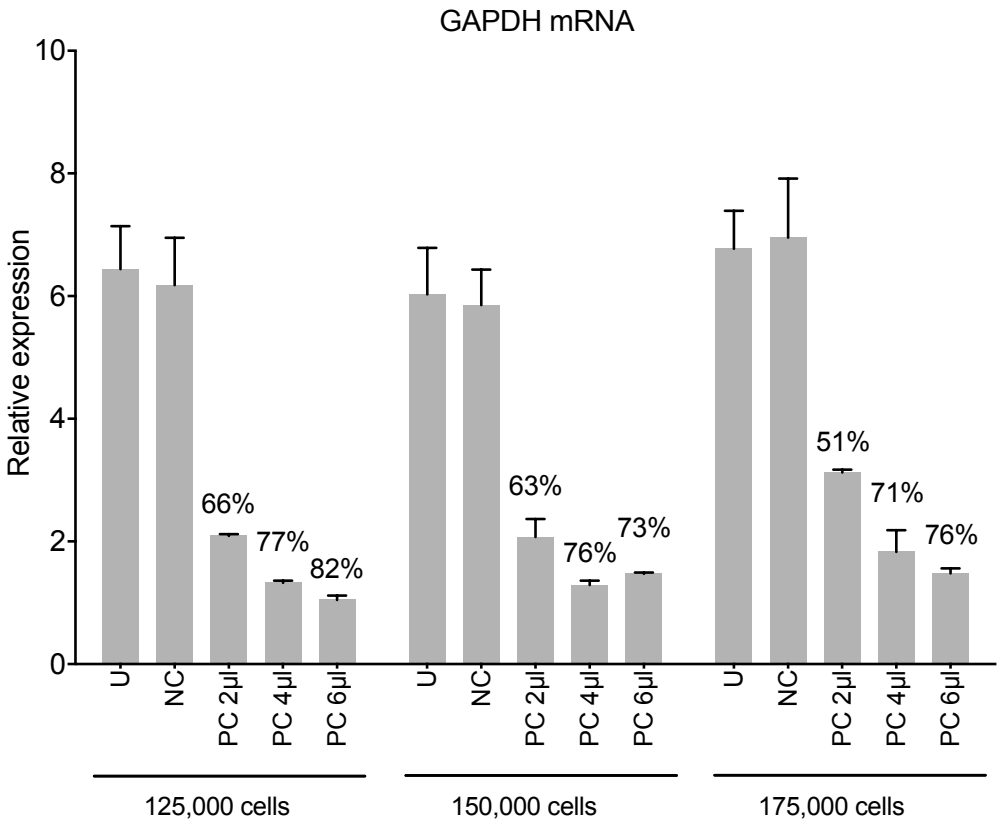


Figure 15. Relative GAPDH expression across three different MCF7 cell densities (125,000, 150,000 and 175,000 cells/ well), using positive control (PC) siRNA targeting GAPDH, and different volumes of DharmaFECT transfection reagent (2µl, 4µl and 6µl). Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. There was a significant difference for all 3 DharmaFECT volumes tested, relative to the NC for the corresponding cell number ($p=<0.0001$, one-way AVONA with Tukey's multiple comparisons test). There was no difference in knockdown achieved between the DharmaFECT volumes within the cell number groups (one-way ANOVA with Tukey's multiple comparisons test). U: untreated cells. Results are expressed as mean±SD.

3.3.2.1.1.2 Target siRNA optimisation

Once the optimum cell density (135,000 cells/ well) and DharmaFECT volume (4µl/ well) had been determined, a further optimisation experiment was performed to identify the optimal target siRNA (ESR1 and ESR2) concentrations using 5 different final concentrations (15nM – 100nM). PC and NC siRNA was used at 25nM final concentration. At 24hrs, the cell morphology was viewed

using a light microscope; the cells transfected with ESR2 siRNA at a concentration of 70nM and 100nM were contracted, irregular in shape and lifting off the base of the well.

Relative GAPDH mRNA expression was knocked down by 78% ($p<0.0001$) in MCF7 cells treated with the PC siRNA, demonstrating efficient transfection (Figure 16).

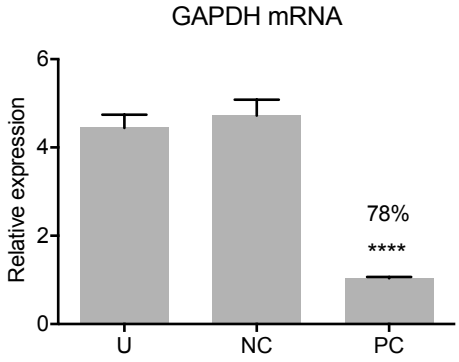


Figure 16. Relative GAPDH expression using positive control (PC) siRNA targeting GAPDH, in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of 78% was statistically significant (p=<0.0001, t-test). U: untreated cells. Results are expressed as mean±SD.

Relative ESR1 and ESR2 mRNA expression was reduced across all siRNA final concentrations by 72 – 80% and 44 – 65%, respectively. The knockdown of ESR1 expression relative to the NC was significant (p=<0.0001) across all siRNA concentrations and there was no significant difference in knockdown between the different concentrations of siRNA (Figure 17). Knockdown of ESR2 expression relative to the NC was also significant across all siRNA concentrations and more significant for cells treated with 25nM and 40nM of siRNA (p=<0.0001) (Figure 17). A final concentration of

25nM was selected as it was the lowest concentration to give the most statistically significant knockdown using the ESR2 siRNA, and the use of the same final concentration across all siRNAs used (including ESR1, PC and NC) simplified the protocol reducing potential errors.

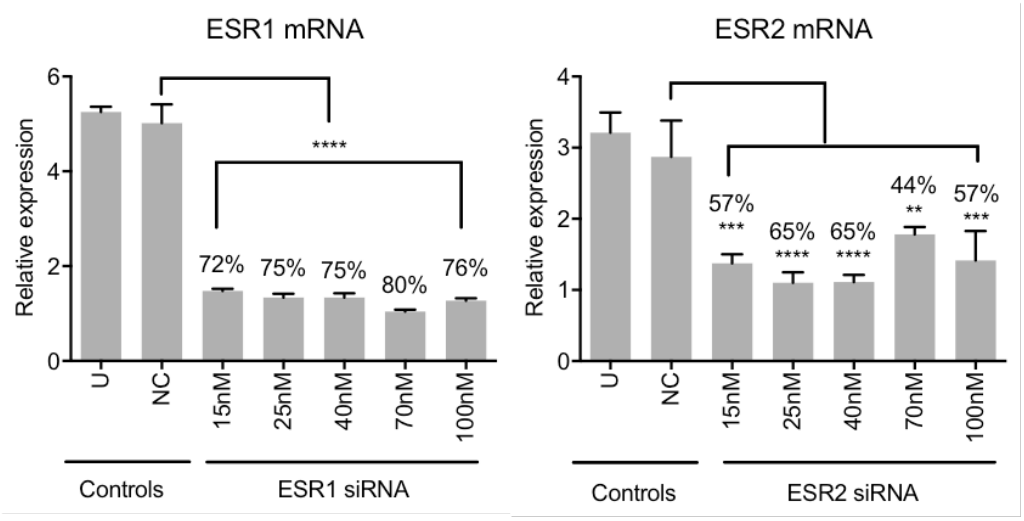


Figure 17. Relative ESR1 and ESR2 (expression, following treatment with different concentrations of ESR1 and ESR2 siRNA in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression and was significant for all ESR1 and ESR2 concentrations (**** p=<0.0001, *** p=0.0002, **p=0.0023, one-way ANOVA with Tukey's multiple comparisons test). For ESR1 siRNA there was no significant difference in knockdown between the different siRNA concentrations (one-way ANOVA with Tukey's multiple comparisons test). U: untreated cells. Results are expressed as mean±SD.

3.3.2.1.2 Simultaneous mRNA and protein knockdown

Once the optimum siRNA experimental conditions had been determined, further experiments were conducted to demonstrate simultaneous mRNA and protein ER knockdown. As well as individual ESR1 and ESR2 siRNA treated cells, both siRNAs (25nM final concentration of each siRNA) were combined with the aim of achieving knockdown of both receptors.

GAPDH knockdown in MCF7 cells using the PC siRNA was 72% (p=<0.0001) and 89% (p=0.0041), for mRNA and protein expression respectively, demonstrating efficient transfection (Figure 18).

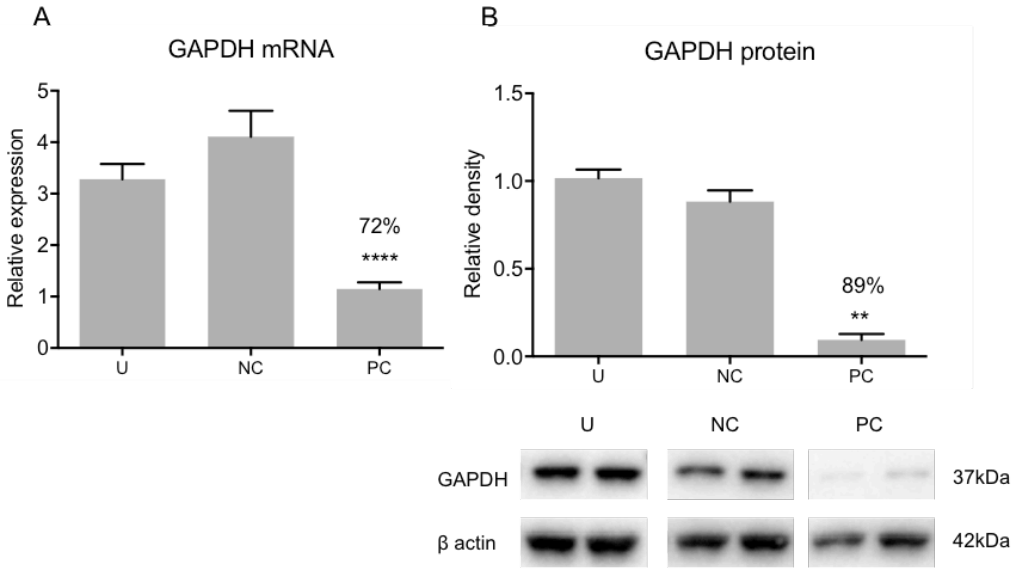


Figure 18. Relative GAPDH mRNA expression (A) and protein expression (B) using positive control (PC) siRNA targeting GAPDH in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of mRNA and protein was 72% (**** p=<0.0001, t-test) and 89% (** p=0.0041, t-test), respectively. U: untreated cells. Results are expressed as mean±SD.

ESR1 mRNA knockdown in MCF7 cells using ESR1 siRNA only, and ESR1 and ESR2 siRNA in combination, was 48% (p=<0.0001) and 32% (p=<0.0001) respectively, both of which were statistically significant compared to the NC. There was no significant knockdown of ERα protein using ESR1 only or ESR1 and ESR2 siRNA in combination, although there was a trend towards knockdown (Figure 19).

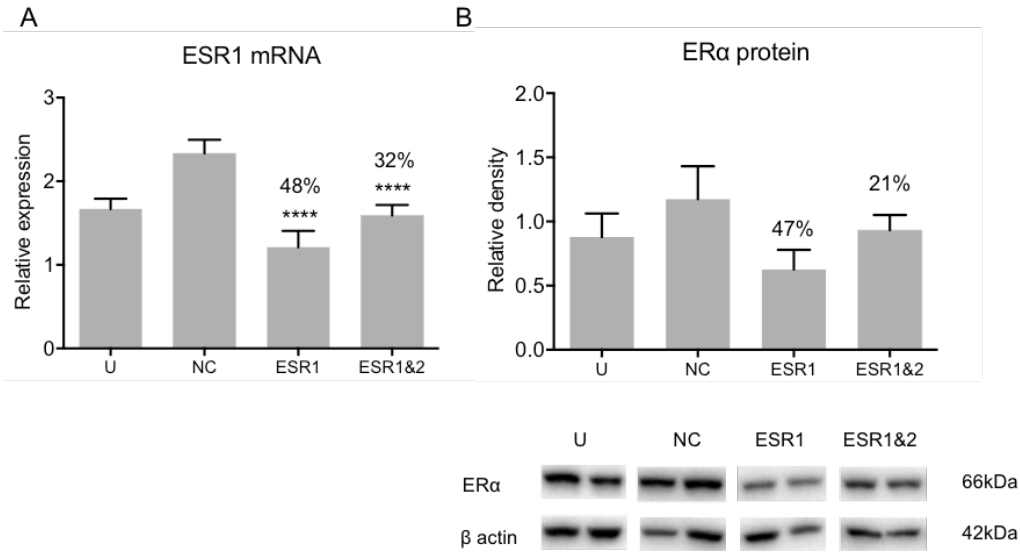


Figure 19. Relative ESR1 mRNA expression (A) and protein expression (B) using ESR1 siRNA and ESR2 siRNA in combination, in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. ESR1 mRNA knockdown in MCF7 cells using ESR1 siRNA only, and ESR1 and ESR2 siRNA in combination, was 48% (p=<0.0001) and 32% (p=<0.0001) respectively. There was no significant knockdown of ERα protein using ESR1 only or ESR1 and ESR2 siRNA in combination. U: untreated cells. Results are expressed as mean±SD.

ESR2 mRNA knockdown in MCF7 cells using ESR2 siRNA only, and ESR1 and ESR2 siRNA in combination was 42% (p=0.0001) and 44% (p=<0.0001) respectively. There was no significant ERβ protein knockdown in cells treated with ESR2 only, or ERS1 and ESR2 siRNA in combination using antibody ab3577 (Figure 20).

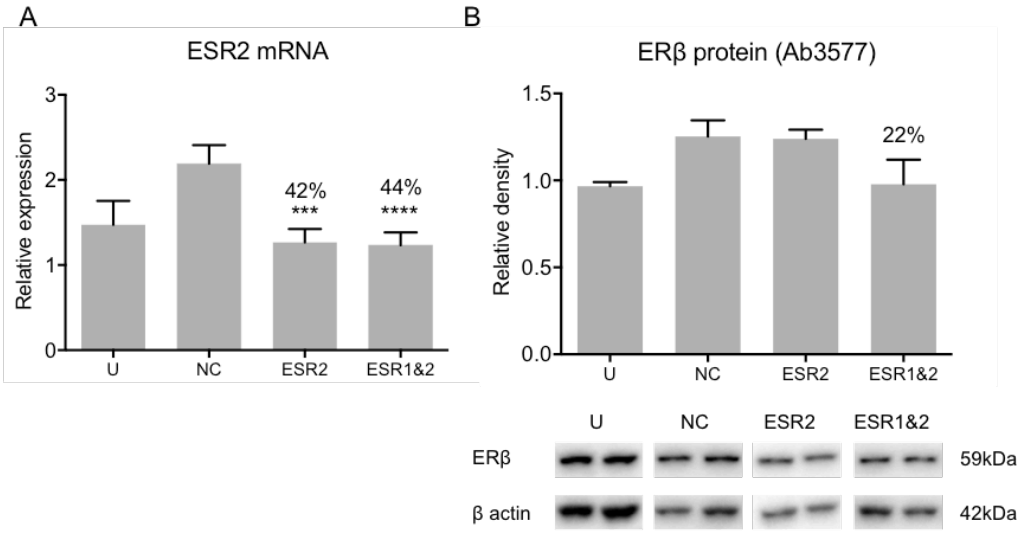


Figure 20. Relative ESR2 mRNA expression (A) and protein expression (B) using ESR2 siRNA and ESR1 and ESR2 siRNA in combination, in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. ESR2 mRNA knockdown in MCF7 cells using ESR2 siRNA only, and ESR1 and ESR2 siRNA in combination was 42% (p=0.0001, t-test) and 44% (p=<0.0001 t-test) respectively. There was no significant ERβ protein knockdown in cells treated with ESR2 only, or ERS1 and ESR2 siRNA. U: untreated cells. Results are expressed as mean±SD.

WB of the extracted protein was then repeated using a second antibody to ERβ (ab133467). ERβ protein knockdown in MCF7 cells using ESR1 and ESR2 in combination was 30% (p=0.0086) (Figure 21). There was only one ESR2 treated protein available due to technical error and so it wasn't possible to perform statistical analysis.

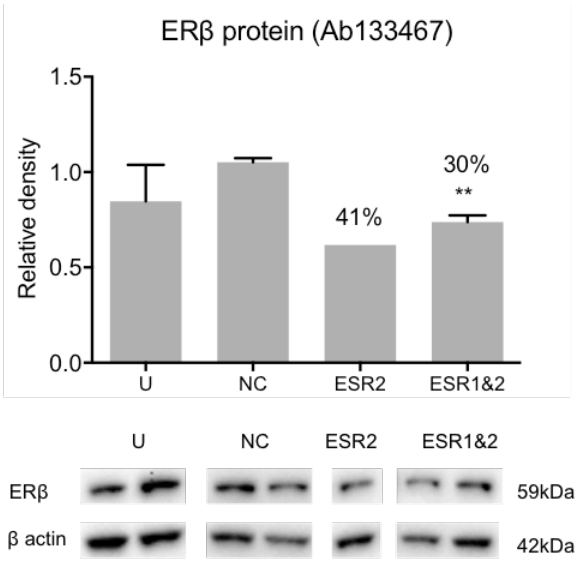


Figure 21. ERβ protein expression in MCF7 cells treated with ESR1 and ESR2 siRNA in combination using a second antibody to ERβ (ab133467), was 30% (**p=0.0086, t-test). Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. There was only one ESR2 treated protein available due to technical error and so it wasn't possible to perform statistical analysis. U: untreated cells. Results are expressed as mean±SD.

Although knockdown of GAPDH mRNA expression using the PC siRNA suggested efficient transfection (72%), the knockdown of ESR1 and ESR2 mRNA of 48% and 42% respectively, was not as high as achieved during the optimisation experiment using the same final concentration of siRNA of 75% and 65%, respectively. The MCF7 cells were of an earlier passage than those used for the optimisation experiments, which may have resulted in the formation of a different MCF7 subline generated by different culture conditions [310]. The above experiments were therefore repeated using the same passage number of MCF7 cells used for the optimisation experiments.

GAPDH mRNA knockdown in MCF7 cells using the PC siRNA was 62% (p=<0.0001) (Figure 22).

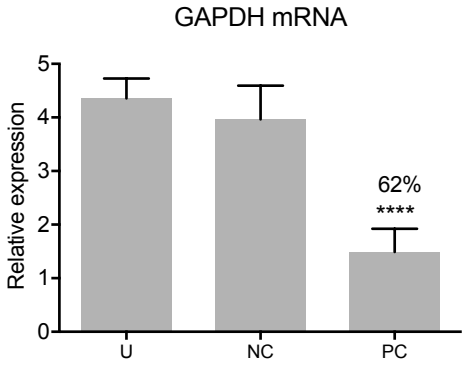


Figure 22. Relative GAPDH mRNA expression using positive control (PC) siRNA targeting GAPDH in MCF7 cells. Knockdown (expressed as a percentage) was 62% (p=<0.0001, t-test) and calculated relative to negative control (NC) expression. U: untreated cells. Results are expressed as mean±SD.

ESR1 mRNA knockdown in MCF7 cells using ESR1 siRNA was 70% (p=<0.0001). The mean ERα protein knockdown of the 6 samples tested was 43% ±9.5, p=0.0002) when normalised to the NC and U expression (Figure 23).

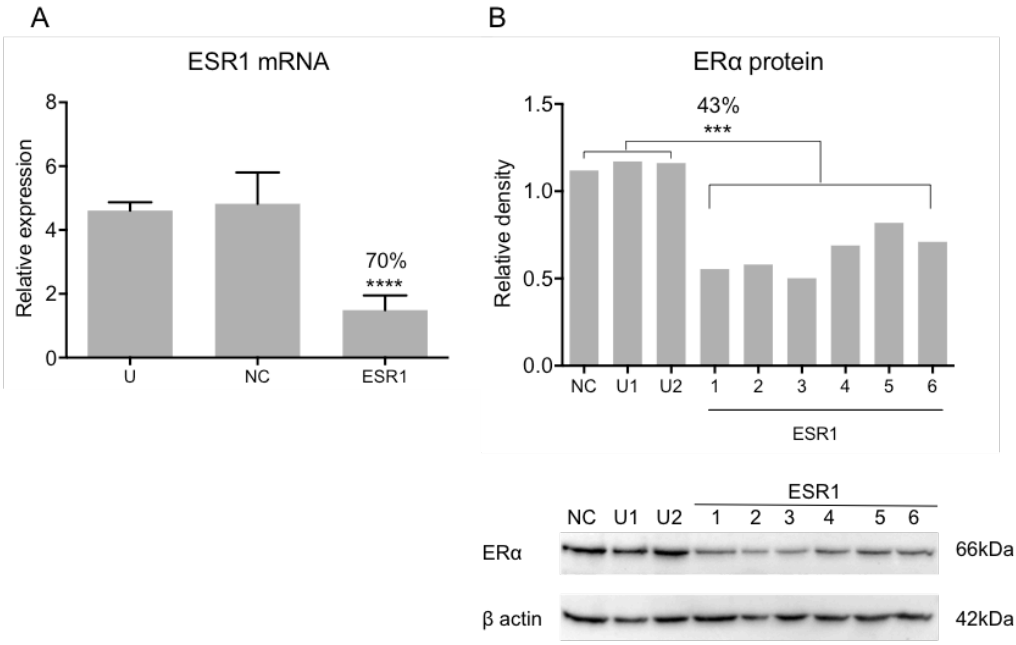


Figure 23. Relative ESR1 mRNA expression (A) and ERα protein expression (B) using ESR1 siRNA (A), in MCF7 cells. Knockdown (expressed as a percentage) for mRNA expression was calculated relative to negative control (NC), and for protein expression to NC and untreated (U). Knockdown of ESR1 mRNA and ERα protein was 70% (****p=<0.0001, t-test) and 43%±9.5 (***p=0.0002, paired t-test), respectively. Results are expressed as mean±SD.

ESR2 mRNA knockdown in MCF7 cells using ESR2 siRNA was 71% (p=0.0006). ESR2 siRNA however, failed to demonstrate ERβ protein knockdown by WB using antibody ab3577 (Figure 24).

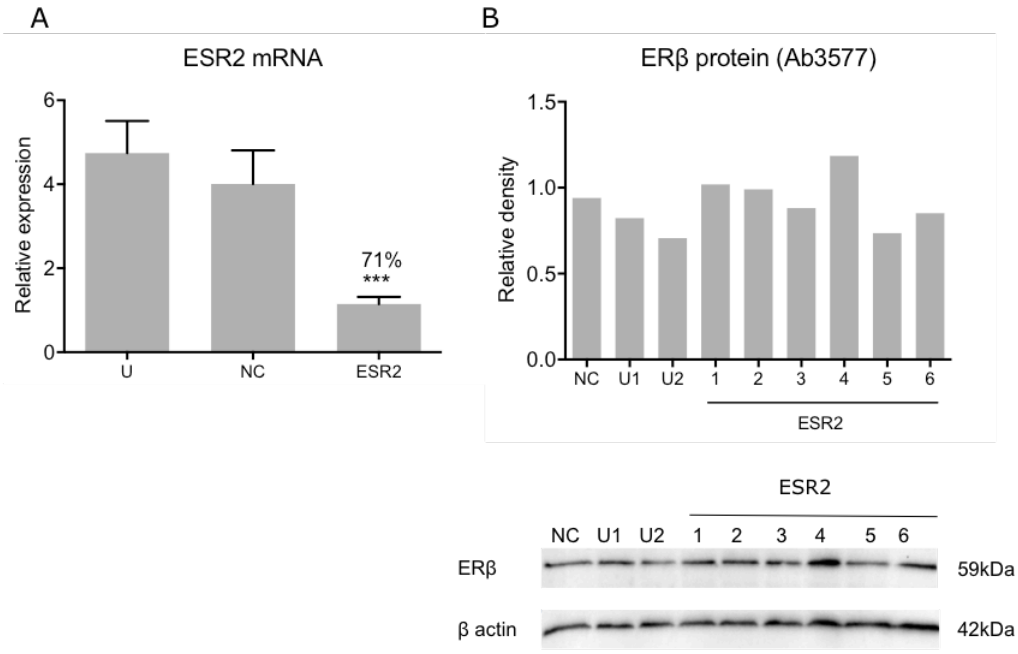


Figure 24. Relative ESR2 mRNA expression (A) and protein expression (B) using ESR2 siRNA (B), in MCF7 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of ESR2 mRNA 71% (***p=0.0006, t-test) but failed to demonstrate ERβ protein knockdown (B). U: untreated cells. Results are expressed as mean±SD.

WB of the extracted protein was then repeated using a second antibody to ERβ (ab133467). There was a mean reduction in the expression of ERβ protein in the six treated samples when normalised to the NC, however, statistical analysis was not possible due to loss of one of the NC samples (Figure 25).

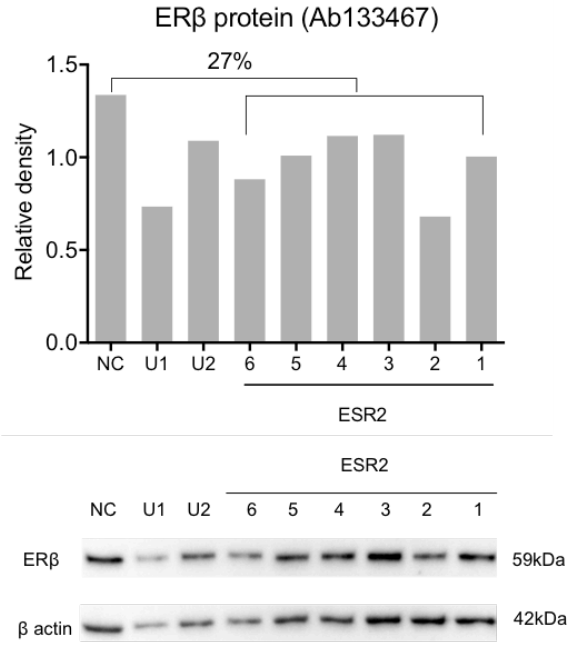


Figure 25. Mean ERβ protein expression knockdown in MCF7 cells treated with ESR2 siRNA (B) was 27% using a second antibody to ERβ (ab133467) although statistical analysis was not possible. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. U: untreated cells.

A summary of ER knockdown in MCF7 cells is presented in the table below (Table 4).

Table 4. A table summarising each experiment using MCF7 cells, showing the mRNA and protein knockdown following treatment with the respective siRNA, expressed as a percentage.

	mRNA % knockdown		**Protein % knockdown**		
Experiment	**ESR1**	**ESR2**	**ERα**	**ERβ (1)**	**ERβ (2)**
Optimisation	76 (n=5)	58 (n=5)	N/A	N/A	N/A
Experiment 1	40 (n=4)	43 (n=4)	34 (n=4)	11 (n=4)	36 (n=4)
Experiment 2	70 (n=2)	71 (n=2)	43 (n=6)	0% (n=6)	27 (n=6)
Mean ±SD	61.6 ±18.1	54.7 ±11.9	39.4 ±9.8	4.4±9.3	30.4±9.7

3.3.2.2 Oesophageal cancer cell lines

3.3.2.2.1 Optimisation of the oesophageal cancer cell lines

Initial experiments were performed on both OE19 and OE33 cell lines. In the OE19 cell lines, relative GAPDH mRNA expression was knocked down by 44% (*p=0.0104) in cells treated with the PC siRNA. Treatment with both ESR1 and ESR2 siRNA failed to knockdown mRNA expression with both 4µl and 6µl of DharmaFECT per well (Figure A1.6-8). The knockdown achieved in the OE19 cell line in the initial experiment was not as high as that achieved in the OE33 cell line and further experiments were therefore focussed on the OE33 cell line.

3.3.2.2.1.1 Optimisation of DharmaFECT volume

Transfection conditions for the OE33 cell line were optimised using 150,000 cells/ well to achieve a cell confluency of 50% at 24 hours, which is the optimum for the MCF7 cell line. Two DharmaFECT volumes (4 and 6µl) and two target siRNA final concentrations (25nM and 40nM) were tested.

Relative GAPDH mRNA expression was knocked down by 62% (p=0.0032), using 4µl of DharmaFECT in OE33 cells treated with the PC siRNA, demonstrating efficient transfection (Figure 26).

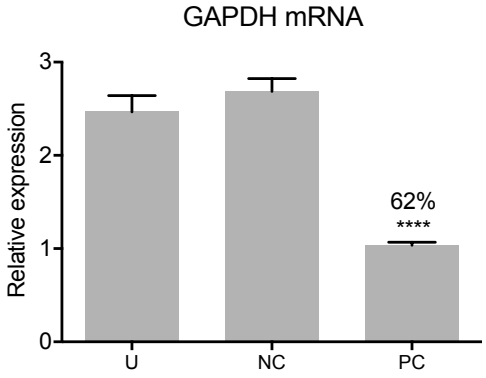


Figure 26. Relative GAPDH expression using positive control (PC) siRNA targeting GAPDH, in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of 62% was statistically significant (**** p=<0.0001, t-test). U: untreated cells. Results are expressed as mean±SD.

Treatment of OE33 cells with ESR1 siRNA at two DharmaFECT volumes (4µl and 6µl) failed to demonstrate significant knockdown of ESR1 mRNA expression (Figure 27).

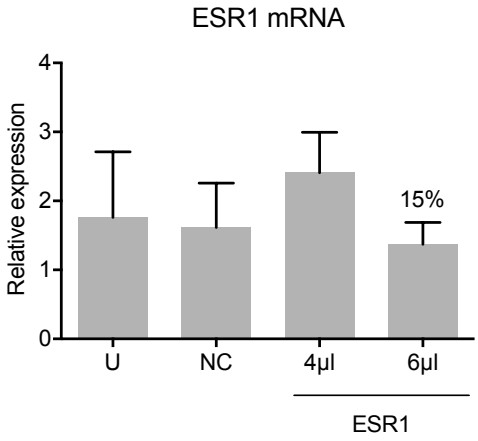


Figure 27. Relative ESR1 expression using ESR1 siRNA, with 4µl or 6µl per well, in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. There was no significant knockdown of ESR1 mRNA expression (one-way AVONA with Dunnett's multiple comparisons test). U: untreated cells. Results are expressed as mean±SD.

Relative ESR2 mRNA expression was knocked down in OE33 cells by 56% (p=0.0011) and 48% (p=0.0025), using ESR2 siRNA 4µl and 6µl of DharmaFECT per well, respectively (Figure 28).

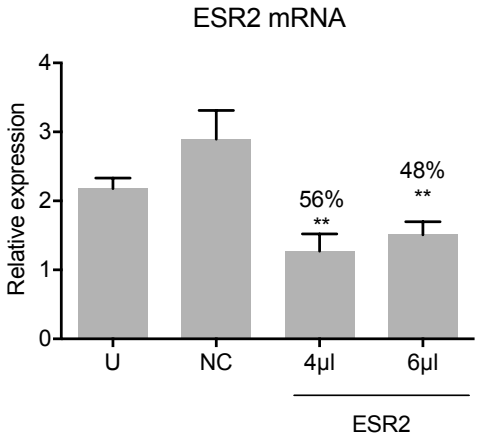


Figure 28. Relative ESR2 expression using ESR2 siRNA, with either 4µl or 6µl per well, in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. ESR2 knockdown was 56% (**p=0.0011, one-way AVONA with Dunnett's multiple comparisons test) and 48% (**p=0.0025), with 4µl and 6µl of DharmaFECT per well, respectively. U: untreated cells. Results are expressed as mean±SD.

3.3.2.2.1.2 Target siRNA optimisation

Once the optimum DharmaFECT volume (4µl/ well) had been determined for the OE33 cell line, a further optimisation experiment was performed to identify the optimal target siRNA (ESR1 and ESR2) concentrations, using 3 different final concentrations (15nM – 70nM). PC and NC siRNA was used at 25nM final concentration.

Relative GAPDH mRNA expression was knocked down by 67% (p=<0.0001) in OE33 cells treated with the PC siRNA, demonstrating efficient transfection (Figure 29).

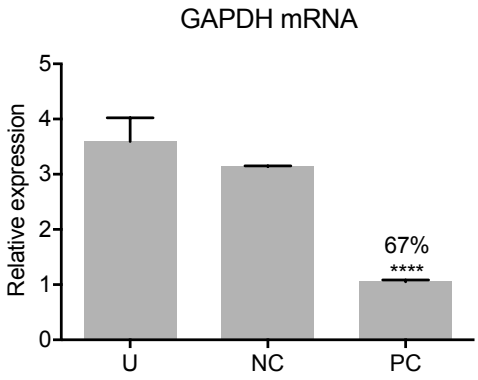


Figure 29. Relative GAPDH expression using positive control (PC) siRNA targeting GAPDH, in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of 67% was statistically significant (****p=<0.0001, t-test). U: untreated cells. Results are expressed as mean±SD.

In OE33 cells treated with ESR1 siRNA (25nM-70nM), there was no significant ESR1 mRNA knocked down, although a trend towards knockdown was suggested, relative to NC expression (Figure 30).

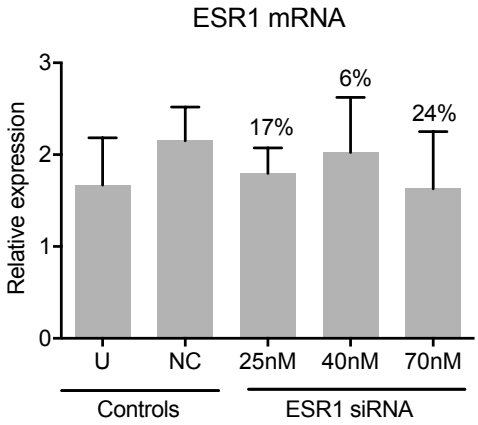


Figure 30. Relative ESR1 expression following treatment with different concentrations of ESR1 siRNA in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. The % knockdown was not statistically significant (one-way AVONA with Dunnett's multiple comparisons test). U: untreated cells. Results are expressed as mean±SD.

In OE33 cells treated with ESR2 siRNA, relative ESR2 mRNA expression was reduced across all siRNA final concentrations by 37 – 42% (p=0.0190, Figure 31). A final concentration of 25nM was selected as the optimum concentration because higher concentrations did not result in a greater increase in knockdown.

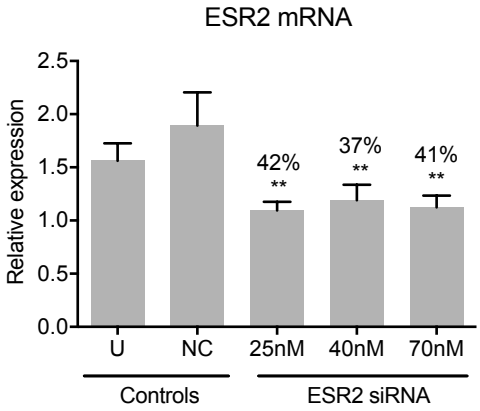


Figure 31. Relative ESR2 expression following treatment with different concentrations of ESR2 siRNA in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. The % knockdown for 25nM, 40nM and 70nM concentration was 42% (**p=0.0002), 37% (**p=0.0044) and 41% (**p=0.0025, one-way AVONA with Dunnett's multiple comparisons test) respectively.

3.3.2.2.2 Simultaneous mRNA and protein knockdown

Once the optimum siRNA experimental conditions had been identified for the OE33 cell line, further experiments were conducted to demonstrate simultaneous mRNA and protein ER knockdown. Despite failure to knockdown ESR1 mRNA using ESR1 siRNA in OE33 cells, which may be secondary to the low copy number, further experiments using ERS1 siRNA were performed to try and detect ERα protein knockdown. As well as individual ESR1 and ESR2 siRNA treated cells, both siRNAs (25nM of each) were combined with the aim of achieving knockdown of both receptors.

GAPDH knockdown in OE33 cells using the PC siRNA was 60% (p=<0.0001) and 100% (p=0.0021), for mRNA and protein expression, respectively, demonstrating efficient transfection (Figure 32).

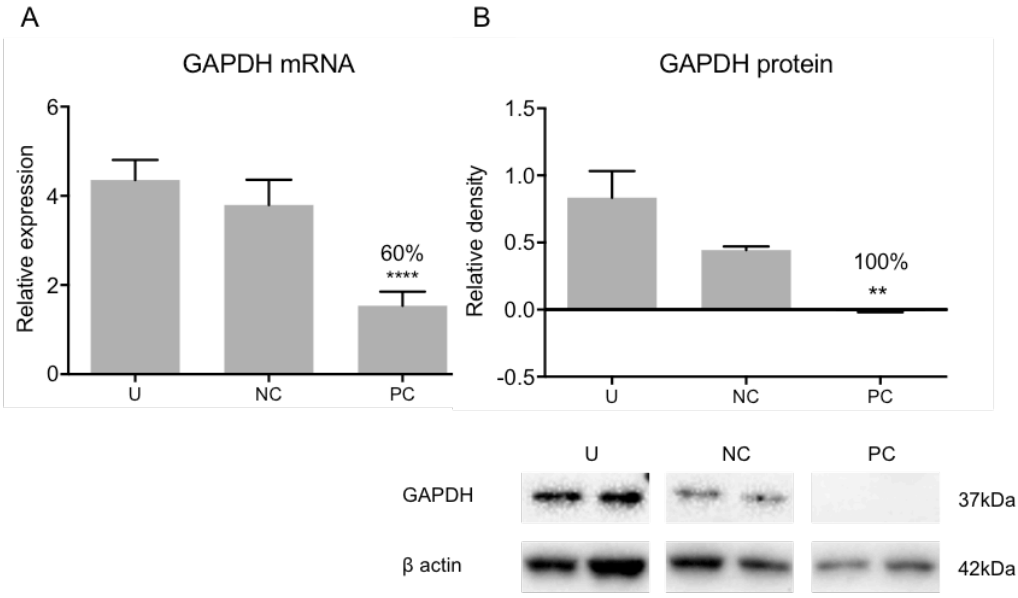


Figure 32. Relative GAPDH mRNA expression (A) and protein expression (B) using positive control (PC) siRNA targeting GAPDH in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of mRNA and protein was 60% (****p=<0.0001, t-test) and 100% (**p=0.0021, t-test), respectively. U: untreated cells. Results are expressed as mean±SD.

Treatment of OE33 cells with ESR1 siRNA failed to demonstrate knockdown of ESR1 mRNA. Interestingly, treatment with ESR1 and ESR2 siRNA in combination resulted in a significant increase in expression of ESR1 mRNA (p=0.0431). There was no significant change in ERα protein expression (Figure 33).

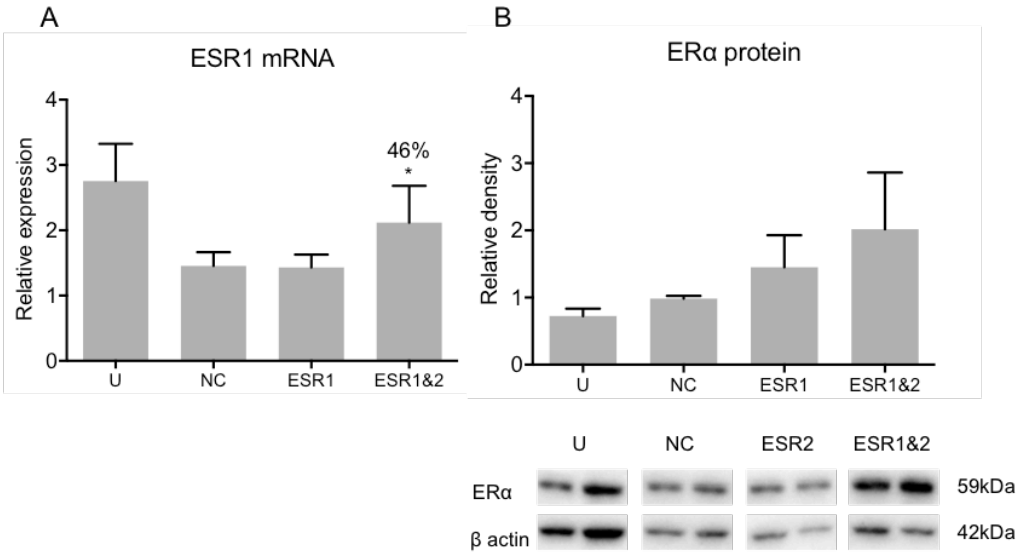


Figure 33. Relative ESR1 mRNA expression (A) and ERα protein expression (B) using ESR1 siRNA only, or ESR1 and ESR2 siRNA in combination in OE33 cells. Relative to the negative control (NC), ESR1 siRNA failed to demonstrate knockdown of ESR1 mRNA or ERα protein. Cells treated with ESR1 and ESR2 siRNA in combination up-regulated ESR1 mRNA expression (*p=0.0431, t-test). U: untreated cells. Results are expressed as mean±SD.

ESR2 mRNA expression was knocked down in OE33 cells by 34% (p=0.0004) and 30% (p=0.0003) using ESR2 siRNA only, and ESR1 and ESR2 siRNA in combination, respectively. There was no significant ERβ protein knockdown in siRNA treated cells using antibody ab3577 (Figure 34).

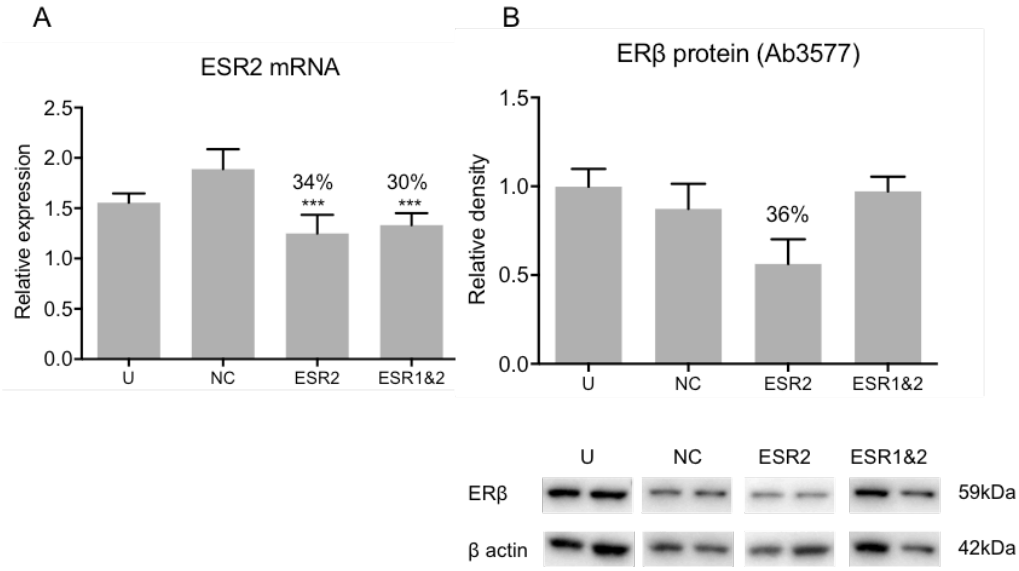


Figure 34. Relative ESR2 mRNA expression (A) and ERβ protein expression (B) using ESR2 siRNA and ESR1 and ESR2 siRNA in combination, in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of ESR2 mRNA and ERβ protein using ESR2 siRNA alone was 34% (***p=0.0004, t-test) and 36% (not significant), respectively. Knockdown of ESR2 mRNA and ERβ protein using ESR1 and ESR2 siRNA in combination was 30% (***p=0.0003, t-test) and 0%, respectively. U: untreated cells. Results are expressed as mean±SD.

Although significant knockdown of GAPDH mRNA and protein expression using the PC siRNA suggested efficient transfection of the OE33 cell line, the knockdown of ESR2 mRNA of 32% was not as high as achieved during the optimisation experiment (42%) using the same final concentration of siRNA. ESR1 siRNA, again, failed to demonstrate statistically significant ESR1 mRNA knockdown or ERα protein knockdown. One possible explanation is that the OE33 cell line had only been put through one passage cycle after being recovered from liquid nitrogen and was not growing satisfactorily. The above experiments were therefore repeated.

Relative GAPDH mRNA expression was knocked down by 55% (p=<0.0001) in cells treated with the PC siRNA, demonstrating efficient transfection (Figure 35).

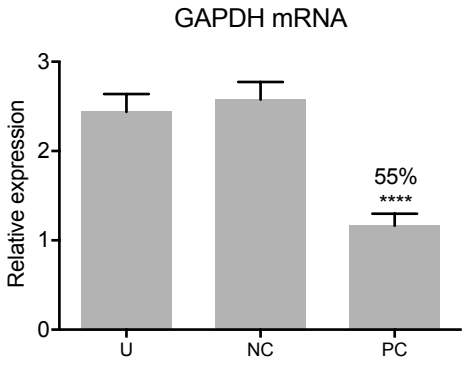


Figure 35. Relative GAPDH mRNA expression using positive control (PC) siRNA targeting GAPDH in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of mRNA was 60%, and was statistically significant (****p=<0.0001, t-test). U: untreated cells. Results are expressed as mean±SD.

In OE33 cells treated with ESR1 siRNA there was no significant ESR1 mRNA knockdown and interestingly ERα protein expression was significantly increased (p=0.0224) (Figure 36).

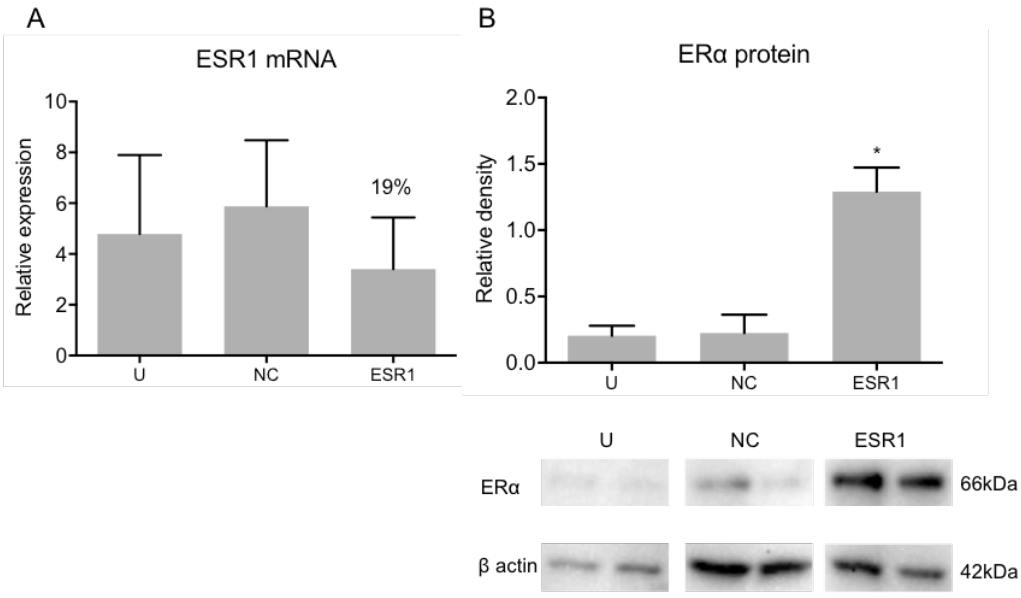


Figure 36. Relative ESR1 mRNA expression (A) and ERα protein expression (B) using ESR1 siRNA in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. There was no significant knockdown of ESR1 mRNA was (19%, t-test) and ERα protein expression was increased (*p=0.0224, t-test). U: untreated cells. Results are expressed as mean±SD.

Relative ESR2 mRNA expression was knocked down by 60% (p=<0.0001) in OE33 cells treated with the ESR2 siRNA, but failed to knockdown ERβ protein expression using antibody ab3577 (Figure 37).

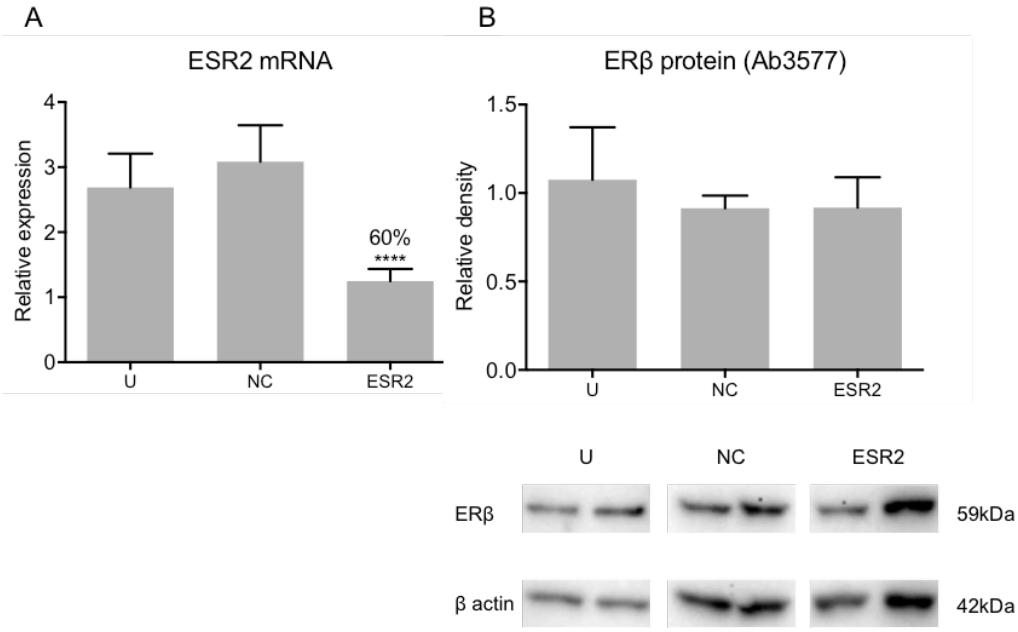


Figure 37. Relative ESR2 mRNA expression (A) and ERβ protein expression (B) using ESR2 siRNA in OE33 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. Knockdown of ESR2 mRNA was 60% (****p=<0.0001, t-test). There was no evidence of ERβ protein knockdown using ab3577. U: untreated cells. Results are expressed as mean±SD.

WB of the harvested protein was then repeated using a second antibody to ERβ (ab133467), which still failed to demonstrate ERβ protein knockdown (Figure 38).

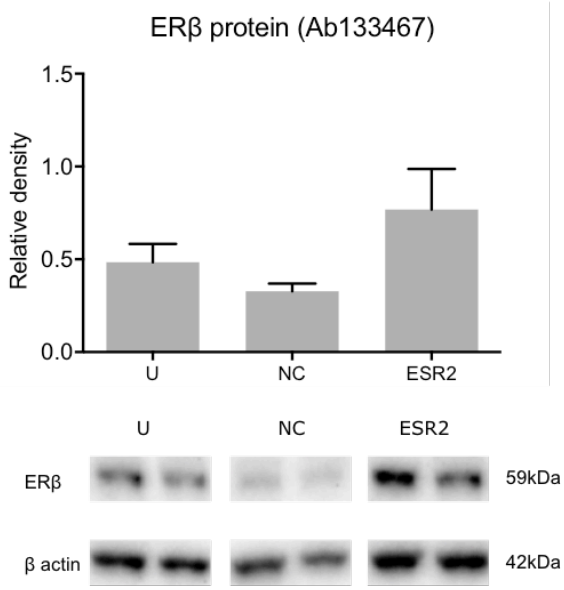


Figure 38. Relative ERβ protein expression using ESR2 siRNA in OE33 cells using antibody ab133467, which still failed to demonstrate ERβ protein knockdown. U: untreated cells. Results are expressed as mean±SD.

3.3.2.2.2.1 ERβ protein expression at different time points

The above experiments on the OE33 cell line failed to demonstrate ERβ protein knockdown in two separate experiments using two different antibodies to ERβ, despite significant ESR2 mRNA knockdown. One possible explanation is that harvesting the protein on day 3 post transfection, may not be the optimal time to detect knockdown, dependent on the ERβ protein turnover. To test this, a further experiment was conducted and the protein harvested daily from day 2 to day 5 post transfection. Eight wells transfected with ESR2 siRNA were prepared, and two samples harvested for protein on each day

WB of the protein harvested from OE33 cells using ab3577 demonstrated a trend towards protein knockdown at day 2, day 3 and day 4, although statistical analysis was not possible as there was only one NC sample, as the other was lost as a result of experimental error (Figure 39).

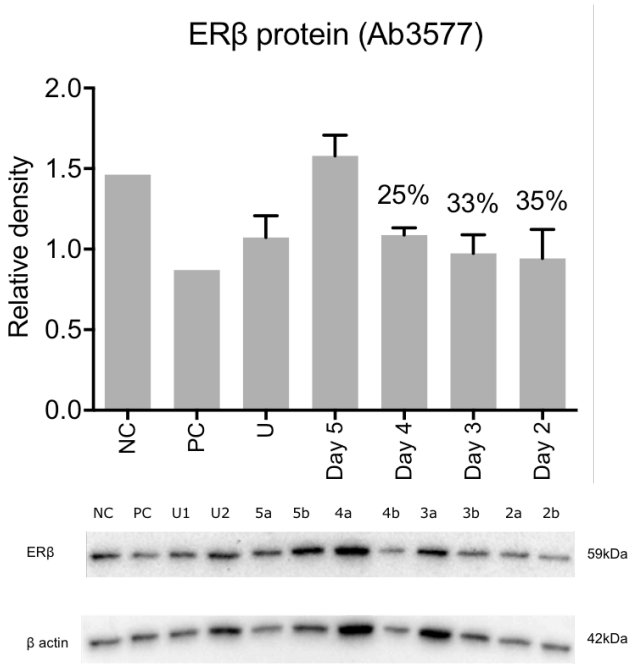


Figure 39. Relative ERβ protein expression (using ab3577) in OE33 cells following 2 – 5 days treatment with ESR2 siRNA. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. U: untreated cells. Results are expressed as mean±SD.

WB of the harvested protein using ab133467 demonstrated a trend towards protein knockdown at day 2, day 3, day 4, and day 5 although statistical analysis was not possible.

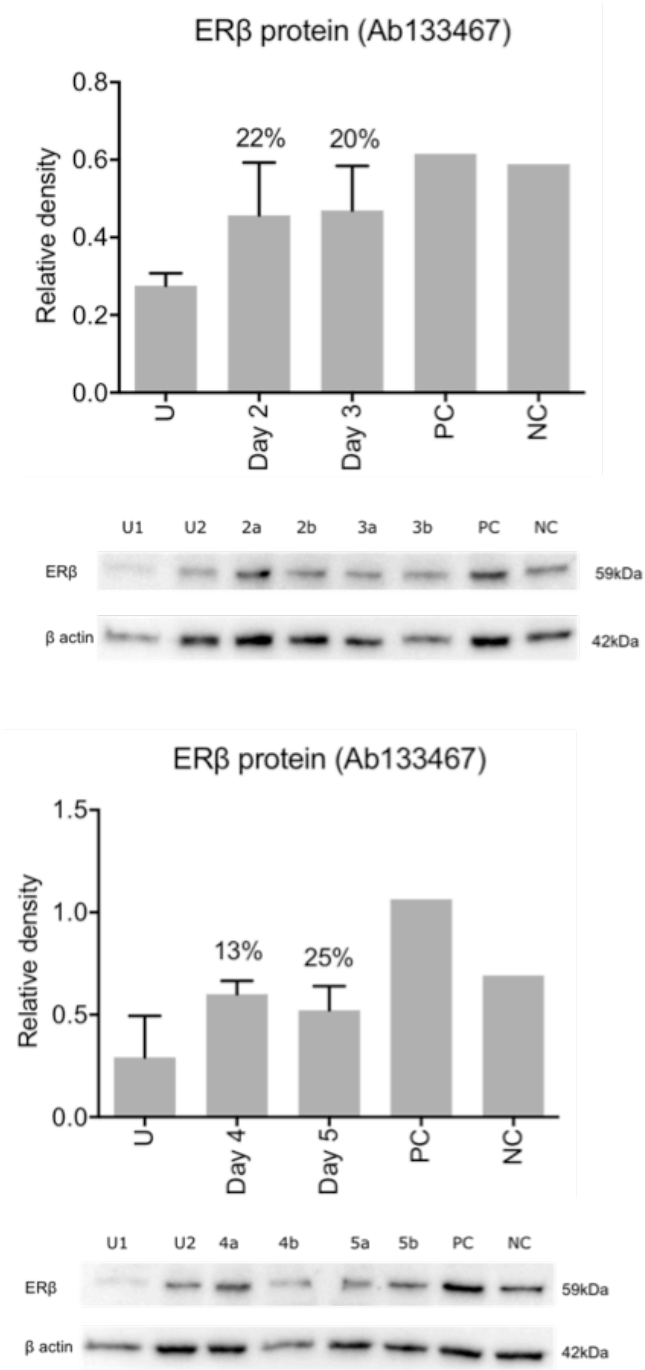


Figure 40. Relative ERβ protein expression (using ab133467) in OE33 cells following 2 – 5 days treatment with ESR2 siRNA. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. U: untreated cells. Results are expressed as mean±SD.

A summary of ER knockdown in OE33 cells is presented in the table below (Table 5).

Table 5. A table summarising each experiment using OE33 cells, showing the mRNA and protein knockdown following treatment with the respective siRNA, expressed as a percentage.

	mRNA % knockdown		**Protein % knockdown**		
Experiment	**ESR1**	**ESR2**	**ERα**	**ERβ (1)**	**ERβ (2)**
Optimisation 1	7.5 (n=2)	52 (n=2)	N/A	N/A	N/A
Optimisation 2	16 (n=3)	40 (n=3)	N/A	N/A	N/A
Experiment 1	0 (n=4)	32 (n=4)	0 (n=4)	18 (n=4)	N/A
Experiment 2	19 (n=2)	60 (n=2)	0 (n=2)	0 (n=2)	0 (n=2)
Experiment 3	N/A	N/A	N/A	23 (n=8)	20 (n=8)
Mean ±SD	9.1 ±9.7	42.9 ±11.5	0	18.4 ±16.9	16.0 ±9.4

3.3.3 Changes in expression of proliferation-associated proteins

To determine the role of the ER subtypes in oesophageal cancer, the effect of ER knockdown on the expression of the following proliferation-associated proteins was examined by WB: Ki67, E-cadherin, Cyclin D and PCNA. Quantification of the of the ER knockdown (mRNA and protein) of the protein used in the following experiments for the MCF7 cell line is available in Table 4 (experiment 2) and for the OE33 cell line in Table 5 (experiment 2).

All antibodies were tested to ensure they produced a strong signal with minimal background at the predicted sizes (Figure A1.9). The Ki67 antibody necessitated additional optimisation because of its high molecular weight; the transfer time was increased (2 hours

instead of 1 hour) and a higher concentration of protein was loaded (20µl instead of 10µl). Ki67 antibody detects a double band of polypeptides at WB (~ 395kD and 345kD) [311] and so both bands were used when calculating protein density. Only up-regulation or down-regulation of >10% for all proliferation-associated proteins are reported to allow ease of interpretation.

3.3.3.1 Positive control cell line (MCF7)

Based on the consensus in the literature on the roles of the individual ER subtypes in breast cancer; ERα being associated with proliferation and ERβ with growth suppression [95, 163, 174], knockdown of ERα in MCF7 cells would be expected to down-regulate Ki67, Cyclin D and PCNA expression and up-regulate E-cadherin expression. Indeed, a study of breast cancer cell lines, ERα knockdown reduced the expression of Cyclin D and PCNA [175]. In contrast, knockdown of ERβ expression would be expected to up-regulate Ki67, Cyclin D and PCNA expression and down-regulate E-cadherin expression; a previous study using breast cancer cell lines did in fact demonstrate reduced E-cadherin expression with ERβ knockdown [312].

There was no significant change in expression in any of the proliferation-associated proteins in ERα or ERβ knocked down MCF7 cells (Figure 41).

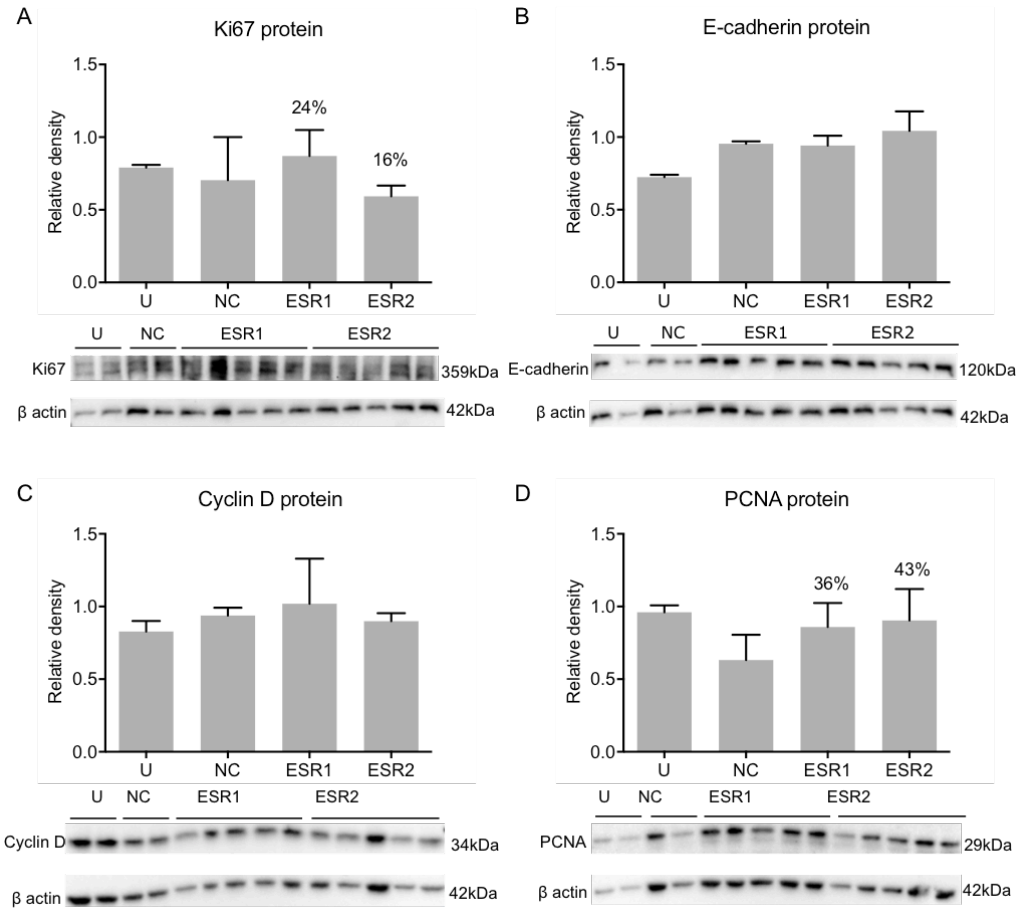


Figure 41. Relative Ki67 (A), E-cadherin (B), Cyclin D (C) and PCNA (D) protein expression using ESR1 and ESR2 siRNA treated protein to knockdown the expression of ERα and ERβ respectively, in MCF7 cells. Knockdown or up-regulation (expressed as a percentage) was calculated relative to negative control (NC) expression. For the second ESR1 treated protein samples, it was not possible to distinguish between the two Ki67 protein bands and it was therefore excluded from the analysis U: untreated cells. There was no significant change in the expression of any of the proliferations-associated proteins (t-test). Results are expressed as mean±SD.

3.3.3.2 OE33 cell line

Cyclin D expression was down regulated in ERβ knocked down cells (46%, p = 0.0461, Figure 42C), however it was of borderline significance. There was no significant change in expression in any of the proliferation-associated proteins in ERα or ERβ knocked down OE33 cells (Figure 42).

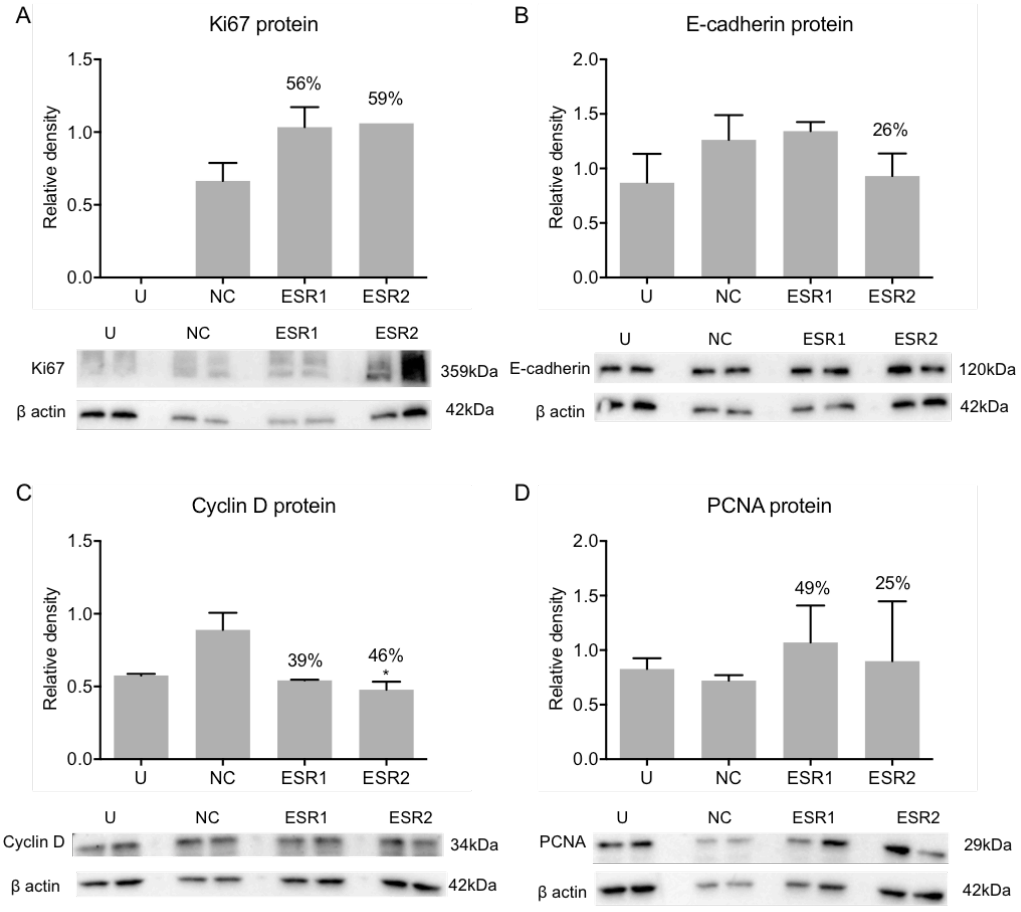


Figure 42. Relative Ki67 (A), E-cadherin (B), Cyclin D (C) and PCNA (D) protein expression using ESR1 and ESR2 siRNA treated protein to knockdown the expression of ERα and ERβ respectively, in OE33 cells. Knockdown or up-regulation (expressed as a percentage) was calculated relative to negative control (NC) expression. For the Ki67 protein, it was not possible to calculate the relative density for the two untreated samples because of failure of protein transfer and the second ESR2 treated sample as it was not possible to distinguish between the protein bands. U: untreated cells. The down-regulation of Cyclin D in cells treated with ESR2 siRNA was statistically significant (*p = 0.0461, t-test). The changes in expression of all other proliferations-associated proteins were not statistically significant (t-test). Results are expressed as mean±SD.

A summary of changes in the expression of proliferation-associated proteins following ERα and ERβ knockdown is presented in the following table (

Table 6).

Table 6. A table summarising the trends in the expression of proliferation-associated proteins with ERα and ERβ knockdown following treatment with ESR1 and ESR1 siRNA. The down-regulation of Cyclin D in cells treated with ESR2 siRNA was of borderline significance (*p = 0.0461, t-test). The changes in expression of all other proliferations-associated proteins were not statistically significant.

	MCF7		**OE33**	
ER knockdown	ERα	ERβ	ERα	ERβ
Proliferation protein				
Ki67	↑	↓	↑	↑
E-cadherin	→	→	→	↓
Cyclin D	→	→	↓	↓*
PCNA	↑	↑	↑	↑

3.4 Discussion

The aim of the research described in this chapter was to manipulate the expression of the two ER subtypes to clarify their role in oesophageal cancer. Firstly, the expression of ERα and ERβ in the OE19 and OE33 cell lines was demonstrated at the mRNA and protein level. As expected, the expression of ESR1 mRNA and ERα protein in the MCF7 cell line was higher than in the OE19 and OE33 cell lines. The expression of ESR2 mRNA and ERβ protein, however, was higher in the OE33 cell line compared to the MCF7 cell line. The MCF7 cell line is known to have a high ERα/ ERβ ratio [313], which may be explained by the known anti-proliferative role of ERβ in breast cancer [95, 163, 174], resulting in loss of expression during establishment of a breast cancer cell lines [314]. The expression of ERα and ERβ protein, detected by WB, in OE33 and OE19 cell lines, has been previously reported by another research group [55] who demonstrated a higher expression of ERα and ERβ protein in the MCF7 cell lines compared to OE19 and OE33. In addition, the expression of ERα and ERβ protein in the OE19 and OE33 cell lines were similar. The different ERβ expression in the MCF7 cell lines between these results and published reports may be because of the use of different sublines, generated by different culture conditions [310].

Secondly, we attempted to knockdown the expression of ERα and ERβ using commercially available ESR1 and ESR2 siRNA. Successful knockdown of ERα and ERβ using siRNA in MCF7 cells has been reported by numerous authors [175, 308, 315]. In the above experiments

on the MCF7 cell line, we achieved significant ESR1 mRNA (61.6% ±18.1) and ERα protein knockdown (39.4% ±9.8), and ESR2 mRNA (54.7% ±11) knockdown, but failed to demonstrate ERβ protein knockdown (Table 4). Regarding the OE33 cell line, despite significant GAPDH mRNA knockdown using the PC siRNA (59.3% ±4.4) and ESR2 mRNA (42.9% ±11.5) knockdown, we failed to demonstrate significant ESR1 mRNA, ERα protein or ERβ protein knockdown. The most plausible reason for failure to achieve ESR1 mRNA and ERα protein knockdown in OE33 cells is due to the low expression of ERα in the OE33 cell line, because knockdown was achieved in the MCF7 cell line, which has a higher ERα expression.

Finally, we attempted to define the individual roles of the ER subtypes in oesophageal cancer by observing the effect of ER subtype knockdown on the expression of proliferation-associated proteins. MCF7 cells were used as the positive control cell line as the roles of the ER subtypes in breast cancer are well described [95, 163, 174]. ERα is associated with proliferation, and knockdown of ERα therefore, should theoretically down-regulate Ki67, Cyclin D [175] and PCNA[175] expression and up-regulate E-cadherin expression. The role of ERβ is the opposite to ERα, and so with ERβ knockdown, Ki67, Cyclin D and PCNA expression should be up-regulated and E-cadherin down-regulated [312]. We failed to demonstrate significant up or down regulation of proliferation-associated proteins in MCF7 cells following ER-subtype knockdown (

Table 6). Other authors have however, reported appropriate up- or down-regulation of the above proliferation-associated proteins in breast cancer cells following ER subtype knockdown; in one study using mammary epithelial cells, ERα knockdown using siRNA, down-regulated Cyclin D and PCNA protein expression assessed by WB [175]. In another study using MCF7 cells, ERα and ERβ knockdown up-regulated and down-regulated E-cadherin protein expression ($p = <0.05$, assessed by WB), respectively [312]. As knockdown in the MCF7 cell line failed to change the expression of the proliferation-associated proteins as expected, it is likely that the ER knockdown using the siRNA was inadequate and is discussed in detail below. For this reason, the results obtained in the OE33 cell cannot be used to draw any valid conclusions. Regardless, other than a borderline significant decrease in expression of Cyclin D following knockdown of ERα and ERβ there was no significant change in the expression of proliferation-associated proteins (
Table 6). Following review of the raw data by the manufacturer, they agreed the siRNA failed to fulfil their performance guarantee that states the SMARTpool siRNA reagent will silence target gene expression by at least 75% at the mRNA level and they subsequently provided a refund. The original aim was to determine the effect of knockdown on the expression of proliferation-associated proteins as described above and also by investigating the effect of knockdown on proliferation directly, using growth assays. However, as the knockdown was inadequate, downstream studies using growth assays were not performed.

Knocking down ERs in OE19 and OE33 cell lines is technically difficult, not only because the ERs are expressed at low levels, but also because ERs are coded by a large number of alternatively spliced transcript variants, making them difficult to target with siRNA [140-142, 151-154]. The complexity of ER expression is further exacerbated by the challenge of detecting the protein products of the splice variants as they give rise to numerous ER isoforms, and because of high sequence homology, it is difficult to identify and distinguish between them; often the transcripts alone, not proteins of the variants are identified [316]. These caveats make targeting knockdown and detecting receptor knockdown difficult [316, 317]. In addition, the RT-PCR primers and antibodies designed to target particular sequences and isoforms will also identify a number of spliced isoforms and variants, without discriminating between or quantifying them [318], potentially 'diluting' any knockdown achieved using the siRNA. There is no sequence information published in the literature or on databases such as the Human Epigenome Browser [319319319319319319319319319319319318318318318317317316316316316316315315315314313313313312] and the Human Protein Atlas [320] on which splice variants or isoforms are expressed in OE19 or OE33 cells. There is only one study on the expression of different ERβ isoforms in oesophageal cancer tissue, which demonstrated expression of isoforms 1, 2, 3 and 5 but failed to demonstrate a prominent isoform [216]. Because the isoforms and splice variants of the ERs expressed in the OE33 cell lines are unknown, SMARTpool siRNA was used which includes 4

target sequences. Four of the ESR2 siRNAs target isoform 1, three target isoform 2, 4, 5, 6 and 9, two target isoform 7 and one of the siRNAs target isoform 3 and 8. Because isoform 3, 7 and 8 are targeted by only one or two siRNAs they may be insufficiently knocked down, if these are the isoforms detected by the RT-PCR primers or antibodies, they may fail to demonstrate effective knockdown, diluting evidence of successful knockdown of the other isoforms. The ESR1 and ESR2 PCR primers used detect all the transcripts targeted by the siRNA. Given that the expression of isoforms and splice variants in the OE33 cell lines is unknown, the PCR primers were also selected as they provide 'best coverage' targeting the maximum number of gene transcripts, the benefit being that they have a higher chance of detecting the presence of the unknown splice variants expressed within the OE33 cell line, but my interpretation is that this also has the disadvantage of 'diluting' the knockdown effect.

Although knockdown of ESR2 mRNA using ESR2 siRNA was achieved in MCF7 and OE33 cells, we failed to demonstrate significant knockdown of the ERβ protein in both cell lines (Table 4 and Table 5). As previously mentioned, there are numerous ERβ protein isoforms coded from different promoters, differential 3' splicing and insertions [151]. The inability to detect ERβ protein knockdown may be due to failure to detect the ERβ isoforms for a variety of reasons; firstly, the ERβ antibody may be detecting a non-specific epitope or be directed to an epitope not present on the isoforms expressed in

the cell line, which is a commonly acknowledged limitation of ERβ antibodies resulting in unreliable quantification of endogenous ERβ protein isoforms in cell lines [313, 321]. Moreover, the antibody may detect an isoform that is not targeted by the ESR2 siRNA; this is unlikely however, because the ERβ antibody ab3577 recognises all ERβ isoforms as it is targeted to the N-terminal, when most isoforms have a deletion in the C-terminal [151, 315]. To address this issue, a second antibody was also used, ab133467, which targets a sequence close to the C-terminal. This antibody only recognises isoforms 1 and 7, and as mentioned previously, 4 siRNAs in the SMARTpool target isoform 1, therefore knockdown of this isoform should not be diluted by detection of the other isoforms, which are not targeted by as many siRNA's. WB using this antibody however, still failed to detect significant ERβ protein knockdown. Another cause for failure to detect ERβ protein knockdown, despite being able to demonstrate mRNA knockdown could be due to differences in receptor turnover; the effect of short-term mRNA knockdown may not be translated into detectable levels of protein knockdown if the protein turnover is long. In an attempt to account for the unknown ERβ protein turnover, OE33 cell lines transfected with ESR2 siRNA were harvested for protein at 2-5 days post transfection, but still failed to demonstrate significant protein knockdown (Figure 39). Lastly, failure to demonstrate ERβ protein knockdown, despite a knockdown in ESR2 mRNA may be because of a lack of protein translation. A large amount of scepticism exists as to whether protein products of the splice variants are translated, because often,

only transcripts, not proteins of the variants are detected [316]. ER expression is controlled by a complex interplay between transcriptional, post-transcriptional and translational regulation. A study investigating the correlation of ERα mRNA expression and protein expression, assessed by RT-PCR and immunohistochemistry, respectively, in 274 breast cancer patients, reported only fair to moderate agreement (estimated using the Kappa coefficient) [322]. Moreover, up to 25% of ESR1 mRNA-positive tumours, lacked immunohistochemical protein staining [322]. Reasons for this discrepancy are failure of mRNA translation to protein due to alternate splicing, alternative polyadenylation, translational silencing, rapid degradation of the protein receptor or post-translational mechanisms. Another study of 167 breast cancer specimens reported ERα protein was closely related to mRNA expression (Chi-square, $P<0.001$), but ERβ protein expression was not related to the corresponding mRNA [323], findings reported by another study of 61 breast cancer specimens [324]. Complex regulatory mechanisms exist for ERs, and regulatory translation mechanisms that alter the efficiency of translation from mRNA to protein are likely to make a significant contribution to ER protein expression. There are countless ways in which the process of translation in this context can be modified; one research group, investigating the discrepancy between ESR2 mRNA and ERβ protein expression in MCF7 cells, reported that differences in the length of the 5' untranslated region of mRNA, which is the key step to initiating protein translation, makes a significant contributing

factor[325] [321]. Differences in the 5' untranslated region of ESR1 mRNA have also been shown to modulate translation efficiency and mRNA turnover, affecting ERα expression [138]. Alternation in the 3' untranslated region of ESR1 has been shown to influence mRNA stability, with destabilising sequences resulting in rapid mRNA degradation [326]. In prostate cancer, it has been proposed that post-transcriptional regulation affecting mRNA structure is thought to account for the discrepancy between ERβ mRNA and protein expression, as the aberrant mRNA structure affects transcript stability and the mechanics of translation [327]. *In-vitro* work on prostate cancer cells also reported that ERβ mRNA translation is controlled primarily by 5' untranslated exons [327].

As far as we are aware this is the first study to attempt ERα and ERβ knockdown in oesophageal cancer cells. There is an abundance of studies in the literature reporting successful ERα and ERβ knockdown in breast cancer cell lines using siRNA [175, 308, 328-331]. Our ability to demonstrate ERα mRNA and protein knockdown in the positive control MCF7 cells is in concordance with other reports published in the literature, including one study which used the same ON-TARGET plus SMART pool siRNA [308]. As well as breast cancer cell lines, there are reports of the use of ESR1 and ESR2 siRNA in other cell lines such as endothelial cell lines [332], and ESR2 siRNA in prostate cancer and lung cancer cell lines [333-335]. The degree of receptor knockdown necessary to provide the desired effect is dependent on numerous factors such as the receptor expression and

turnover, and its role in the pathway being investigated. In a study of breast cancer and prostate cancer cell lines which used the same ESR2 siRNA (Dharmacon's siGENOME SMART pool), ESR2 mRNA expression was knocked down by >85% in both cell lines. The study, however, failed to report whether ERβ protein knockdown was assessed [333]. Another study using prostate cancer cell lines, reported that the ESR2 mRNA was knocked down significantly at 72 hours post transfection (PCR consisted of 35 cycles for ESR2) but the % knockdown was not reported and neither was evidence of protein knockdown [334]. In another study of lung cancer cell lines, the relative expression of ESR2 was reduced by approximately 80% following transfection with ESR2 siRNA. Despite the 80% knockdown, however, no statistically significant effect on proliferation or changes in cell signaling or apoptosis-related molecules was observed. This study also failed to report evidence of ERβ protein knockdown [335]. Of those studies that did report ERβ protein knockdown as a measure of transfection efficiency, the authors failed to quantify the knockdown or report the time the protein was harvested [175, 330, 332, 336, 337]. Moreover, none of the studies reviewed above reported knockdown at both the mRNA and protein level.

Interestingly, one study of ERβ knockdown in MCF7 cells using small hairpin RNA (shRNA) which is used to 'block' gene expression for a longer period, as opposed to gene 'knockdown' with siRNA, experienced the same pitfalls as we encountered; although they

achieved 80% ESR2 mRNA knockdown, with significant effects during downstream studies, they were unable to detect ERβ protein knockdown, despite efforts to optimise the WB protocol and antibodies [338].

As discussed above, low expression of ERβ in oesophageal cancer cells and the unknown expression of ERβ isoforms and spliced variants, makes it difficult to knockdown and to accurately detect receptor knockdown. Other scientific approaches are available to manipulate ERβ expression such as up-regulation using a viral vector [183, 204, 205]. ER up-regulation has been attempted previously in oesophageal squamous cell carcinoma cells (ECGI-10) following transfection with expression vectors, which achieved a significant increase in the respective mRNA assessed by RT-PCR [217].

Other methods could be used to knockdown expression of ERs by the use of shRNA as mentioned previously [338], or the CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas system, which can be used to cut the gene from the genome, completely abolishing transcription of the gene [339]. Other gene delivery methods which are not reliant on cell division could be used, such as nucleofection, which delivers siRNA into the cell using an electrical pulse [340, 341]. However, delivery of the siRNA into the cell does not appear to be the problem during the above experiments, as adequate knockdown of GAPDH with the PC siRNA was achieved and siRNA-mediated knockdown of other target

proteins using lipofectamine transfection reagent have been reported in oesophageal cell lines (TE-1, Eca109 and EC9706) [342]. Another approach to knockdown ER expression in oesophageal cancer cell lines would be to characterise the isoforms and splice variants of the ERs in the OE19 or OE33 cell lines and custom-make siRNA designed to target those variants for use in future studies.

In this study, we were able to demonstrate the expression of ERα and ERβ at the mRNA and protein level in oesophageal cancer cell lines. As far as we are aware, this is the first study to attempt ER knockdown using siRNA. We were able to demonstrate ESR2 mRNA knockdown in OE33 cells but failed to demonstrate significant ERβ protein knockdown. However, unfortunately, we were unable to clarify the role of ERβ in oesophageal cancer cell development as it failed to have any significant effect on the expression of proliferation-associated proteins. Although the data presented in this Chapter does not prove a direct role between ERs and oesophageal cancer, this cannot be excluded given the pitfalls of the scientific technique. To investigate the potential functional interaction between ERs and oesophageal cancer, the next Chapter describes *in-vitro* experiments using the ER ligand, tamoxifen, to determine any effect on oesophageal cancer cell growth.

4 Chapter Four

The effect of tamoxifen on oesophageal cancer cell lines

4.1 Introduction

As shown in Chapter 3, there is compelling evidence that ERs are expressed in oesophageal cancer cell lines. The functional consequence of ER expression in oesophageal cancer remains to be determined. Their presence however, warrants further investigation into whether treatment with ER ligands and/ or SERMs like tamoxifen might be used as a therapeutic tool. Tamoxifen is classified as a SERM, as it has both oestrogenic and anti-oestrogenic actions, depending on the target tissue (Chapter 1, section 4). Tamoxifen behaves as an antagonist on breast tissue and has been used in the treatment for ER positive breast cancer for over 30 years[227]. Tamoxifen has also been used at high doses in clinical trials of other solid tumours such as prostate cancer and lung cancer [235, 244, 246]. To our knowledge, only one research group has published results on the effect of tamoxifen on oesophageal cancer cell lines (OE33 and OE19), where tamoxifen induced apoptosis, increased cell death, reduced the expression of the proliferation marker Ki67 and increased the expression of the cell-adhesion protein, E-cadherin [55, 219]. In addition, work conducted by our research group also showed that tamoxifen and highly selective ER antagonists (MPP and PHTPP), significantly inhibited oesophageal cancer cell proliferation *in-vitro* ($p < 0.05$) [54, 279]. This Chapter aims to take this study further by investigating the effect of tamoxifen on oesophageal cancer cell proliferation and on the expression of the following proliferation associated proteins; Ki67, E-cadherin, Cyclin D and PCNA.

As previously discussed (Chapter 3, section 1), both Ki67 and PCNA are commonly used proliferative markers. Ki67 has been identified as a biomarker to predict neoplastic progression of patients with BM [343-347], and dysplasia, in the context of oesophageal SCC [348, 349]. PCNA has also been identified as a biomarker, able to distinguish dysplasia from invasive oesophageal cancer [350-353]. Moreover, the expression of Ki67 and PCNA have been used to detect response to chemotherapy in patients with oesophageal cancer [354, 355].

Cyclin D is a cell cycle G1 cyclin that forms a complex with cyclin-dependant kinases, thereby facilitating cell cycle progression. Although the precise sequence of molecular events in the development of invasive oesophageal cancer from normal mucosa is not well characterised, dysregulation of cell cycle proteins has been reported. Numerous authors have reported increased Cyclin D expression in oesophageal AC and ACC compared to normal tissue [349, 356-361], and in BM, a Cyclin D positive biopsy is associated with a statistically significantly increased risk of progression to oesophageal AC [362]. Moreover, anti-oestrogens, including tamoxifen, have been shown to reduce Cyclin D expression in breast cancer cells *in-vitro* and down-regulation of Cyclin D is suggested as a key mechanism via which tamoxifen has its anti-proliferative effect in breast cancer [363-366].

E-cadherin is a cell adhesion molecule [294] and loss of expression is implicated in cancer progression, invasion and metastasis [295].

Mutations of the gene encoding E-cadherin have been identified in diffuse gastric carcinoma, lobular breast carcinoma and ovarian carcinoma [367]. In oesophageal cancer, although a common gene mutation has not been identified [368], reduced E-cadherin expression has been reported in both BM, oesophageal AC and SCC [369-371]. Moreover, E-cadherin expression is associated with lymph node metastases and a poor prognosis [372-374]. Tamoxifen has also been shown to increase E-cadherin expression at the mRNA and protein level in breast cancer cells *in-vitro* [375-378].

The effect of tamoxifen on the expression of the proliferation markers, Ki67 and PCNA, will provide further evidence to support the findings from previous studies that tamoxifen has an anti-proliferative effect on oesophageal cancer cells [55, 219]. Both Cyclin D and E-cadherin were selected as suitable biomarkers to assess tamoxifen efficacy in our study as they have been used to assess tamoxifen efficacy in the context of breast cancer previously [363-366, 375-378] and may provide information on the potential mechanism of action of tamoxifen in oesophageal cancer. The aim of this chapter is to determine the effect of tamoxifen on oesophageal cancer cell proliferation *in-vitro* and to identify a potentially suitable biomarker for tamoxifen efficacy in oesophageal cancer, which can be used in *in-vivo* studies.

4.2 Methods

Methodology for cell culture, the BrdU assay, WB, antibodies used and details of statistical analysis are available in Chapter 2.

The MCF7 breast cancer cell line was used as the positive control, as the effect of tamoxifen on breast cancer cell growth is well defined.

4.3 Results

A series of optimisation experiments were undertaken to ensure the optimum cell density for both cell lines, which was 2,000 cells/well of a 6-well plate (see Appendix 2).

The BRdU proliferation assay data presented below is representative of at least two separate experiments.

4.3.1 Effect of different concentrations of tamoxifen on cell growth

The effect of tamoxifen on MCF7 and OE33 cell proliferation was investigated at five different concentrations (1 – 10,000nM). Tamoxifen treatment resulted in a dose-dependent reduction in proliferation for the MCF7 cell line; a concentration of 10nM significantly reduced proliferation ($p=0.0196$), although a concentration of >100nM resulted in a greater reduction compared to untreated cells ($p=0.0005$), Figure 43.

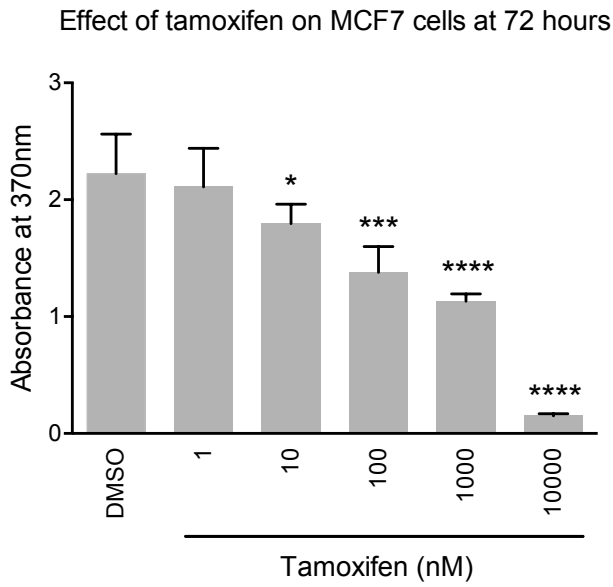


Figure 43. The effect of different tamoxifen concentrations (1–10,000nM) on MCF7 cell proliferation, assessed using the BrdU assay at 72 hours. 10–10,000nM concentrations significantly inhibited proliferation compared to the DMSO control. *p=0.0196, *** p=0.0005, **** p=<0.0001

For the OE33 cell line, a dose-dependent reduction in proliferation with tamoxifen treatment was demonstrated; a concentration of 10nM significantly reduced proliferation (p=0.0002), with a concentration of >100nM resulted in a greater reduction compared to untreated cells (p=<0.0001), Figure 44.

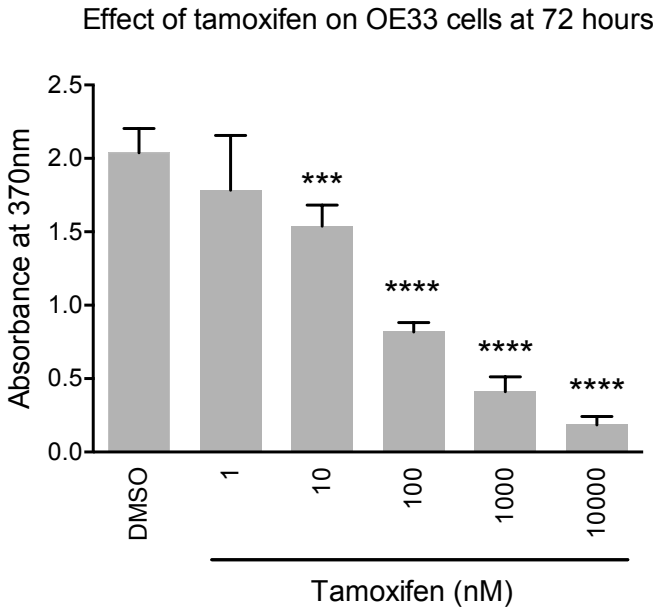


Figure 44. The effect of different tamoxifen concentrations (1–10,000nM) on OE33 cell proliferation, assessed using the BrdU assay at 72 hours. 10–10,000nM concentrations significantly inhibited proliferation compared to the DMSO control. *** p=0.0002, **** p=<0.0001

A tamoxifen concentration of 100nM was selected for future experiments for both cell lines, as it is the lowest concentration that had a highly significant (p=<0.0001) effect on cell proliferation for the OE33 cell line. Moreover, 100nM is the tamoxifen concentration achievable *in-vivo* with standard dose tamoxifen [255-259], which will be the administered dose for further *in-vivo* studies (Chapter 5).

4.3.2 The effect of tamoxifen on the expression of proliferation-associated proteins

To determine the effect of tamoxifen on oesophageal cancer cells, changes in the expression of the following proliferation-associated proteins were investigated by WB: Ki67, PCNA, Cyclin D and E-cadherin.

Details of the cell culture protocol are available in Chapter 2, section 6. Cells were seeded in 6-well plates and harvested on day 3, day 4 and day 5 of tamoxifen treatment for WB analysis. The aim of the initial experiment was to identify the optimal day for harvest, with the intention of repeating the experiment with 3 treated samples to allow for statistical analysis. Cells were treated for 5 days and no longer because the cells would have required trypsinisation and re-seeding to avoid over-confluence, which would have introduced further variability.

All antibodies had previously been tested to ensure they produced a strong signal with minimal background at the predicted sizes. Full images of the blots of the antibodies optimisations in western blots are presented in Appendix 1 Figure A1.11.

4.3.2.1 Positive control cell line (MCF7)

Based on the consensus in the literature on the inhibitory effect of tamoxifen on breast cancer growth, treatment with tamoxifen would be expected to doen-regulate Ki67 and PCNA expression [229-233] and up-regulate Cyclin D expression [363-366]. In these experiments however, tamoxifen failed to significantly change the expression of Ki67, PCNA or Cyclin D. The expression of E-cadherin protein, however, was significantly up-regulated after 4 and 5 days of tamoxifen treatment ($p=0.0022$), consistent with published studies [375-378] (Figure 45).

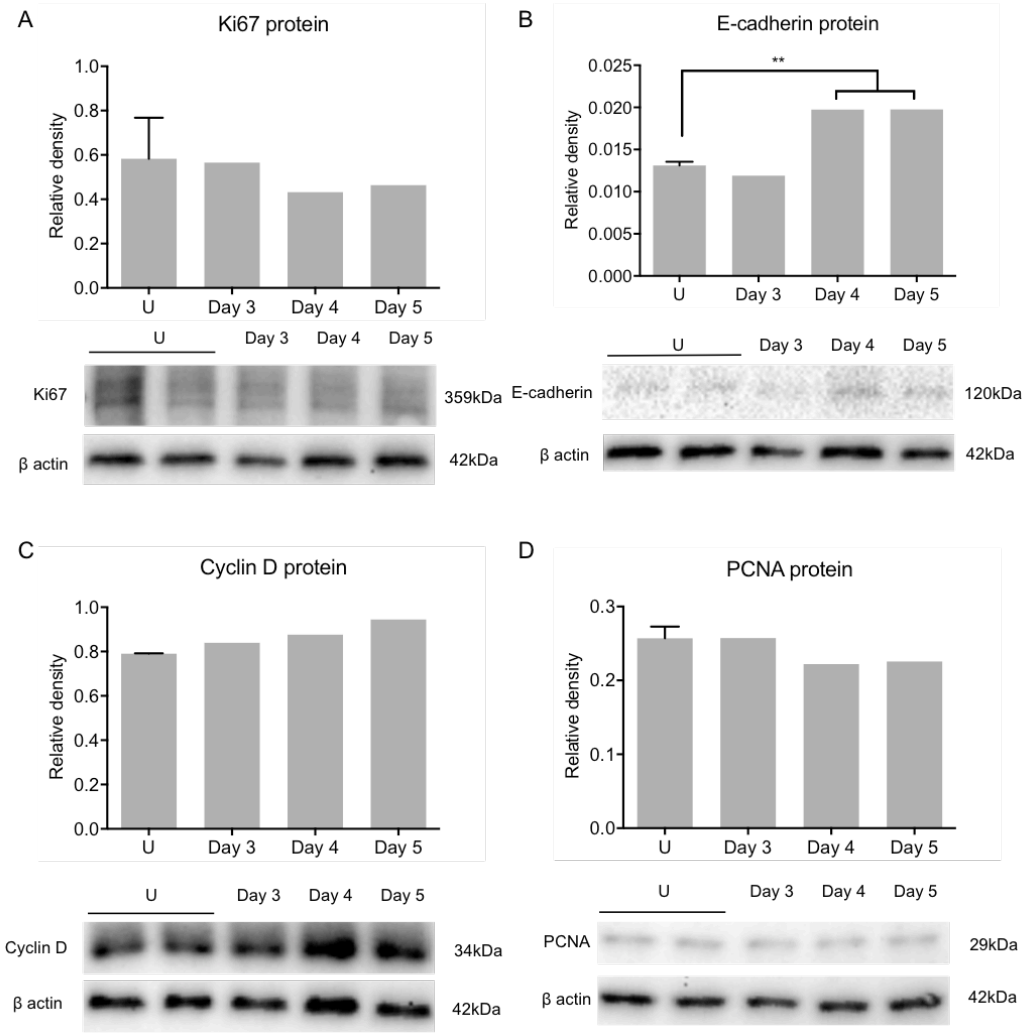


Figure 45. Relative Ki67 (A), E-cadherin (B), Cyclin D (C) and PCNA (D) protein expression following treatment of MCF7 cells with 100nM of tamoxifen for 3, 4 or 5 days. Down or up-regulation was calculated relative to untreated cells (U). In this optimisation experiment, only 1 treatment sample was harvested per day, hence there are no error bars for treated samples. There was no significant change in the expression of Ki67, PCNA or Cyclin D. E-cadherin expression was significantly up-regulated on day 4 and day 5 of tamoxifen treatment p=0.0022 (t-test).

4.3.2.2 OE33 cell line

Tamoxifen treatment of the OE33 cell line failed to demonstrate a significant change in Ki67 expression, unlike the previously published study of tamoxifen treatment of OE33 cell lines, which demonstrated a significant down-regulation in Ki67 protein expression detected by WB [55]. Likewise, treatment with tamoxifen failed to change the expression of PCNA or Cyclin D protein

expression. Treatment with tamoxifen did however, significantly down-regulate the expression of E-cadherin (p=0.0063). Down-regulation of E-cadherin suggests tamoxifen has tumour-promoting effects and is not consistent with previously published work on the OE33 cell line which demonstrated tamoxifen up-regulated the expression of E-cadherin detected by WB [55], Figure 46.

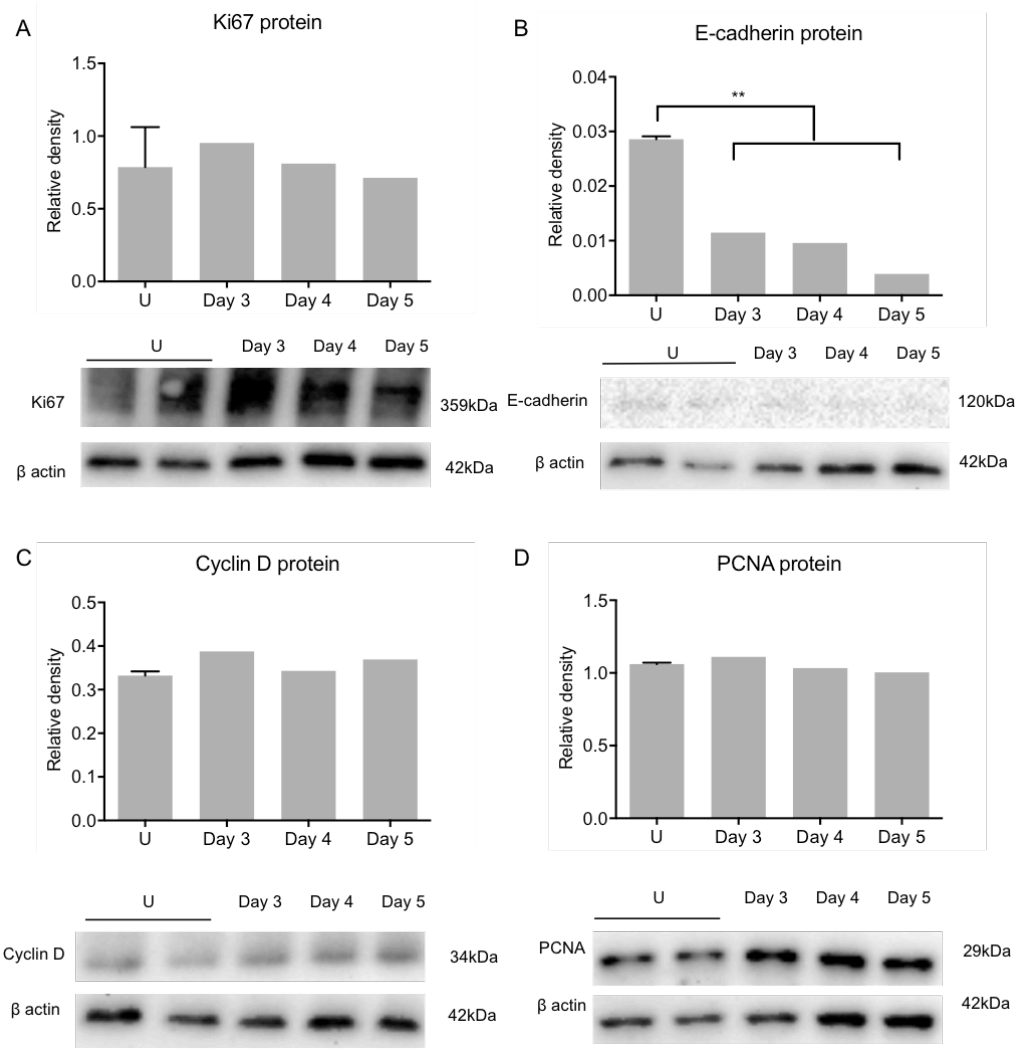


Figure 46. Relative Ki67 (A), E-cadherin (B), Cyclin D (C) and PCNA (D) protein expression following treatment of OE33 cells with 100nM of tamoxifen for 3, 4 or 5 days. Down or up-regulation was calculated relative to untreated cells (U). In this optimisation experiment, only 1 treatment sample was harvested per day, hence there are no error bars for treated samples. E-cadherin expression was significantly down-regulated on day 3, 4 and 5 of tamoxifen treatment p=0.0063 (t-test). There was no change in the expression of the other proliferation-associated proteins

4.3.2.3 E-cadherin expression - repeat

The initial WB of E-cadherin expression produced a blot with poor protein bands. The WB was repeated using the same protein loaded at a higher concentration. For the MCF7 cell line, treatment with tamoxifen failed to significantly change E-cadherin protein expression. The first untreated specimen demonstrated an unusually high E-cadherin expression, which was excluded from the graph. Treatment of OE33 cells with tamoxifen, down-regulated E-cadherin protein expression on days 4 and 5 of treatment, although the down-regulation was not significant (Figure 47).

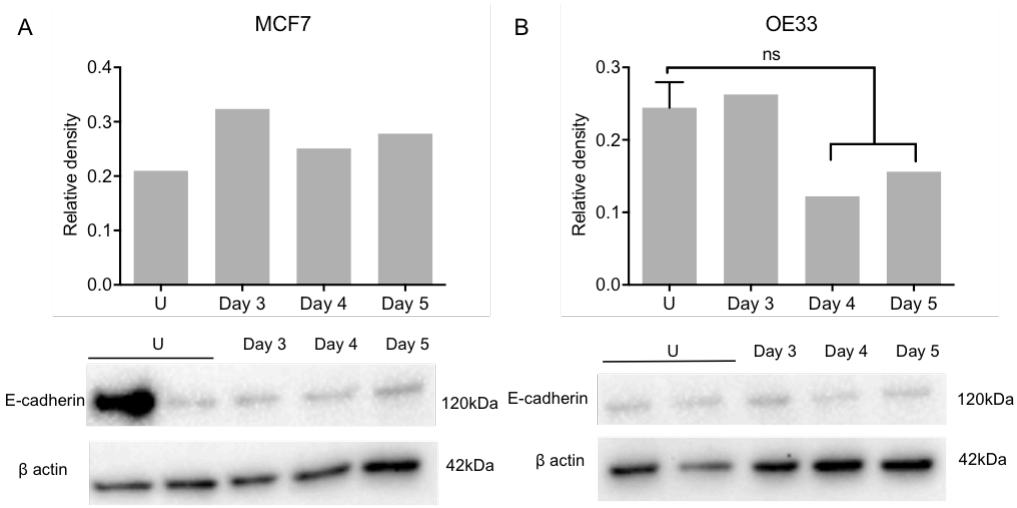


Figure 47. Relative E-cadherin protein expression following treatment of MCF7 and OE33 cells with 100nM of tamoxifen for 3, 4 or 5 days. Down or up-regulation was calculated relative to untreated cells (U). In this optimisation experiment, only 1 treatment sample was harvested per day, hence there are no error bars for treated samples. For the MCF7 cell lines, one untreated sample was excluded as it was considered as not representative. For the MCF7 cell line, treatment with tamoxifen failed to significantly change E-cadherin protein expression. For the OE33 cell line, E-cadherin expression was down-regulated on day 4 and 5 of tamoxifen treatment, although it was not significant (t-test).

4.4 Discussion

The aim of this chapter was to investigate the growth inhibitory effects of tamoxifen on oesophageal cancer cells and to identify a potential biomarker for tamoxifen efficacy in the context of

oesophageal cancer, which can be used for *in-vivo* studies. Tamoxifen inhibited proliferation of the MCF7 and OE33 cell lines, in a dose-dependent manner (Figure 43 and Figure 44). Importantly, a tamoxifen dose of 100nM, the concentration achievable *in-vivo* with standard dose tamoxifen administration, significantly inhibited OE33 cell proliferation ($p=<0.0001$).

Tamoxifen treatment of the positive control cell line, MCF7, significantly up-regulated the expression of E-cadherin as expected but failed to significantly change the expression of Ki67, PCNA or Cyclin D (Figure 45). For the OE33 cell line, tamoxifen treatment failed to change the expression of Ki67, PCNA or Cyclin D (Figure 46). E-cadherin expression, however, was significantly down-regulated with tamoxifen treatment, although a significant down-regulation was not reproducible (Figure 47). E-cadherin down-regulation suggests tamoxifen has cancer-promoting effects on oesophageal cancer cells which is contradictory to the previous study on OE33 cell lines, wherein 14 days of 10nM tamoxifen up-regulated E-cadherin expression [55]. This study, however, was performed in a different laboratory with OE33 cells cultured under different conditions. In the current study, the western blots of two separate experiments were poor (Figure 45, Figure 46 and Figure 47), and the protein bands were not clear enough to allow reliable and accurate density measurements. The E-cadherin antibody had been optimised and used in previous experiments (Appendix 1 Figure 1.11, Chapter 3) producing clean blots, and so the antibody

may have degraded with repeated freeze/thaw cycles. Loss of E-cadherin expression with tamoxifen treatment has been demonstrated in tamoxifen-resistant MCF7 cells compared to endocrine-responsive MCF7 cells, suggesting loss of E-cadherin expression plays a significant role in the development of tamoxifen resistance in breast cancer [379, 380]. Tamoxifen resistance is unlikely to account for E-cadherin down-regulation in this study as the OE33 cells used had only been exposed to tamoxifen treatment for the purpose of this experiment (3 days). In this study, the proliferation assays did demonstrate a decrease in OE33 cell growth upon tamoxifen treatment, demonstrating tamoxifen has an overall inhibitory effect. The growth inhibitory mechanism of action of tamoxifen in these experimental culture conditions may not be dependent on E-cadherin expression or the unexpected changes in E-cadherin expression may be due to technical failure such as antibody degradation. To clarify this further, experiments using a new aliquot of the same antibody could be performed, and/ or changes in the expression of E-cadherin mRNA transcripts detected by RT-PCR could be investigated. In addition, given the limitation of immortalised cell lines, future studies on primary oesophageal cells could be performed to further dissect the molecular mechanism of action.

There are a number of potential reasons for failure to demonstrate a change in proliferation-associated proteins that supports the hypothesis that tamoxifen inhibits oesophageal cancer cell growth. Firstly, pure tamoxifen was used in this study, rather than a

metabolite of tamoxifen, such as 4-Hydroxytamoxifen, which is more potent (Chapter 1, section 4.1). Both tamoxifen and its metabolites are often used in *in-vitro* studies and there is no evidence that *in-vitro* 4-Hydroxytamoxifen treatment is significantly more efficacious than tamoxifen for short-term treatment (<7 days) [381, 382]. Although, it is argued that as tamoxifen is metabolised by mitochondrial P450 enzymes, the use of 4-Hydroxytamoxifen allows for more reliable dosing [219].

Secondly, a tamoxifen concentration of 100nM for 5 days may not have been sufficient to result in a measurable change in expression of the proliferation-associated proteins. A dose of 100nM was selected because it is achievable *in-vivo* with standard dose tamoxifen and the growth proliferation assay demonstrated a concentration of 100nM significantly inhibited proliferation compared to untreated cells (p=<0.0001, Figure 44). Previous studies reported in the literature demonstrated growth inhibition of OE33 cells following treatment with 14 days of 10nM tamoxifen [55] and 48 hours of 7,200nM 4-Hydroxytamoxifen [219]. Moreover, work conducted by a predecessor in the same laboratory on the OE33 cell line, demonstrated a significant reduction in cell proliferation with a wide range of tamoxifen concentrations (10 – 100,000nM) at 72 hours (p<0.005), assessed using the MTS proliferation assay [54]. There are numerous complex methodologies available to calculate the drug *in-vitro – in-vivo* correlation based on the *in-vitro* drug dissolution rate and the *in-vivo* bioavailability [383, 384]. In the case of

tamoxifen, there is tremendous interpatient variability in drug concentrations achieved *in-vivo* [248, 385] and therefore, another approach may be used to calculate the most suitable *in-vitro* drug testing concentration such as the half maximal effective concentration (EC_{50}) which refers to the concentration where 50% of the maximal effect is observed. The calculated EC_{50} values in this study for the OE33 and MCF7 cell lines were 1nM and 0.99nM, respectively (Appendix 2 Figure A2.9 and A2.10).

Finally, the technique of WB used to detect changes is the expression of proliferation-associated proteins may not be sensitive enough, especially for the larger proteins (>80kDa) as they do not resolve well and exhibit poor electrotransfer [311, 386]. Ki67 is a large protein (395kDa and 345kDa) and the WB protocol using the Ki67 antibody was therefore optimised to account for this by using a gradient (4-15%) acrylamide gel and an increased transfer time (Chapter 3, section 3.3.3). Use of the gradient gel still did not allow for adequate separation of the protein bands, whilst ensuring the β-actin control remained on the gel, and increased transfer time had the drawback of causing the gel to stick to the membrane. In hindsight, Ki67 protein expression would be more appropriately investigated by immunohistochemistry, which is considered the gold standard [387, 388]; this would have the additional advantage of being more readily transferrable to the assessment of tamoxifen *in-vivo,* as Ki67 immunohistochemical analysis of human tissue samples is routinely carried out in clinical pathology laboratories [389, 390].

This *in-vitro* study on oesophageal cancer cells demonstrated that tamoxifen inhibits cell growth in a dose dependant manner, supporting the findings of previous authors [54, 55, 219]. The mechanism of action of tamoxifen is complex and can be either ER-mediated or ER independent, and its effect on cell growth is determined by the expression of coregulatory proteins, ER subtypes and isoforms in the target tissue (Introduction, section 1.4.1.2). The mechanism via which tamoxifen exerts its anti-proliferative effect in the context of oesophageal cancer is still unknown and unfortunately, this study failed to demonstrate changes in selected proliferation-associated proteins. Nevertheless, the data does demonstrate, along with findings from other authors [54, 55, 219], that oesophageal cancer cell proliferation is suppressed with tamoxifen treatment and as tamoxifen is a safe and well tolerated drug used in the treatment of breast cancer, a pilot study of tamoxifen in patients with oesophageal cancer is warranted and is reported in Chapter 5.

5 Chapter Five

A pilot study of tamoxifen in oesophageal cancer

5.1 Introduction

Oesophageal cancer is the 8th most common cancer worldwide, and the UK has the world's highest incidence of oesophageal adenocarcinoma [4]. The 5-year survival for patients diagnosed with oesophageal cancer during 2010-2011 in England and Wales was 15% [2]. Only one-third of patients are amenable to curative treatment at presentation, with the remaining receiving palliative treatment, or best supportive care [391].

In the UK, the management of patients diagnosed with oesophageal cancer is discussed at a multidisciplinary team (MDT) meeting, where a collective decision is made on the most suitable treatment option; either potentially curative or palliative treatment. The decision takes into consideration disease extent (cancer stage) and patient factors (e.g. fitness, comorbidities, patient preference). Potentially curative treatment includes surgical resection (with or without neoadjuvant chemotherapy), radical radiotherapy/ chemoradiotherapy or endoscopic interventions (endoscopic mucosal resection or radiofrequency ablation for intramucosal cancers). Palliative treatment consists of palliative chemotherapy, palliative radiotherapy, or endoscopic interventions such as stenting, argon beam coagulation, laser therapy and brachytherapy [63]. The principle aim of palliative treatment is to maintain quality of life with pain and symptom control. The UK National Oesophago-Gastric Cancer Audit, which includes over 21,000 patients diagnosed between April 2011 and March 2013, found that 37% of patients underwent potentially

curative treatment, 43% underwent palliative treatment and 20% underwent no active treatment/ best supportive care [391]. Palliative chemotherapy was the most utilised palliative treatment option for 50% of patients; of these patients, 50% completed the course of chemotherapy, 18% developed progressive disease, 13% discontinued treatment because of toxicity, 12% died during treatment, and 8% of patients withdrew from treatment [392]. Older patients and those with poorer performance status were the least likely to complete treatment [392]. This study focuses on patients who are not candidates for potentially curative management, in the hope that tamoxifen may offer an effective and tolerable treatment option for the future.

We have demonstrated that the treatment of oesophageal cancer cell lines with tamoxifen inhibits oesophageal cancer cell proliferation *in-vitro* (Chapter 4); findings consistent with reports published by other research groups [54, 55, 219]. To date, there are no published studies of tamoxifen in patients with oesophageal cancer. We designed a pilot study to determine the biological effect of short-term tamoxifen on oesophageal cancer *in-vivo.* The biological response to treatment was assessed by measuring the immunohistochemical expression of Ki67, a surrogate marker of proliferation [291], chosen as it has been used as a marker of tamoxifen efficacy in numerous *in-vivo* studies [317, 393-409].

5.1.1 Study aims and objectives

The aims of this pilot study are two-fold: (1) to determine the effect of tamoxifen on oesophageal cancer cell growth *in-vivo* using Ki67 expression as a biological endpoint, and (2) to assess the feasibility of the study protocol to determine if a larger clinical trial would be both achievable and warranted.

The primary objective was to observe the effect of tamoxifen on Ki67 expression by comparing the pre and post tamoxifen biopsy specimens. The secondary objective was to observe the effect of tamoxifen on ERα an ERβ expression and to determine if there is a correlation between Ki67 response and ER expression.

5.2 Methods

5.2.1 Study protocol

I researched and designed the pilot study; the rationale for the study design is described in the study protocol. The study protocol was approved by the National Research and Ethics Committee (REC) East Midlands - Nottingham 2, in May 2015 (reference: 15/EM/0225), and by the NHS Research and Development department, Royal Derby Hospital, in November 2015 (Appendix 3).

In summary, this was a single arm, feasibility study in which patients with oesophageal cancer received tamoxifen tablets or tamoxifen liquid (80mg for the first 4 days and 20mg thereafter) daily for 4 weeks. Oesophageal cancer biopsies were taken before and after treatment via endoscopy using standard-sized biopsy forceps (2.2mm outer diameter). Patients with oesophageal cancer who were not candidates for curative management or further active palliative treatment were eligible for inclusion. A detailed list of the inclusion and exclusion criteria is summarised in Table 7.

Table 7. Study inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
- Patients with oesophageal cancer who are not candidates for surgical resection or further chemotherapy. - Men aged 18 - 95. - Women aged 55 – 95. - The patient agrees and is able to return to the same research facility for the gastroscopy. - The patient has been informed of the nature of the study, agrees to its provisions, and has provided written informed consent as approved by the Institutional Review Board/Ethics Committee (IRB/EC) of the respective clinical site.	- Chemotherapy within 4 weeks of entry into the trial. - Life expectancy <4 weeks - Concurrent hormonal therapy e.g. Any SERMs, Hormone Replacement Therapy, Oral Contraceptive, or within 4 weeks of entry into the trial. - Concurrent warfarin treatment. - Concurrent aminoglutethimide, and droperidol drugs. - Previous thromboembolic disease (DVT/ PE/ stroke). - A positive family history (first-degree relative) of confirmed idiopathic venous thromboembolic events (DVT/ PE). - Bleeding diathesis or thrombocytopenia. - Women of childbearing age/ pre-menopause (<55 years of age). - Any other medical condition, which in the opinion of the investigator should be excluded from the study.

5.2.1.1 Study amendments

During the study, two amendments were approved by the REC committee to aid recruitment: (1) allowing a pre tamoxifen biopsy to be taken if there was no suitable biopsy stored in the pathology department, and (2) allowing recruitment of patients taking any anticoagulation therapy, other than warfarin.

5.2.2 Immunohistochemistry

Preparation and staining of the biopsy samples were undertaken by the Pathology department at the Royal Derby Hospital, according to their protocol used for routine clinical practice, which is regulated by the UK National External Quality Assessment Scheme for immunohistochemistry. The specimens were fixed in formaldehyde, processed into paraffin blocks and cut into 4μm thick sections. Routine H&E staining was performed and the slides reviewed by the

trial pathologist, Dr Rahul Deb, to identify the most representative area of tumour. The sections were then stained for Ki67, and ERα and ERβ expression using the Leica BOND automated machine. Details of the antibodies used and dilutions are available in Table 8. The Ki67 antibody is routinely used in the pathology laboratory for prognostic scoring of lymphoma, carcinoid, and multiple myeloma, and the ERα antibody is used in breast cancer. The ERβ antibody was purchased specifically for the study and optimised using breast cancer tissue and different dilutions and antigen-retrieval times. Negative control (without primary antibody) and positive control tissues were included in all experiments to ensure the quality of staining. Positive control tissue for the respective antibody was present on each histology slide as an internal control (Table 8).

Table 8. Primary antibodies used for immunohistochemistry and the corresponding positive control tissue.

Antibody	**Dilution**	**Source (Cat. No.)**	**Positive control**
Ki67 (MIB-1 clone)	1:200	Dako (M7240)	Tonsil tissue
ERα (SP-1 clone)	Pre-diluted	Ventana, Roche (790-4325)	ERα positive breast tissue scoring negative, moderate and strong
ERβ (PPG5/10 clone)	1:20	Dako (M7292)	Breast tissue

5.2.2.1 Biomarker scoring

All slides were examined and scored together by Dr Rahul Deb and I, using a standard light microscope with a dual viewing attachment (Nikon Eclipse 80i). All samples were anonymised and selected at random for scoring. Assessment was carried out on the entire tumour represented in the section with a minimum of 1 high power field of tumour. Only strong, distinct nuclear staining of invasive tumour cells was considered as positive. Ki67 scoring was quantified

using the Labeling Index, which is the percentage of positively stained tumour cells among the total number of tumour cells present; the percentage was derived by estimation and was not counted manually.

ER expression was scored using the Allred scoring system, which involves combining scores of the proportion of positive cells (1-5) and the intensity of staining (1-3), to give a maximum score of 8 [410, 411]. The proportion score represents the percentage of positive staining tumour cell nuclei (0 = none; 1 = <1%, 2 = 1-10%, 3 = 11-33%, 4 = 34-66%, 5 = >67%). The intensity score represents the estimated average staining intensity of positive tumour cell nuclei (0 = none, 1 = weak, 2 = intermediate, 3 = strong).

5.2.3 Statistical analysis

The power calculation was performed with the help of Dr Andrea Venn (Honorary Associate Professor, School of Medicine, University of Nottingham) using nQuery v4.0 software; 20 patients were required to detect a change in mean Ki67 level of 10%, with 90% power and a two-sided 0.05 significance level, within a SD of 12.9. The study was powered to detect a change in mean Ki67 expression of 10%, based on previously published studies [400, 407]. The larger SD was chosen to be more cautious. Statistical analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). The results were parametrically distributed and therefore expressed as mean±SD. The paired t-test (two-sided) was used for

comparative analysis of paired samples and Pearson's correlation co-efficient was used to measure the correlation between the change in Ki67 and ERβ expression. Only patients who received the full course of tamoxifen treatment and completed the trial by having a post tamoxifen biopsy were included in the analysis.

5.3 Results

One of the aims of the pilot study was to assess feasibility of the study protocol, to allow for a larger clinical trial. The outcome of patient identification and recruitment is described below.

5.3.1 Patient identification and screening

Patients suitable for inclusion were identified from the following different sources to maximise recruitment:

- **Weekly upper GI cancer MDT meeting** - A total of 19 meetings were attended during a 6-month recruitment period.
- **Outpatient clinics** - The upper gastro-intestinal surgical cancer, oncology and palliative care outpatient clinic lists were reviewed for potential patients using iSOFT Clinical Management System; a total of 152 clinic lists were reviewed. Oesophageal cancer patients were screened for eligibility by reviewing clinic letters and test results.
- **Upper GI cancer database** - The database was provided by the hospital cancer care audit department and includes all new cancer diagnoses. The database consisted of 568 patients diagnosed with upper GI cancer between 2012 and 2016. Oesophageal cancer patients were screened for eligibility, which identified an additional 21 eligible patients.

5.3.2 Patient recruitment

Patients were recruited between December 2015 and June 2016; 67 eligible patients were identified from the sources described above. 38 patients were seen in the outpatient department, 28 were contacted over the telephone, and 1 patient was on the hospital ward during an inpatient admission. I verbally introduced the study to all eligible patients and provided the Patient Information Leaflet (Appendix 3). 54 patients refused participation and 13 patients consented for recruitment into the study; 1 patient subsequently withdrew consent prior to the initial biopsy, 2 patients died during treatment and a further 2 patients were excluded due to lack of cancer in the pre tamoxifen biopsy (Figure 48). Of the patients who did not have cancer in the pre tamoxifen biopsy, 1 patient had undergone radical chemoradiotherapy and in response to this, the study protocol was amended to exclude patients who had undergone curative radical chemoradiotherapy (study number: 03). 4 of the 13 recruited patients died during or within 8 weeks of the study end date. The median number of days after the second tamoxifen biopsy to death was 37.5 days (range: 21 - 175). Of those who completed the trial, the median duration of tamoxifen treatment was 30 days (range: 28 - 45 days). Two of the patients (02, 07) were commenced on liquid tamoxifen due to dysphagia. Incidentally, both of these patients died before completion of the study and so were excluded from the analysis. Tamoxifen treatment was well tolerated and none of the patients reported side effects. Compliance was reported as 100%.

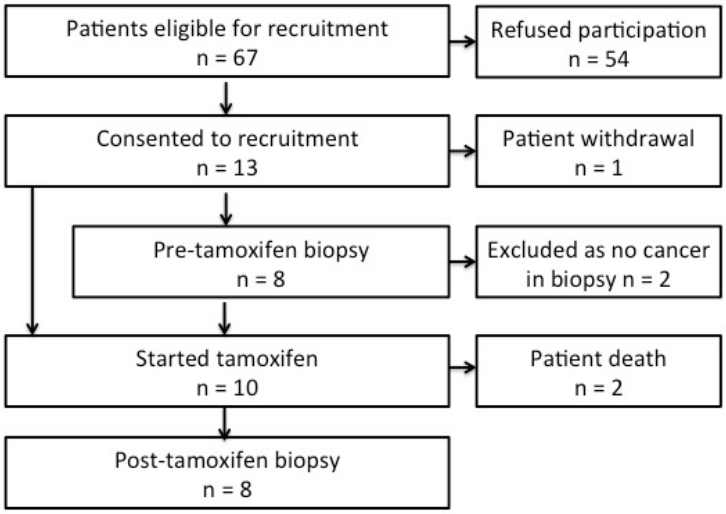


Figure 48. Patient recruitment flow-chart

5.3.2.1 Patient characteristics

Of the 13 patients who consented to the study, 10 were male and 3 were female. 11 patients had adenocarcinoma (AC) and 2 patients had squamous cell carcinoma (SCC). The median age was 82 years, with a range of 61–94 years (mean: 79 years, Table 9).

Table 9. Demographic characteristics of the study participants and treatment received prior to study enrollment. AC: adenocarcinoma, SCC: squamous cell carcinoma. Death is recorded within 8 weeks of the study end date. * denotes patients excluded from statistical analysis as they did not complete study.

Patient number	Sex	Age	Histology	Treatment	Death
01*	M	76	AC	Palliative chemotherapy	N
02*	M	78	AC	None	Y
03*	M	60	AC	Radical chemoradiotherapy	N
04	M	86	AC	None	Y
05	M	94	AC	Palliative Radiotherapy	N
06	F	82	SCC	Palliative Radiotherapy	N
07*	M	79	AC	None	Y
08	M	61	AC	Palliative chemotherapy	N
09*	F	80	AC	Palliative chemotherapy	N
10	F	69	SCC	Palliative chemoradiotherapy	N
11	M	69	AC	Palliative chemotherapy	Y
12	M	87	AC	None	N
13	M	81	AC	None	N

5.3.3 Immunohistochemistry results

5.3.3.1 Ki67 expression

The mean Ki67 Labelling Index in the pre tamoxifen biopsies was 65%±29.2 and was not significantly different to the post tamoxifen biopsy scores (mean: 65.63%±31.9). Of the paired samples, the mean expression increased by 0.625 but this was not significant ($p = 0.962$), the 95% CI (confidence interval) ranged from -29.3 to 30.55 (Figure 49). The expression of Ki67 decreased as expected in 3 cases, 2 of which were female with SCC (patient number 06 and

10, depicted in red on the graph, Figure 49). The third patient who demonstrated a reduction in Ki67 was male with AC (patient number 08). Representative samples of paired pre and post tamoxifen biopsies are available in

Figure 50. In all tumours, Ki67 staining was nuclear and confined to tumour cells with lack of staining in adjacent stromal cells.

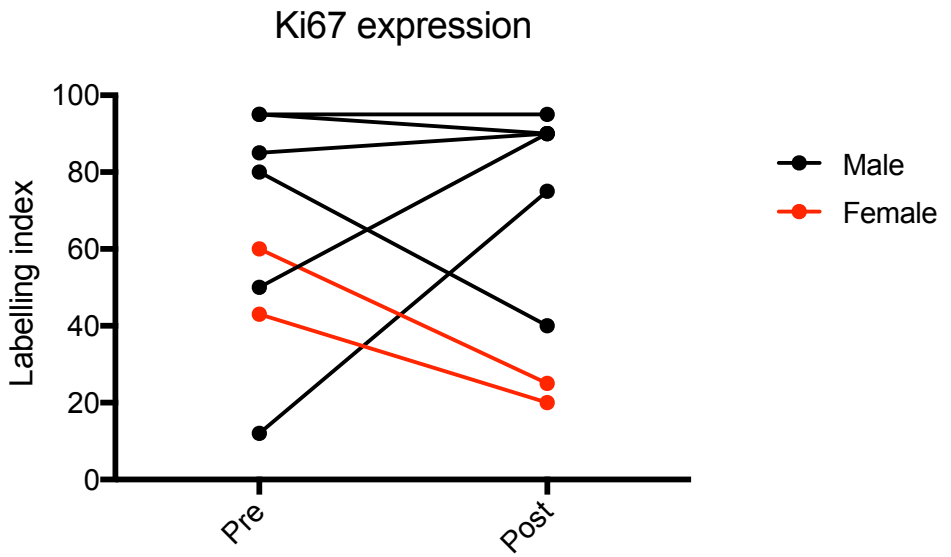


Figure 49. The difference in Ki67 expression between paired oesophageal cancer biopsies taken pre and post tamoxifen, which was not statistically significant (p = 0.962, paired t-test).

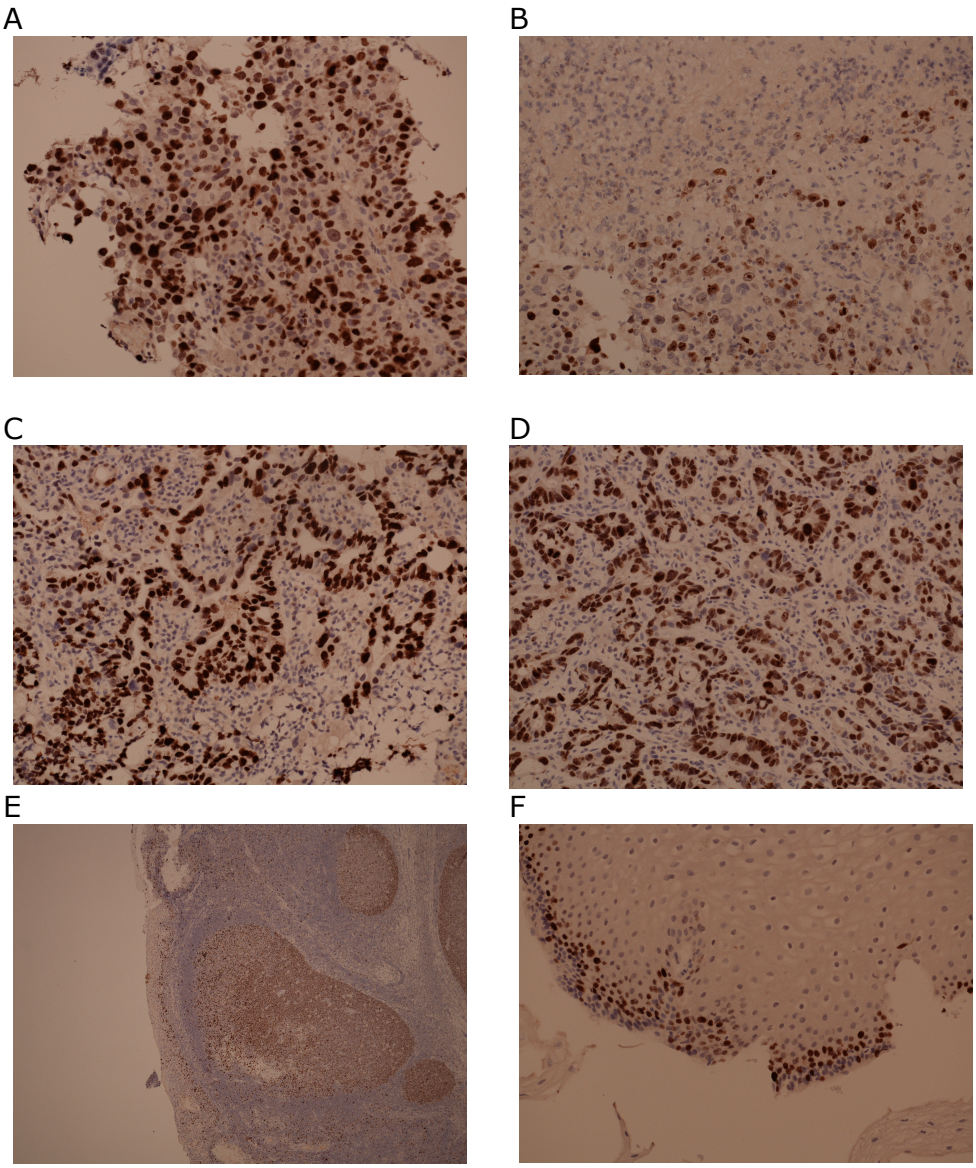


Figure 50. Ki67 expression in: A) Pre tamoxifen biopsy for patient 08 which scored 80%, B) Post tamoxifen biopsy for patient 08 which scored 40%, C) Pre tamoxifen biopsy for patient 04 which scored 95%, D) Post tamoxifen biopsy for patient 04 which scored 95%, E) Positive control tonsillar tissue showing positive and graduated staining in the lymphoid follicle, F) Pre tamoxifen biopsy for patient 03, which failed to show any evidence of cancer in the specimen and Ki67 expression in the mucosal cells only. All images are 200X magnification except the positive control (E), which is 40X.

Two of the cases demonstrated an increase in Ki67 following tamoxifen treatment. These histology slides were re-scored 4 weeks later and there was so significant difference in the score assigned,

demonstrating reproducibility (Figure 51). For both cases, the pre tamoxifen biopsy tissue demonstrated variable staining, with some high/ low scoring areas. The post tamoxifen biopsy volumes for these two cases were small, and therefore, if a high-staining area had been biopsied this may account for the increased Ki67 expression.

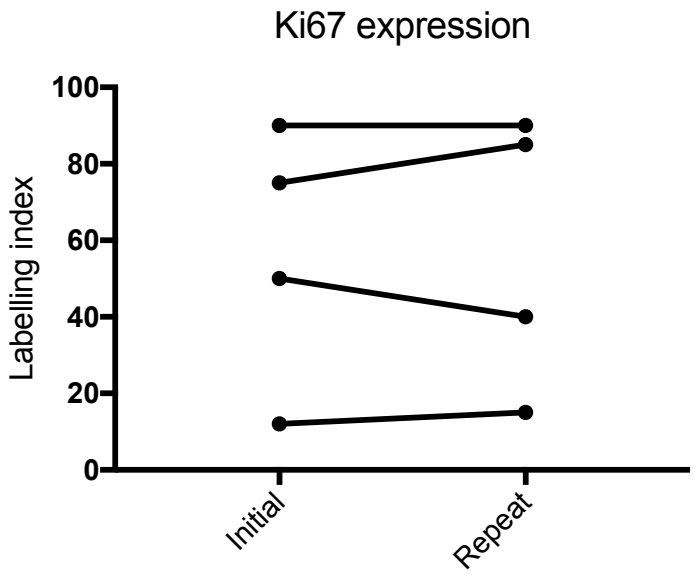


Figure 51. Ki67 Labelling Index scores of the two patients who demonstrated an increase in expression following treatment. The initial scoring was repeated and there was no significant difference between the initial and repeated scoring (p = 0.962, paired t-test).

5.3.3.2 ERα expression

ERα expression was negative for all pre and post tamoxifen biopsies, despite positive scoring of the control breast tissue, which was present on each histology slide (Figure 52).

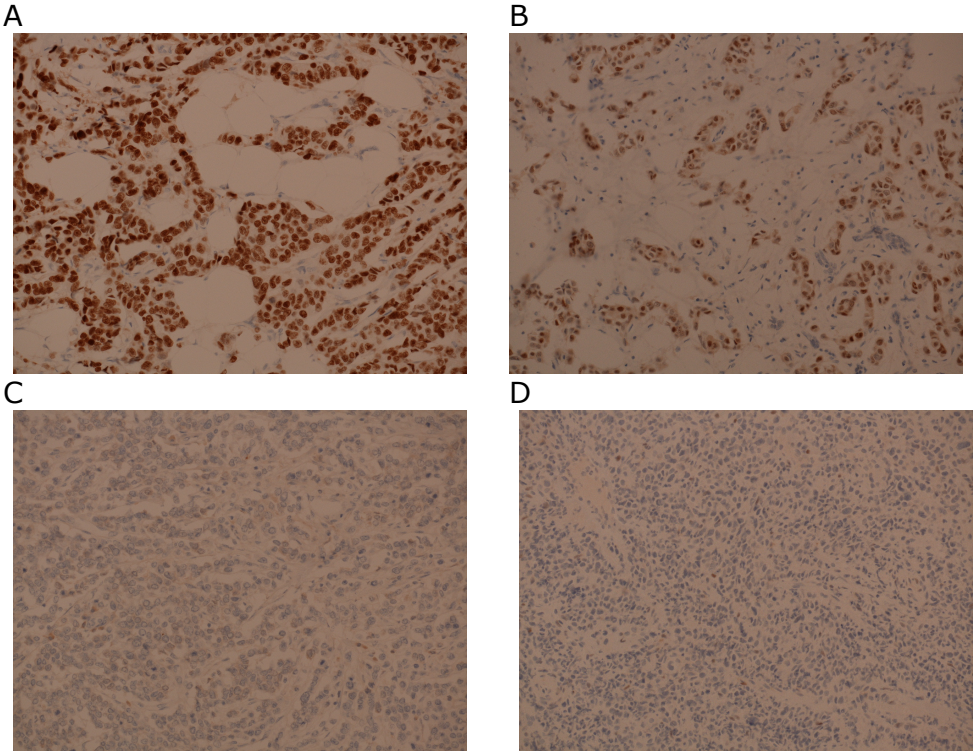


Figure 52. ERα expression in: A) Breast tissue control - strong immunoreactivity, B) Breast tissue control - medium immunoreactivity, C) Breast tissue control – negative, D) Oesophageal cancer tissue (patient 08), negative. All images are 200X magnification.

5.3.3.3 ERβ expression

ERβ expression was positive for all oesophageal cancer biopsies. The mean ERβ expression in the pre tamoxifen biopsies was 6.25±1.28 and was not significantly different to the corresponding post tamoxifen biopsies (7±1.31, p=0.0796). Of the paired biopsies, the mean expression increased by 0.75 and the 95% CI ranged from -0.12 to 1.62 (Figure 53). The pattern of immunohistochemical staining for both high and low Allred scores are demonstrated in Figure 54. ERβ was also expressed in the normal oesophageal cancer tissue if present in the tumour specimen, although the expression was not scored.

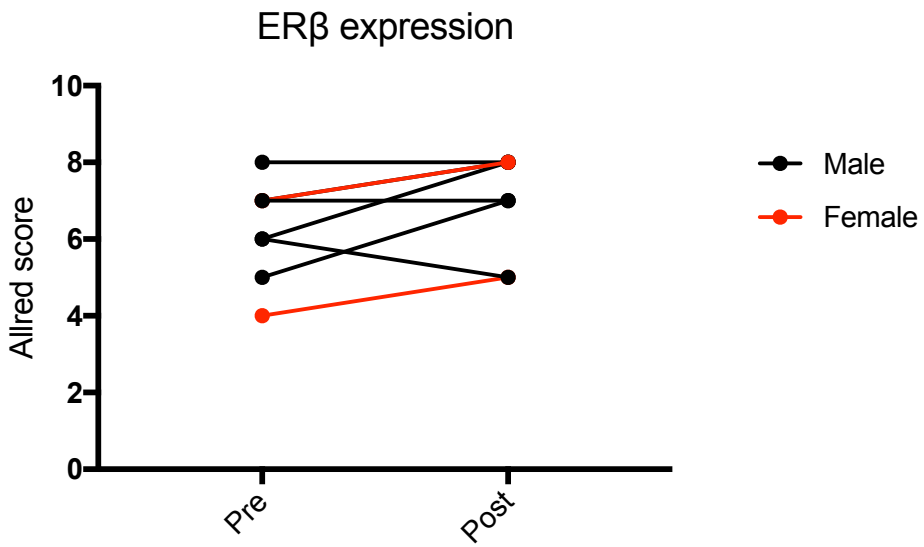


Figure 53. The difference in ERβ expression between paired oesophageal cancer biopsies taken pre and post tamoxifen, which was not statistically significant (p = 0.0796, paired t-test).

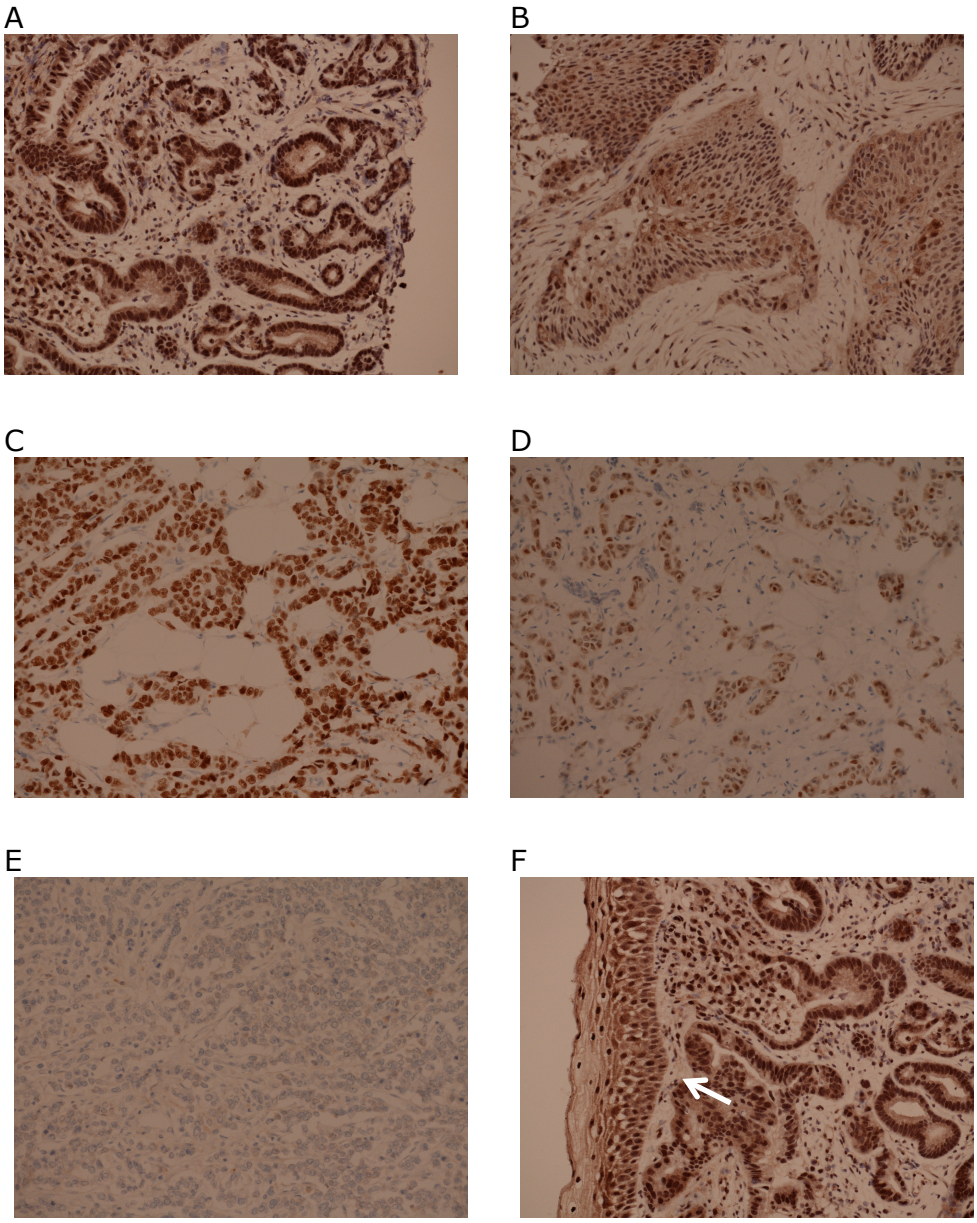


Figure 54. ERβ expression in: A) Oesophageal cancer tissue (patient 07, Allred score 5+3=8), B) Oesophageal cancer tissue (patient 06, Allred score 3+1=4), C) Breast tissue control - strong immunoreactivity (Allred 7/8), D) Breast tissue control - medium immunoreactivity (Allred 5/6), E) Breast tissue control – negative (Allred 0), F) Oesophageal cancer tissue and normal oesophageal tissue expressing ERβ (arrow). All images are 200X magnification.

5.3.3.3.1 Change in ERβ expression in patients who demonstrated a reduction in Ki67 expression with treatment

Three patients demonstrated a reduction in Ki67 expression following tamoxifen treatment (Patient 06, 08, 10) and in each of these patients, ERβ expression increased by an Allred score of 1 (Figure 55, Table 10). It was not possible to perform statistical analyses because there was no variation in the changes, as the SD was 0.

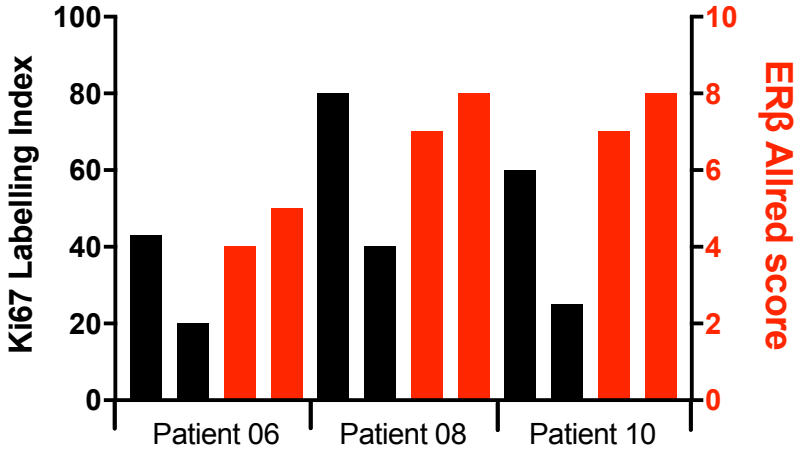


Figure 55. Ki67 expression (black bar) in pre and post tamoxifen biopsies of those patients who demonstrated a reduction with treatment, plotted against changes in ERβ expression (red bar) pre and post treatment; ERβ expression increased by an Allred score of 1 in all 3 patients who demonstrated a reduction in Ki67 expression. The pre tamoxifen biopsy is plotted first, before the corresponding post tamoxifen biopsy.

5.3.3.4 Correlation between change in Ki67 and mean ERβ expression

The relationship between the change in Ki67 expression and the degree of ERβ positivity was investigated (Figure 56). There was no significant correlation demonstrated between Ki67 expression and ERβ expression although the correlation trend was negative (r = -

0.2272), which may imply patients with a higher ERβ expression responded to tamoxifen therapy by a reduction in Ki67 expression.

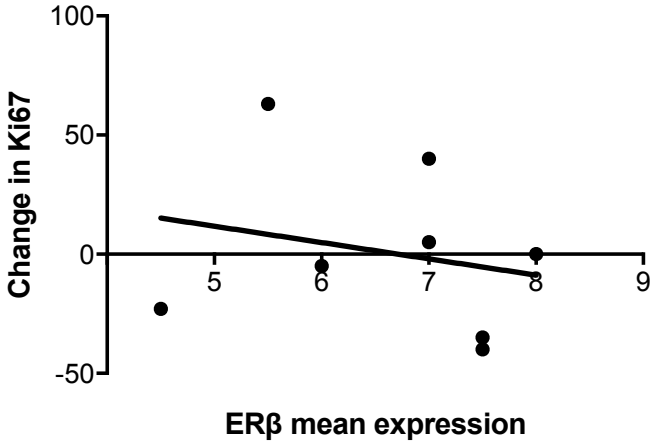


Figure 56. Linear regression analysis showing the relationship between change in Ki67 expression with treatment and the degree of ERβ positivity. A negative correlation was demonstrated although it was not significant. (r = 0.05163, p = 0.5884, Pearson's correlation co-efficient).

5.3.3.5 Correlation between clinicopathological characteristics and biomarker scoring

5.3.3.5.1 Initial Ki67 score and death

Four patients died during the study or within 8 weeks of the study end-date (study number: 02, 04, 07, 11). Patients who died within 8 weeks of the study end-date had a mean pre tamoxifen Ki67 labelling index score of 86.25±6.3%, compared to 56.67±29.16% in patients who were still alive. The difference between the mean of each group was of borderline significance (p=0.0555, t-test). The majority of patients included in this study had metastatic cancer which may be responsible for the increased Ki67 expression and given the small number of patients included this may have occurred

by chance. Indeed, Ki67 has been identified as a prognostic biomarker in oesophageal AC and SCC, although it is not used during routine clinical practice [348, 412-415].

5.3.3.5.2 Ki67 change according to sex and histological subtype

Two out of the 8 patients who completed the study were female and both of the female patients demonstrated a reduction in Ki67 expression with treatment. The female patients demonstrated a median Ki67 change following tamoxifen treatment of -29%±8.49, compared to 10.5%±36.21 in the male patients. The difference between the mean of each group was statistically significant (p=0.0483, t-test). In addition, the two female patients were the only two patients included who had SCC compared to AC carcinoma in the male patients. Although the difference between the male and female patients was statistically significant, only two female patients were included and this finding may have occurred by chance alone.

5.3.3.5.3 ERβ staining score and sex of the patient

The two female patients had a median Allred ERβ score of 6±2.12, compared to 6.5±1.23 in the male patients. The difference between the mean of each group was not statistically significant (p=0.6670, t-test).

5.3.3.5.4 ERβ score and death

Four patients died during the study or within 8 weeks of the study end-date (study number: 02, 04, 07, 11). Patients who died within 8 weeks of the study end-date had a median pre tamoxifen ERβ score of 7.75+±0.5, compared to 5.83±1.17 in patients who were still alive. The difference between the mean of each group was statistically significant (p=0.0088, t-test).

5.3.4 Summary table

A summary of all pre and post tamoxifen biopsy score results for Ki67, ERα and ERβ expression are available in Table 10.

Table 10. Summary table of Ki67, ERα and ERβ scoring in all oesophageal cancer biopsy specimens. N/A: not applicable, as no tumour specimen available to assess, Neg: negative, * denotes female patients, LI: labelling index - % of positive cells, Allred score: sum of the % number of positive cells (1-5) and the intensity of staining (1-3). Patient 01 is absent as he withdrew from the study prior to endoscopy and biopsy.

	Ki67 (%, LI)			ERα		ERβ (Allred score)		
Patient number	Pre tamoxifen	Post tamoxifen	Change in Ki67	Pre tamoxifen	Post tamoxifen	Pre tamoxifen	Post tamoxifen	Change in ERβ
02	85	N/A	N/A	Neg	N/A	8 (5+3)	N/A	N/A
03	No tumour	N/A	N/A	No tumour	N/A	No tumour	N/A	N/A
04	95	95	0	Neg	Neg	8 (5+3)	8 (5+3)	0
05	50	90	40	Neg	Neg	6 (4=2)	8 (5+3)	2
06*	43	20	-23	Neg	Neg	4 (3+1)	5 (3+2)	1
07	80	N/A	N/A	Neg	N/A	8 (5+3)	N/A	N/A
08	80	40	-40	Neg	Neg	7 (5+2)	8 (5+3)	1
09*	No tumour	N/A	N/A	No tumour	N/A	No tumour	N/A	N/A
10*	60	25	-35	Neg	Neg	7 (5+ 2)	8 (5+3)	1
11	85	90	5	Neg	Neg	7 (5+2)	7 (5+2)	0
12	95	90	-5	Neg	Neg	5 (4+1)	7 (5+2)	2
13	12	75	63	Neg	Neg	6 (4+2)	5 (4+1)	-1

5.4 Discussion

The aim of this study was to determine the biological effect of short-term tamoxifen on oesophageal cancer cell proliferation *in-vivo*, and to assess the feasibility of a larger trial, the results of which, could potentially allow for a further phase II study to determine clinical

response to treatment. 13 patients consented to recruitment over a 6-month period and of these patients, 8 completed the study. Moreover, all biopsy specimens obtained in these 8 patients were large enough to allow for adequate biomarker scoring for the primary and secondary objectives. Tamoxifen was well-tolerated with no reported side effects or adverse events, and none of the patients developed complications of endoscopy and biopsy. It appears therefore, that a phase II study would be both feasible and safe. The question of whether a further study would be warranted and suggestions for a further study is discussed in detail below.

Ki67 is a marker of proliferation, and its change in expression was used as a biological marker of tamoxifen efficacy [290]. Ki67 was selected as the primary outcome measure for a number of reasons; it has previously been validated as a suitable marker for assessing the short-term treatment efficacy of therapeutic agents, before the initiation of large-scale clinical trials [317, 416, 417]. Importantly, it has also been used as a biomarker of tamoxifen therapy in numerous breast cancer studies [317, 393-399, 401-409]; all reported a decrease in Ki67 expression, which was significant in 10 out of 18 studies [317, 393-399, 401-409] after a median of 28 days (range: 14 – 84 days) of 20mg/ day of tamoxifen [317, 393-399, 401-409]. Ki67 has also been used in oesophageal cancer to measure treatment response in clinical trials. In a phase II trial investigating gefitinib (EGFR tyrosine kinase inhibitor) in the treatment of oesophageal AC, they assessed clinical response to treatment radiologically after 28 days of treatment in 27

patients. In a hypothesis-generating analysis, they assessed the change in Ki67 expression in 7 patients for whom pre and post treatment biopsies were available; a significant reduction in the immunohistochemical expression of Ki67 ($P < 0.05$) was observed in 5 of the 7 patients, although the method of Ki67 expression scoring was not reported [418]. The immunohistochemical expression of Ki67 as a marker of proliferation was also used in a study of 65 patients with oesophageal SCC, treated with definitive radiotherapy; a Ki67 LI of ≥45% ($p=0.05$) showed a lower local recurrence rate, possibly attributable to the fact that rapidly cycling cells are more radiosensitive than quiescent cells [419]. A later study by the same research group also showed that the KI67 LI in oesophageal SCC resected surgical specimens was significantly reduced compared with pre-treatment biopsy specimens in patients who had undergone neoadjuvant chemotherapy compared to the control group of patients who underwent resection without neoadjuvant chemotherapy ($p<0.005$) [355]. Importantly, they also demonstrated significant correlation between the LIs of biopsy and surgical specimens for the control group ($p=0.006$). In the context of tamoxifen oesophageal cancer, there are no reports of *in-vivo* studies on patients, however, *in-vitro* studies on oesophageal cancer cell lines by another research group demonstrated a significant decrease in expression of Ki67 with tamoxifen treatment, assessed by WB analysis [55], although, we were unable to reproduce the results using OE33 cell lines in our laboratory (Chapter 4).

In this *in-vivo* study of patients with oesophageal cancer, tamoxifen treatment failed to demonstrate a significant change in Ki67 expression. There are numerous reasons for this; firstly, the study was underpowered, with only 8 patients rather than 20 patients, which was calculated as the required number to identify a change of 10%. Increasing the number of patients, however, is unlikely to yield statistically significant results alone, as only 3 out of 8 patients demonstrated a decrease in Ki67 expression, and in fact, an overall mean increase in Ki67 expression occurred.

A second explanation for failure to demonstrate a reduction in Ki67 expression is that the time of tissue sampling may not have coincided with the maximal anti-proliferative activity, or the treatment dose and duration (20mg/ day for only 4 weeks) may have been insufficient. A dose of 20mg/ day (following a loading dose of 80mg/ day) was selected because it is licenced at this dose in the UK for the treatment of breast cancer and anovulatory infertility [234] and therefore, its safety profile at these doses are well published [420-439]. A duration of 4 weeks was selected for the purpose of the feasibility study, to ensure patients were able to attend for the post tamoxifen biospy, as it is well known that this cohort of patents experience a deterioration in their quality of life [440]. Despite the widespread use of tamoxifen, the optimal *in-vivo* therapeutic concentration range is unknown because of a lack of dose-response studies [252, 253, 441], and a high degree of inter-individual variability in steady-state levels of tamoxifen and its metabolites [242, 248-255]

(Chapter 1, section 1.4.1.1). We did predict however, that the dosing regimen would be sufficient to achieve plasma steady states within the time period based on other published studies; in a study of patients with metastatic breast cancer, a loading dose of 80mg/ day for 7 days followed by 20 mg/ day thereafter, allowed steady-state values to be reached in one week [257]. Importantly, studies describing the pharmacokinetics of standard dose tamoxifen (10-30mg/ day), report steady state serum levels of 100ng/ml to 333ng/ml [250, 255-259], levels sufficient to induce oesophageal adenocarcinoma cell growth arrest *in-vitro,* as reported in Chapter 4 and by other authors [54, 55]. Moreover, the tamoxifen concentration in breast tissue is 5-11 times higher than in the serum; a trial investigating dosing regimens in breast cancer patients reported the median (range) of tamoxifen concentration at 20mg/ day for 28 days was 83.6ng/ml (8.7–134.4) in serum, and 744.4ng/ml (208.6–2556) in breast cancer tissue, respectively.

Another explanation for negative findings is that the outcome measure was not sensitive to the treatment. Failure of the immunohistochemical staining of tissue using the Ki67 antibody is unlikely, as positive control tissues were used and the protocol and antibody have been well validated by the Pathology laboratory for use in routine clinical practice. The tumour biopsy samples taken at endoscopy may not be representative of the entire tumour; quantitative differences in biomarker expression between sequential biopsies of the same tumour due to intratumoural heterogeneity, is

a well-recognised limitation of tissue biomarkers [442, 443], although the previously discussed study in oesophageal biopsy specimens and paired surgical specimens reported the Ki67 LI was significantly correlated (p 0.006, r2 0.62) [355].

Despite numerous reports advocating the use of Ki67 as a biomarker for tamoxifen efficacy [393, 396, 402, 443], it does have limitations and the results reported from other *in-vivo* studies must be interpreted with caution. The definition of staining thresholds used by different studies are often arbitrary [389], the selection of the area of tumour tissue for staining (e.g. centre or periphery, area of highest tumour density, area of highest tumour reactivity) and method of expression scoring (e.g. % area of staining, % of positive nuclei) vary widely between studies [444]. Moreover, the results obtained in other studies are not directly transferrable as they are in breast cancer patients and used different tissue biopsy methodology (core-cut or fine needle aspiration). In addition, fluctuation in biomarker scoring in paired samples without treatment have been reported, although results from the control arm in a randomised study of raloxifene in breast cancer reported this median difference was low (7%) and not significant [445]. Other studies of neoadjuvant tamoxifen treatment in breast cancer have reported paradoxical increases in Ki67 expression for some patients [416, 446]. Moreover, the degree of reduction varies markedly between patients in some trials [447], which may be due to different degrees of dependence on oestrogen between tumours [317]. A study of patients with breast

cancer also failed to demonstrate a reduction in Ki67 following short-term tamoxifen treatment, although the study did demonstrate a clinical response to treatment at long-term follow-up [448]. Other methods to detect a reduction in proliferation could be used in future studies such as thymidine labelling index [449, 450] or ploidy and S-phase fraction [448, 451], which have been used to investigate tamoxifen and other drug treatment in breast cancer. As previously described, the mechanism of action of tamoxifen is complex, involving the induction of cell cycle arrest, and changes in the expression of ER-dependent transcription. Induction of apoptosis is another major determinant of tumour growth and tamoxifen has been shown to inhibit apoptosis in breast cancer *in-vitro* [452, 453] and *in-vivo*[454]. Moreover, numerous *in-vivo* studies have used markers of apoptosis to assess the efficacy of tamoxifen such as Bcl-2 expression (a proto-oncogene which blocks apoptosis) [394, 448] and the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for highlighting apoptotic cells [401, 417].

In the management of patients with breast cancer, assessment of the ERα status is mandatory [444, 455], as it predicts prognosis and response to endocrine therapy. The *in-vivo* breast cancer studies that this pilot study is modelled on, also confirm the biological response to tamoxifen treatment is related to ERα expression [394, 396]. Clinical guidelines therefore, recommend tamoxifen for patients with a minimum of 1% of weakly ERα positive cells (an Allred score of ≥3) [456, 457]. Although the protective role of ERβ in breast cancer has

been described [95, 163, 174] and there is evidence that ERβ expression is associated with better survival and clinical outcomes in breast cancer [458 158 459-461], currently, it is not strong enough to suggest it adds any clinical value over measuring ERα expression alone in clinical practice [462]. The role of ERβ in other cancers have also been described such as in colon cancer and prostate cancer (Chapter 1, section 3), although its expression in these cancers is not involved in clinical decision making.

The expression of ERs in oesophageal cancer and normal oesophageal tissue, detected by immunohistochemistry, has been previously reported [92, 93, 213-217]. ERβ is the predominant subtype [213] and its expression in oesophageal cancer tissue appears to be higher than adjacent normal tissue [213, 215, 216]. The effect of ERβ expression on clinical outcome however is unclear; some studies suggest increased expression is associated with an unfavourable prognosis [217], whilst others have reported contradictory associations with different clinicopathological features [214, 217]. In this study, oesophageal cancer biopsies from 10 patients were examined for ER expression; all biopsies were positive for ERβ expression and negative for ERα expression. The reason for negative ERα expression in all biopsy specimens in this study is unlikely to be due to failure of the staining technique or antibody, as the pathology laboratory regularly performs ERα staining on breast cancer tissue and the process is fully validated and highly regulated. Work on oesophageal cancer tissue performed by colleagues in the same

research group also demonstrated a low number of ERα positive oesophageal cancer specimens. In one study of 30 oesophageal cancer specimens, mild ERα staining was present in only 1/30 cancer specimens, whereas ERβ expression was present for 21/30 specimens [90]. In an earlier study of 67 oesophageal cancer specimens ERα receptors were significantly under expressed ($p=0.01$) compared to ERβ receptor in normal mucosa and cancer tissue [54]. These studies used different antibodies and the slide fixation, preparation and staining was performed manually, not by the hospital pathology department. Reduced ERα compared with ERβ expression in oesophageal cancer and normal epithelium has also been reported in the scientific literature [213-215, 217, 218]. However, the most recent account of ER expression in oesophageal AC specimens by Due *et al*. reported ERα expression was positive in 8/10 specimens and ERβ was positive in 7/10 specimens [219]. The authors recognised the failure of previous studies to identify ERα expression in oesophageal cancer tissue and therefore used a biotin-free polymeric visualization system, which is a more sensitive and specific ERα characterisation in breast cancer [220]. For this pilot study, we also used a biotin-free system and so this does not account for why we were unable to demonstrate ERα expression in any of the specimens; the most plausible reason is that ERα was not expressed in the small number of specimens screened.

As previously mentioned, all oesophageal cancer biopsies examined in this study were positive for ERβ expression. Although the study

was not powered to detect associations between ER expression and clinicopathological features, patients who died had a higher ERβ score than those who survived (p=0.0088) and there was no significant difference in ERβ score between male and female patients. Although non-significant, a weak negative correlation between a negative change in Ki67 expression with treatment (i.e. an appropriate response to tamoxifen treatment by a reduction in proliferation) and the degree of ERβ positivity was demonstrated. It is not possible to draw any formal conclusions based on these findings however because the study was underpowered and so may have occurred entirely by chance. With acknowledgement of these limitations, the above observations may indicate that tamoxifen is exerting an antiproliferative effect through ERβ, in a similar way that tamoxifen exerts its antiproliferative effect in breast cancer via ERα. If tamoxifen is behaving as an agonist via ERβ in oesophageal tissue, this may account for the protective effective of oestrogen in women, which has been suggested as the reason behind the gender bias in oesophageal cancer [11, 13]. This hypothesis, however, does not fit with the finding that patients who died during the study had a higher ERβ expression, unless tamoxifen was behaving as an antagonist, thereby competing with or inhibiting the action of an agonist acting on the ER. The change in ER expression with tamoxifen treatment was also investigated and ERβ expression increased by an Allred score of 0.75, following treatment with tamoxifen for all included patients. An increase in expression with treatment however, does not support the notion that tamoxifen

treatment in oesophageal cancer behaves as an agonist, as one would expect a ligand-dependant down regulation by the cell to attenuate the transcription in response to overstimulation by the agonist[463]. The ER degradation mechanism, however, is not well understood and it is unlikely to be this simplistic [464]. For example, the *in-vivo* studies of tamoxifen in breast cancer, on which this study is modelled, demonstrated ERα upregulation [465], downregulation [402, 443] and no change [393, 396] following short-term tamoxifen treatment. A suggested explanation for the down regulation reported in these studies of breast cancer patients is that it is due to the agonist effects of tamoxifen, even though it is accepted that it behaves as an antagonist in breast tissue [416]. Only one of the breast cancer studies reviewed, investigated the effect of tamoxifen on the expression of ERβ and they reported a significant reduction in Ki67 expression in ERβ positive patients but not in ERβ negative patients. They also failed to show a change in ERβ expression with treatment [317].

A pilot study is a preliminary study intended to aid the design of a larger scale study by assessing aspects of trial design such as the feasibility of recruitment, retention, assessment procedures and treatment implementation [466]. This pilot study has identified numerous areas in which the trial design could be improved to ensure a further study is of high quality. Patient recruitment could be improved via a number of different approaches. The recruitment period was only 6 months but could be increased to 12 months or

more. Patients may be recruited from multiple centres; two oesophageal cancer tertiary referral centres (Nottingham City Hospital and Leicester Royal Infirmary) are located within a 40-mile radius of the trial-coordinating centre making it logistically possible for the lead co-investigator to attend the MDT meetings, which was the most efficient source of patient identification. Oesophageal cancer patients included in the current pilot study were not candidates for surgical resection or further chemotherapy. However, such patients could be included in a future study to increase patient numbers. Patients being actively staged for potentially curative resection could be recruited without resulting in a delay to surgical resection if they were deemed resectable, as the time from cancer diagnosis to surgical resection will be long enough for tamoxifen treatment. Patients who undergo chemoradiotherapy with the aim to downstage the cancer prior to curative resection have a window-period of approximately 6 weeks until surgery and so potentially tamoxifen may be started at the beginning of this window period. This cohort of patients who are being investigated and managed for surgical resection with curative intent will be fitter, younger and possibly more motivated than the cohort included in this study and therefore the proportion expected to enrol is likely to be higher.

Another major limitation of the pilot study is regarding the accuracy of Ki67 in reflecting tumour biology and response to tamoxifen treatment. The issue of random variability in Ki67 expression could be addressed in future study design by taking a larger sample of

tissue at endoscopy using jumbo or large-capacity forceps, or by endoscopic mucosal resection (EMR). The use of jumbo forceps allows larger diameter tissue to be obtained compared with standard-sized forceps, although a therapeutic endoscope, rather than a standard endoscope is required [467]. EMR is a technique used to resect cancer confined to the mucosal layer and for diagnosis, as it increases the yield of diagnosis over random biopsies [468]. EMR however, is a more specialised technique not as readily available, and has higher risks of bleeding and perforation and is therefore not ethically acceptable [469]. A more acceptable alternative would be to take multiple biopsies using standard biopsy forceps. Studies have demonstrated the greater the number of biopsies taken, the higher the diagnostic accuracy. In a study of 200 patients, the percentage of correct diagnoses of oesophageal cancer following the first biopsy was 93% compared with 98% if seven biopsies were taken [470]. As previously suggested, inclusion of patients undergoing surgical resection will not only serve to increase patient numbers but would also allow for the entire cancer resection specimen to be analysed and scored for Ki67 expression, addressing the issue of tumour heterogeneity.

Randomised controlled trials (RCTs) are the gold standard for assessing the efficacy and safety of a new treatment, and along with meta-analyses are considered the highest level of evidence [471]. The next logical step in determining the effect of tamoxifen in oesophageal cancer is to perform a RCT. The effect of tamoxifen

(treatment group) could be compared with placebo (control group). Patients could be randomised to treatment groups by stratified randomisation, ensuring the groups are comparable and confounding factors that may affect response to treatment such as sex and disease stage are divided equally. Alternatively, the study could be single sex, and we propose including male patients only; because of the lower incidence of oesophageal cancer in women we are unlikely to recruit enough women in a mixed-sex trial to make valid conclusions. Both the clinical co-investigators and patients could be blinded to treatment (double-blind) to minimise bias. The primary endpoint for the pilot study was change in Ki67 expression in pre and post tamoxifen biopsy specimens and the secondary endpoints were changes in ERα and ERβ expression. As well as assessing the biological response to tamoxifen treatment using Ki67 expression, clinical response may be evaluated radiologically using CT imaging to assess disease response according to the size of the tumour or disease progression. This could be performed immediately prior to surgical resection for those undergoing surgery and periodically for those patients who are not for further active treatment. A clinical trial of two groups is likely to require a larger sample size, however with the measures discussed previously to improve recruitment, a RCT remains a feasible option.

In conclusion, the pharmacological action of tamoxifen is complex and multifaceted (Chapter 1, section 4) with tissue-specific agonist, antagonist and mixed activity. The role of tamoxifen in oesophageal

cancer is yet to be defined and the findings from this pilot study should be taken forward in the design of a RCT to determine the efficacy of tamoxifen in oesophageal cancer treatment.

6 Chapter Six

Discussion

Despite advancements in the diagnosis and treatment of oesophageal cancer, the prognosis remains dismal. There is a clear male predominance in the incidence of oesophageal cancer, for reasons that remain unexplained. The mechanism driving the gender bias may potentially be exploited in the treatment of this deadly disease. In this thesis, the hypothesis that ERs mediate the gender bias in oesophageal cancer was investigated.

The research was conducted on oesophageal cancer cell lines and investigated the role of ERs in the gender bias in oesophageal cancer. There are numerous limitations to this approach. Firstly, there are other physiological pathways and considerations with respect to the gender bias in oesophageal cancer as discussed in Chapter 1, section 1.1.3, in particular, the role of androgen and ARs. Although out of the scope of this thesis, their role and potential interaction with the ER system warrants further investigation. Secondly, although cell lines have been used as experimental models for cancer research for many years there are obvious limitations and disadvantages; the cell culture environment is different from that of the tumour and the cell culture conditions can change cell morphology, gene expression and hence cellular pathways. Therefore, the behaviour of the cells *in-vitro* may not be representative of cell behaviour *in-vivo*. Nevertheless, the findings from this thesis contribute to the ever-growing evidence of ERs in oesophageal cancer. This research demonstrated the expression of both ERα and ERβ in oesophageal AC cell lines. The expression of

both ER subtypes was higher in the oesophageal cancer cell line established from a female patient with stage II oesophageal AC (OE33), compared with male patient with oesophageal AC of a more advanced stage (stage III, OE19). Moreover, ERβ was the dominant receptor, confirming findings reported by other authors [213-218]. Unlike other organs of the gastrointestinal tract such as the colon and stomach, where loss of ERβ expression is associated with cancer progression [190, 191], changes in the expression of ERβ in normal vs. cancer tissue is conflicting, with the majority of studies reporting increased ERβ expression with oesophageal cancer progression [213-218].

In an attempt to clarify the role of the ER subtypes in oesophageal cancer development, ER expression was knocked-down using siRNA for the first time in oesophageal AC cell lines. Adequate knockdown of ERα expression was not achieved at the mRNA or protein level and work therefore focused on ERβ. Knockdown of ESR2 mRNA was achieved in numerous experiments; however, we were unable to reliably reproduce significant ERβ protein knockdown. Further downstream experiments failed to demonstrate a change in the expression of selected proliferation associated proteins, however, this may be due to inadequate ERβ knockdown and it is not therefore possible to conclude that knockdown of ERβ has no effect on oesophageal cancer cell proliferation. Although the aim of this Chapter was not met, the research did highlight the discordance between ER mRNA and protein expression, also reported by other

authors [316, 322-324], highlighting the complex translational and post-translational regulation of the ER, which themselves, may play a role in carcinogenesis [138, 321, 325-327].

The SERM, tamoxifen, used in the treatment of breast cancer, significantly inhibited the proliferation of oesophageal cancer cell lines in a dose-dependent manner, confirming findings of other authors [54, 55, 219, 279]. The mechanism of action of tamoxifen in oesophageal cancer remains elusive and unfortunately, this research failed to demonstrate any reproducible and significant changes in selected proliferation associated proteins assessed by WB. The ultimate question of the role of tamoxifen in the treatment of oesophageal cancer *in-vivo* was explored. An initial pilot study was conducted to aid the design of a clinical trial and confirmed that such a trial is feasible, although, because the study was underpowered, we were unable to answer the primary objective of whether tamoxifen treatment changes the expression of Ki67 (a surrogate marker for proliferation). Nonetheless, the preliminary findings may be used to generate further hypotheses. Results of the pilot study mirrored the findings of the *in-vitro* work on oesophageal cancer cell lines in that ERβ is the dominant receptor type. Moreover, women appear to be more responsive to tamoxifen treatment than men; of the two women included in the pilot study, both demonstrated a reduction in Ki67 expression. This may also be responsible for the gender bias in oesophageal cancer development; with the natural ER ligand, oestrogen, present in women, acting via

ERβ receptors, having a protective effect (Figure 57). Interestingly, patients who died had a higher ERβ score than those who survived (p=0.0088), which supports the notion proposed by other authors that ERβ expression is associated with oesophageal cancer progression and a worse prognosis [213-218]. In addition, although it was not significant, a correlation was demonstrated between a negative change in Ki67 expression and mean ERβ expression, suggesting the anti-proliferative effect of tamoxifen is mediated through ERβ. If this is indeed confirmed to be true, the expression of ERβ may offer the scope for personalised medicine. Intense research is currently underway to identify key signalling pathways that may be targeted in an attempt to offer additional treatment options in oesophageal cancer [472].

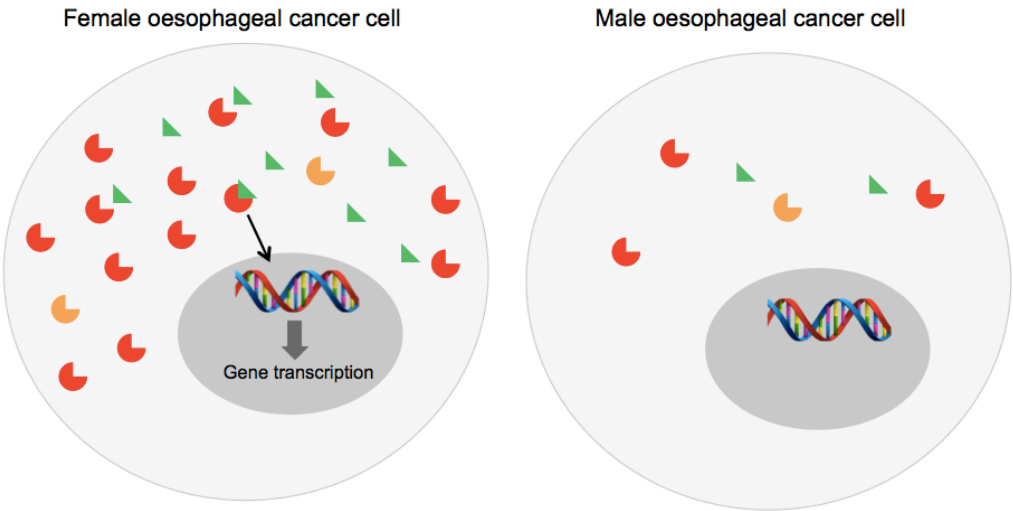


Figure 57. Increased expression of ERβ (red) compared with ERα receptors (orange) in oesophageal cancer cells suggest ER ligands (green) act via ERβ. Increased ERβ expression in female relative to male oesophageal cancer cells may result in reduced proliferation of female oesophageal cancer cells when bound to naturally occurring ligands such as oestrogen, or synthetic ligands such as tamoxifen.

ERβ has been identified as a target for cancer treatment for a number of cancers including breast and colon cancer [473]. *In-vitro*

work using ERβ specific agonists has begun to decipher the role of this receptor subtype in cancer development [474, 475]. *In-vivo* work on a mouse model has also demonstrated a highly selective orally active ERβ agonist significantly reduced inflammation in the HLA-B27 transgenic rat model of inflammatory bowel disease and adjuvant-induced arthritis in the Lewis rat [476]. Moreover, results of phase II studies of the ERα antagonists and ERβ agonist, TAS-180, reported promising results in the treatment of breast cancer [477, 478]. The precise role of ERβ in oesophageal cancer is still unknown and the use of siRNA to knockdown ERβ expression in the OE33 cell line, presented in this thesis, did not allow for further downstream experiments. This question remains of paramount importance and an understanding of ERβ expression, interactions and post-translational modifications may allow for targeted therapy by SERMs such as tamoxifen or other ER ligands.

Given that the precise role of ERβ in oesophageal cancer remains unknown, the experimental use of novel ERβ agonists *in-vivo* is not yet indicated. As tamoxifen is a safe and well-tolerated drug and numerous authors have reported inhibition of oesophageal cancer cell growth *in-vitro* [54, 55, 219, 279], the next logical step would be to further investigate the effects of tamoxifen in patients with oesophageal cancer in a RCT, to determine the efficacy and optimal dose regimen as discussed in Chapter 5. Any future trial should ensure adequate amount of tissue is taken for analysis by performing numerous biopsies or analysing resection specimens,

and patient recruitment could be improved by taking a multicentre approach if necessary, to ensure it is adequately powered.

Future work with respect to determining the role of the ER subtypes in oesophageal cancer is essential to elucidate their role in oesophageal cancer development. As siRNA failed to produce adequate ER protein knockdown in oesophageal cancer cell lines other approaches should be attempted such as the CRISPR /Cas system, which cuts the gene from the genome completely abolishing transcription [339]. To avoid the aforementioned pitfalls of research using cell lines, animal models could be used; oesophageal cancer cells with stable ER subtype knockdown could be injected into immunodeficient mice and the effect of subtype knockdown assessed by measuring tumour volume and other markers of proliferation or tumour progression such as angiogenesis by microvessel density, for example. Moreover an oesophageal cancer transgenic mouse model could be used to examine the effect of tamoxifen on oesophageal cancer growth[479, 480].

In conclusion, the research presented in this thesis supports the hypothesis that ERs play a role in oesophageal cancer development and may be responsible for the gender disparity. The research also suggests that the presence of ER ligands may be used in the treatment of this deadly disease where currently, treatment options are few. Further work is needed to confirm these findings and to

explore additional treatment options for patients with oesophageal cancer.

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8 Appendix

8.1 Appendix 1

Figure A 1.1 ERα antibody (ab32063) optimisation

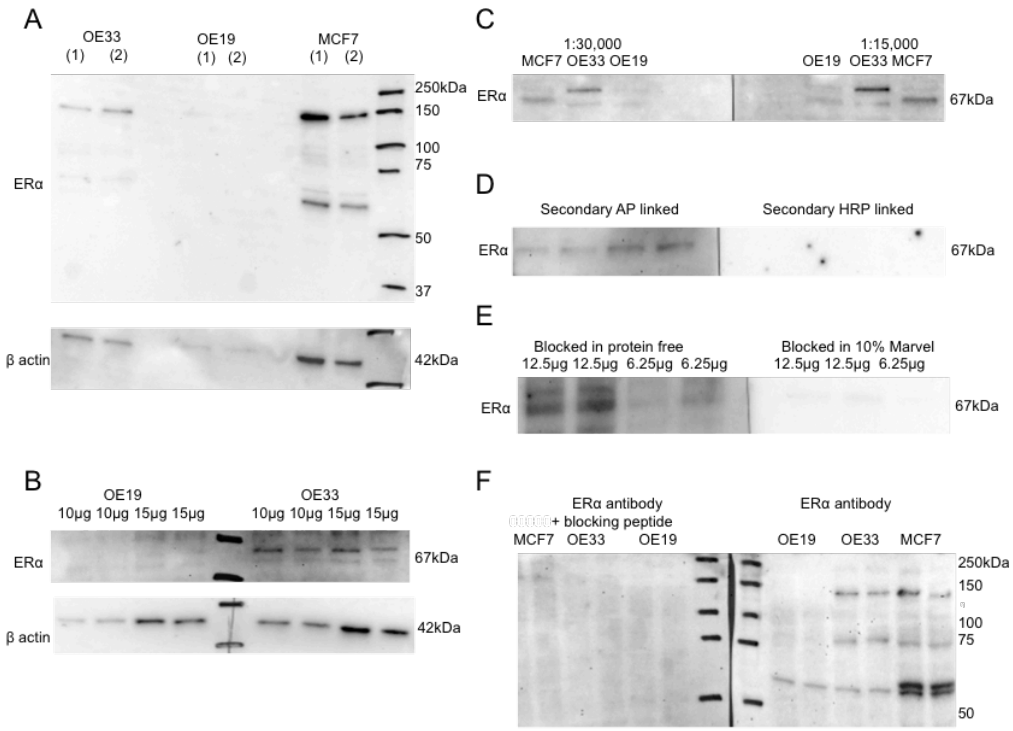


Figure A1.1 WB demonstrating optimisation of ERα antibody (ab32063) using protein from MCF7, OE33, and OE19 cell lines. Unless indicated, 10µg of protein was loaded per well, 10% Marvel was used as the blocking solution and the secondary antibody was Anti-Rabbit IgG (whole molecule) – Alkaline Phosphatase antibody (A3937, Sigma-Aldrich, 1:30,000). A: Identification of the ERα protein using two separate protein lysates from all three cell lines, using a primary antibody dilution of 1:1000. B: Increased concentration of protein (15µg) was loaded and increased concentration of primary antibody was used (1:500). C: Different concentrations of secondary antibody. D: Using HRP linked secondary. E: Using protein-free blocking solution. F: ERα antibody compared with blocking peptide. Not all of the blots were stripped and re-probed for β actin as the purpose of the experiment was optimization of the primary antibody, not quantification.

Figure A1.2 ERα antibody (ab108398) optimisation

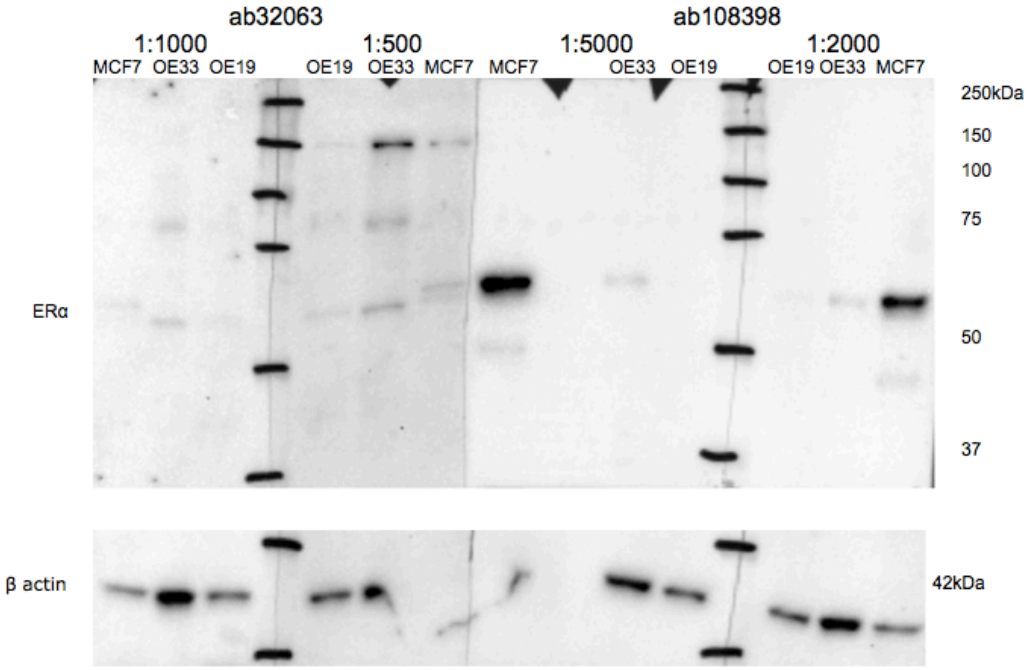


Figure A1.2 WB demonstrating optimisation of anti-ERα antibody (ab108398) at different concentrations and in comparison to original ERα antibody (ab32063). 10µg of protein from OE19, OE33 and MCF7 cell lines was loaded per well, 10% Marvel was used as the blocking solution and the secondary antibody was Anti-Rabbit IgG (whole molecule) – Alkaline Phosphatase antibody (A3937, Sigma-Aldrich, 1:30,000).

Figure A1.3 ERβ antibody (ab3577) optimisation

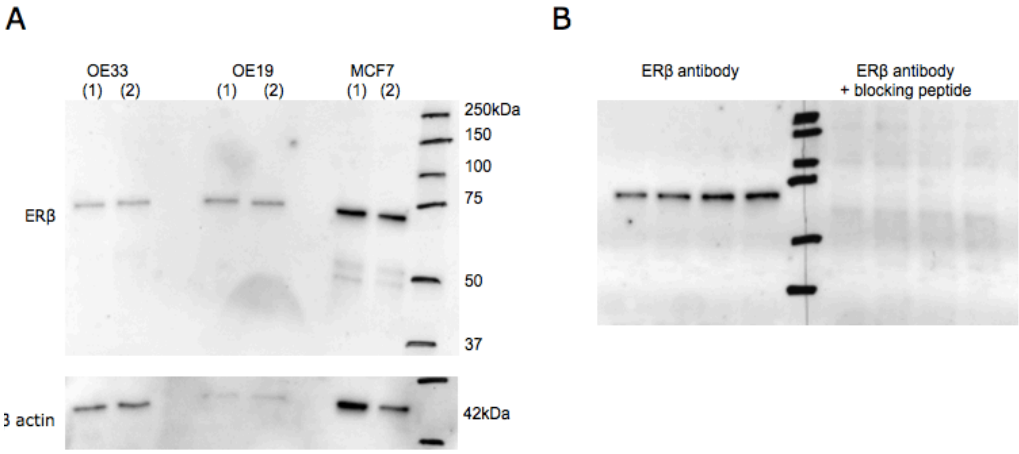


Figure A1.3 WB demonstrating optimisation of ERβ antibody (ab3577) at 1:1,500 dilution using protein from OE19, OE33 and MCF7 cell lines. 10µg of protein was loaded per well, 10% Marvel was used as the blocking solution, and the secondary antibody was Anti-Rabbit IgG Alkaline Phosphatase antibody (A3937, Sigma-Aldrich, 1:30,000). A: Identification of the ERβ protein signal using two separate protein lysates from all three cell lines. B: ERβ antibody signal blocked out using the specific blocking peptide for this antibody (ab3564) in MCF7 protein lysate.

Figure A1.4 ERβ antibody optimisation (ab3576 and ab133467)

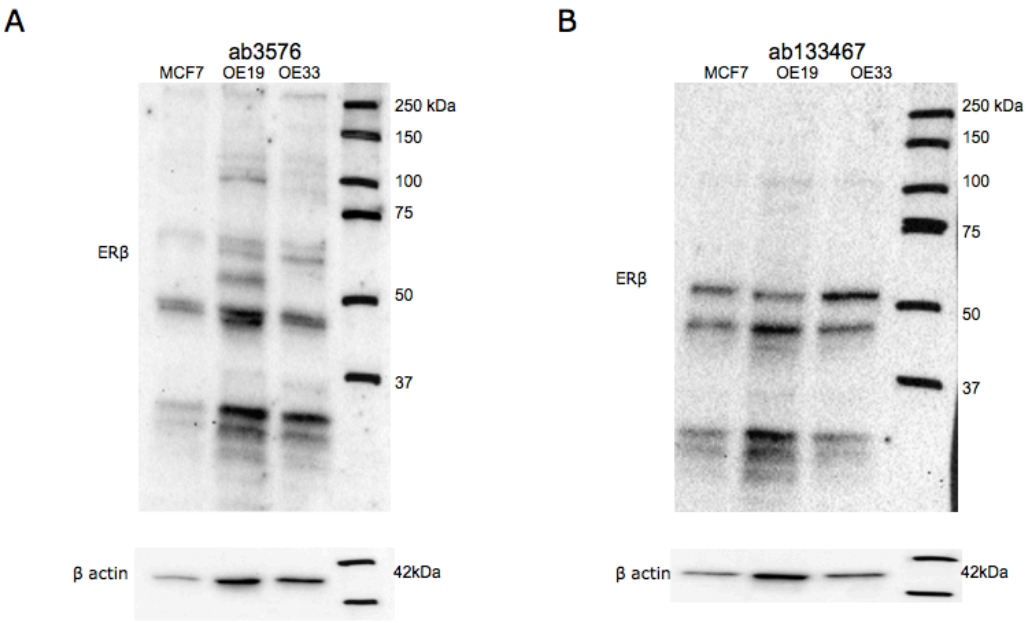


Figure A1.4 WB demonstrating optimisation of ERβ antibody ab3576 (A) and ab133467 (B). 15µg of protein MCF7, OE19 and OE33 protein lysate was loaded per well, 10% Marvel was used as the blocking solution, and the secondary antibody was Anti-Rabbit IgG Alkaline Phosphatase antibody (A3937, Sigma-Aldrich, 1:30,000

Figure A1.5 Initial MCF7 transfection

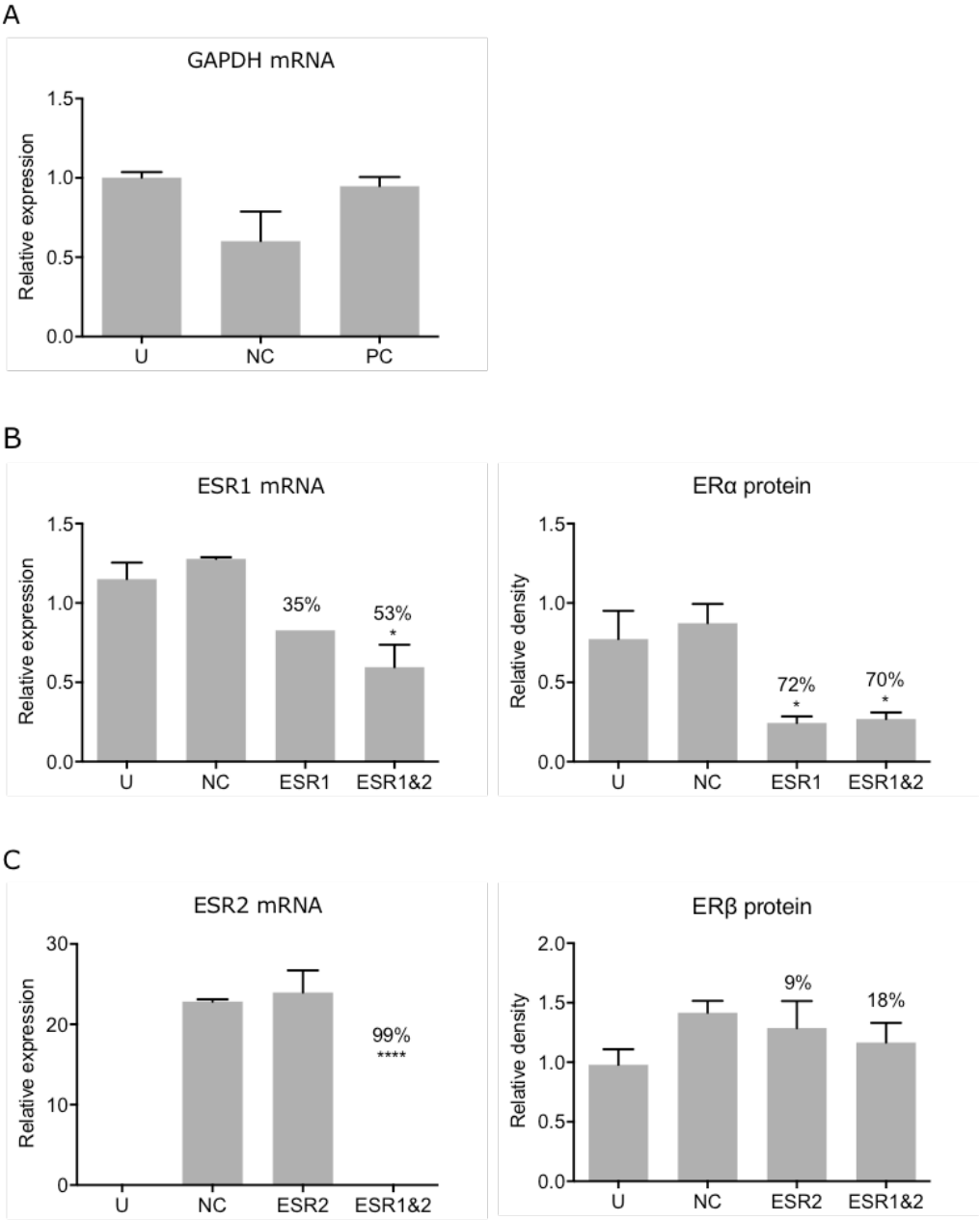


Figure A1.5 Figure showing the relative mRNA and protein knockdown in siRNA treated MCF7 cells. Relative GAPDH mRNA expression was not knocked down (0%) using positive control (PC) siRNA and was up-regulated although this wasn't significant (t-test) (A). In cells treated with ERS1 siRNA, ESR1 mRNA and ER α protein expression was knocked down by 35% and 72% (*p= 0.02, t-test), respectively. In cells treated with ESR2 siRNA, ESR2 mRNA and ERβ protein expression was knocked down by 0% and 9% respectively. In cells treated with both ESR1 and ESR2 siRNA in combination, ESR1 mRNA and ERα protein expression was knocked down by 53% (*p= 0.02, t-test) and 70% (*p= 0.02, t-test), respectively, and ESR2 mRNA and ERβ protein was knocked down by 99% (****p=<0.001) and 18%, respectively. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. U: untreated cells. Results are expressed as mean±SD.

Figure A1.6 OE19 cells treated with Positive control siRNA

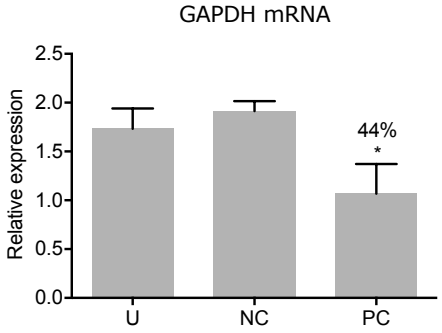


Figure A1.6 Relative GAPDH expression using positive control (PC) siRNA targeting GAPDH, in OE19 cells. GAPDH was knocked down by 44% (*p=0.0104). Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. U: untreated cells. Results are expressed as mean±SD.

Figure A1.7 OE19 cells treated with ESR1 siRNA

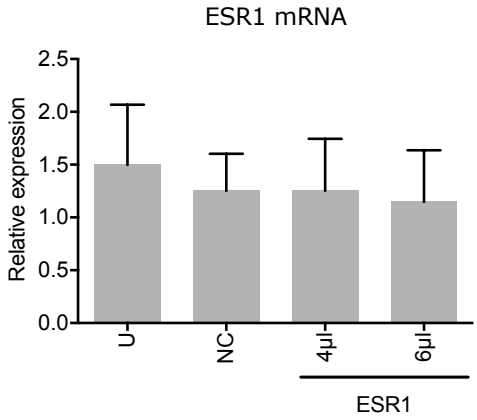


Figure A1.7 Relative ESR1 expression using ESR1 siRNA, with 4µl or 6µl per well, in OE19 cells. There was no knockdown of ESR1 expression using either 4µl or 6µl DharmaFECT. NC: Negative control U: untreated cells. The column bar graph represents the mean and the error bars represent SD.

Figure A1.8 OE19 cells treated with ESR2 siRNA

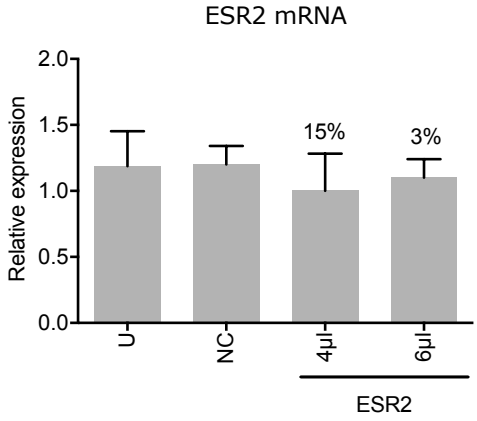
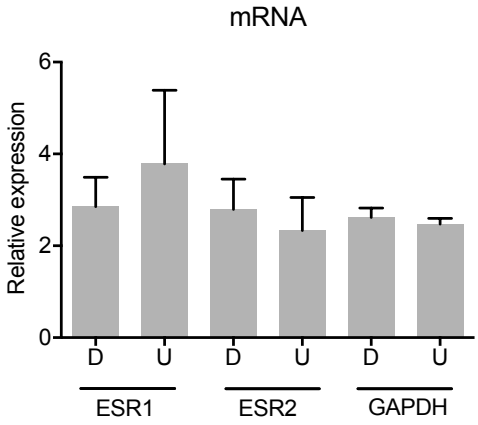


Figure A1.8 Relative ESR2 expression using ESR2 siRNA, with either 4µl or 6µl per well, in OE19 cells. Knockdown (expressed as a percentage) was calculated relative to negative control (NC) expression. ESR2 knockdown was 15% and 3%, with 4µl and 6µl of DharmaFECT per well, respectively. Knockdown was not significant (t-test). U: untreated cells. The column bar graph represents the mean and the error bars represent SD.

Figure A1.9 The effect of DharmaFECT transfection reagent on gene expression

To ensure the DharmaFECT transfection reagent was not affecting the expression of the target genes, the relative mRNA expression in untreated OE33 cells and cells treated with 4µl of DharmaFECT/ well were compared; there was no statistically significant difference between paired samples (Figure A1.9).



FigureA1.9 Relative mRNA expression of ESR1, ESR2 and GAPDH in untreated (U) OE33 cells and cells treated with 4 µl DharmaFECT (D)/ well. There was no significant difference in expression between the treated and untreated cells (t-test). Results are expressed as mean±SD.

Figure A1.10 The effect of trypsinisation on gene expression

Future downstream experiments using growth assays would require the transfected cells (grown in 6-well plates) to be trypsinised and seeded into 96-well plates. The effect of trypsinisation on the expression of the target genes was therefore investigated by comparing the relative mRNA expression in siRNA transfected OE33 cells without and following trypsinisation; there was no statistically significant difference between samples (Figure A1.10).

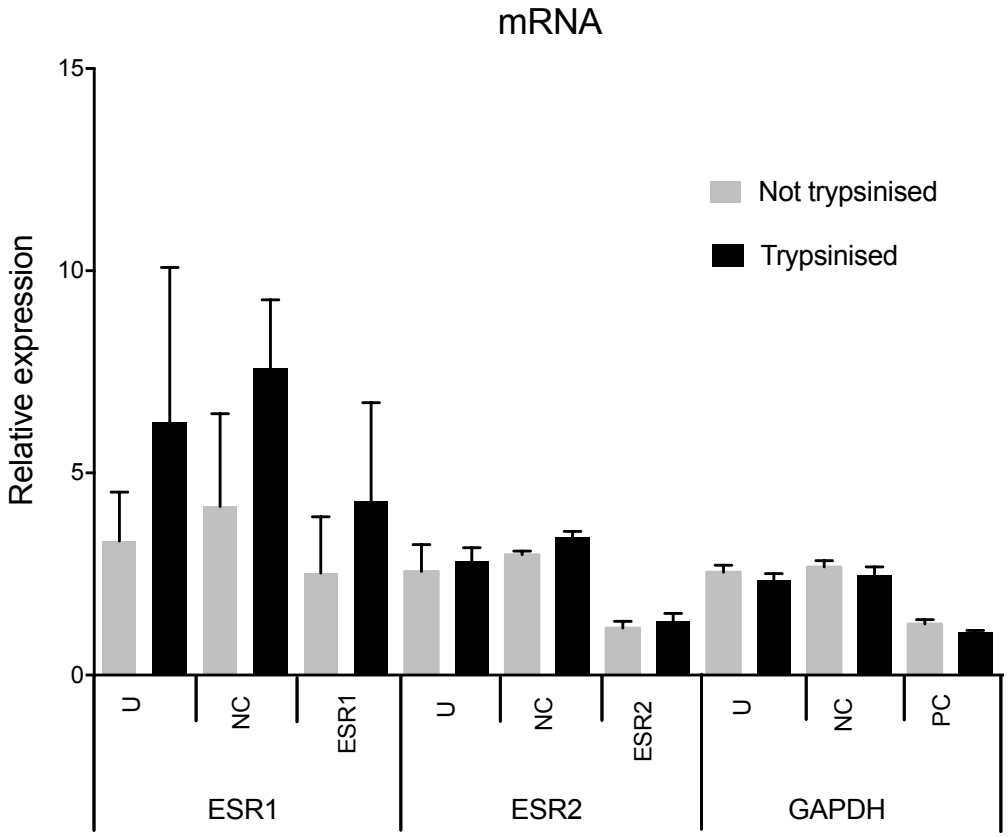


Figure A1.10 The effect of trypsinisation on the expression of target genes on controls (U: untreated, NC: Negative control), and cells transfected with siRNA (ESR1, ESR2, PC: positive control). There was no significant difference in mRNA expression (ESR1, ESR2, GAPDH) between paired samples, one of which had been trypsinised (t-test). Results are expressed as mean±SD.

Figure A1.11 Full blots of proliferation-associated protein antibodies.

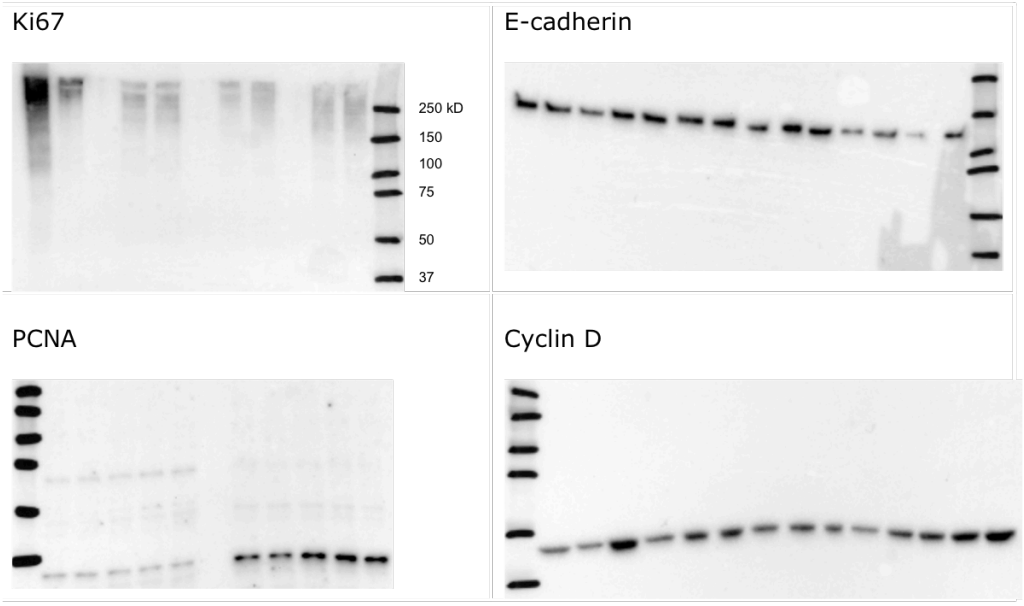


Figure A1.11 Full Western blots of Ki67, E-cadherin, PCNA and Cyclin D antibodies demonstrating clean blots with protein signal at the predicted molecular weights. The molecular weight markers labelled for the Ki67 blot corresponds to the other 3 blots.

8.2 Appendix 2

BrdU assay optimisation

Initial experiment

The BrdU assay was previously optimised using OE33 cell lines in the same laboratory[90] and the initial experiment was therefore conducted according to the following optimised conditions: 5,000 cells/ 96-well, 24hr post-cell seeding incubation time, 2hrs BrdU labelling period and 30 minutes of Substrate solution incubation time (Figure A2.1).

The background control consists of cells and media, which are not incubated with BrdU and therefore provides information on the unspecific binding. According to the manufacturer, the absorbance value should not exceed 0.1nm, in this experiment it was 0.2nm (±0.01), suggesting the cell concentration is too high. Moreover, the absorbance values for the untreated cells (PRF-RPMI and DMSO 0.1%) resulted in absorbance values beyond the measuring range of the plate reader at 24 and 48hrs (2.0nm), therefore reducing the sensitivity of the readings.

The blank control consists of culture medium only and provides information about the unspecific binding of BrdU and the anti-BrdU-POD conjugate. According to the manufacturer, the value should not exceed 0.1nm absorbance. The mean absorbance was 0.08±0.004 and the blank control was performed for each experimental setup and subtracted from all plotted values (Figure A2.1).

The initial experiment demonstrated that 0.1% DMSO had no significant influence on cell growth between cells treated with PRF-RPMI. Nevertheless, cells treated with 0.1% DMSO were used as the control for all subsequent experiments given that this is the final concentration of this solvent for all tamoxifen and etoposide treated cells.

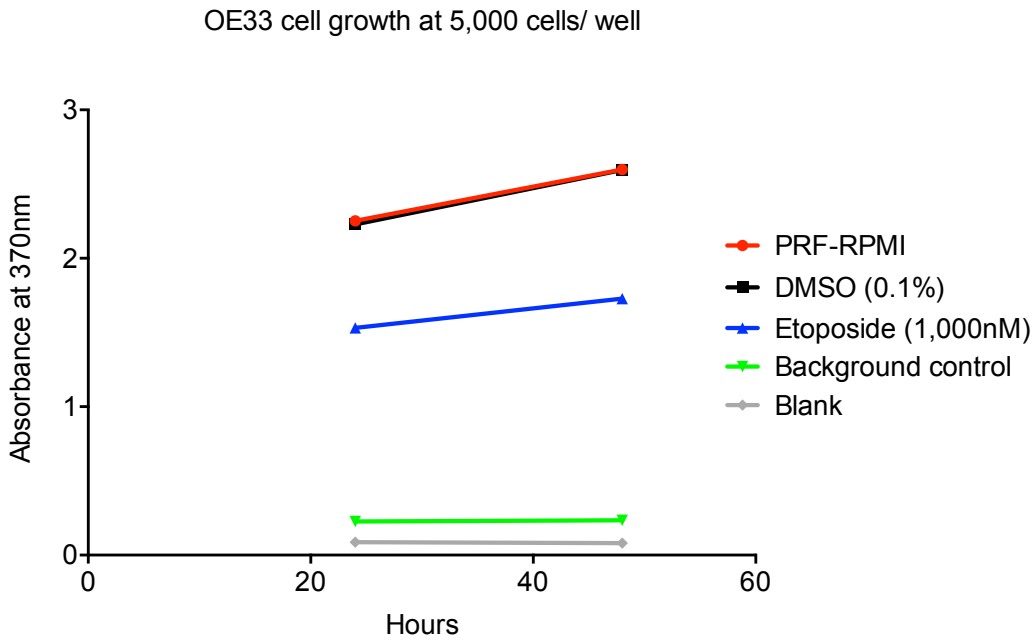


Figure A2.1. The effect of PRF-RPMI, DMSO (0.1%) and etoposide on OE33 cell proliferation, measured by BrdU incorporation at the absorbance of 370nm.

As the absorbance values obtained in the initial experiment using 5,000 cells/ well were too high, the cell density was optimised as follows.

Cell density optimisation

Six different cell densities were tested at 24 and 72hr time points for both cell lines. Cells seeded at 2,000 cells/ well demonstrated the best doubling time (Figure A2.2 & A2.3) and this is on the exponential growth phase of cell growth (Figure A2.4).

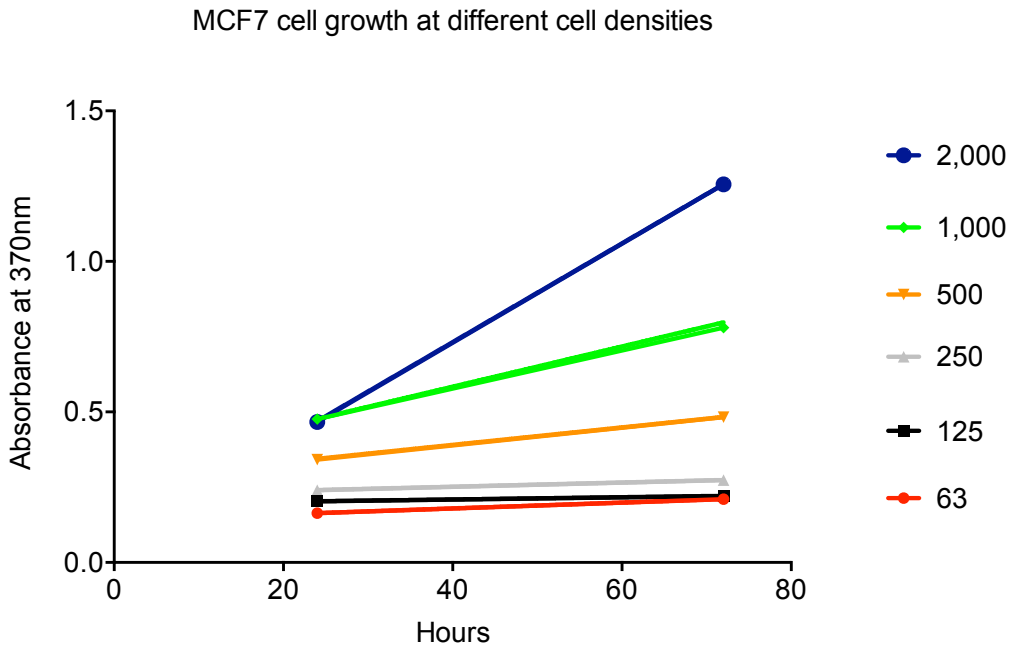


Figure A2.2. The effect of 6 different cell densities (63 - 2,000 cells/ well) on MCF7 cell proliferation at 24 and 72 hours, measured by BrdU incorporation at the absorbance of 370nm.

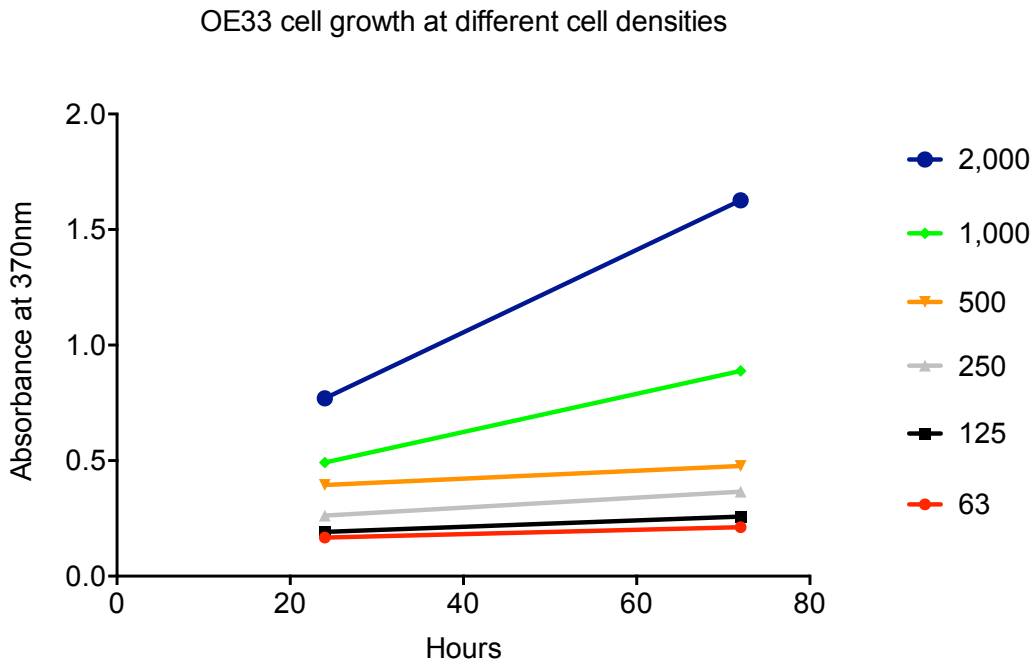


Figure A2.3. The effect of 6 different cell densities (63 - 2,000 cells/ well) on OE33 cell proliferation at 24 and 72 hours, measured by BrdU incorporation at the absorbance of 370nm.

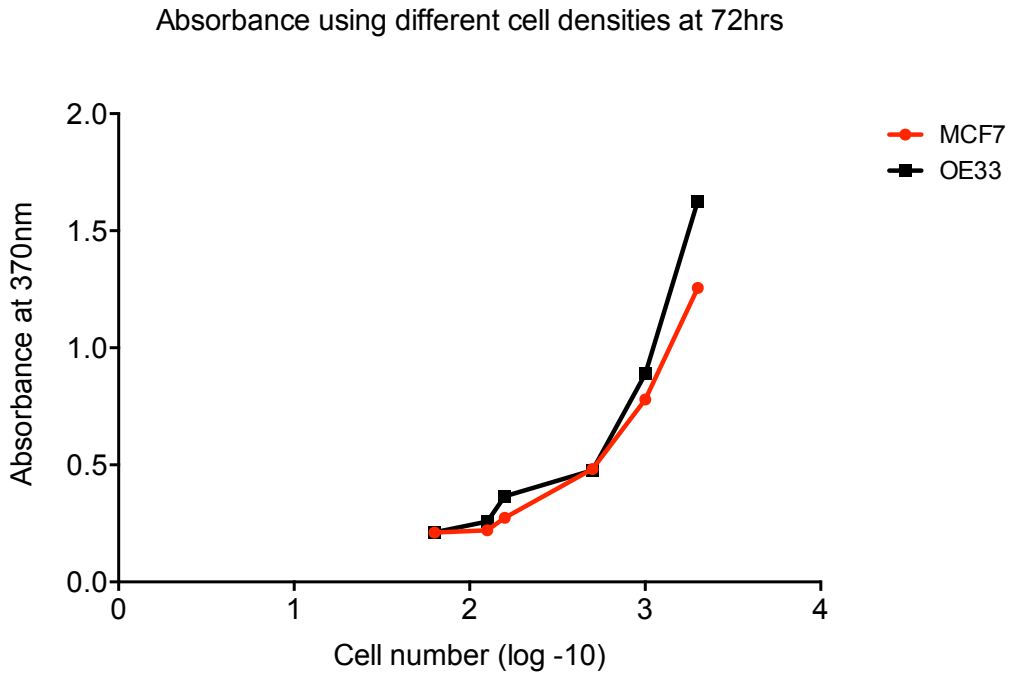


Figure A2.4. Growth curves for MCF7 and OE33 cell lines at different cell densities. Measured by BrdU incorporation at the absorbance of 370nm at 72 hours.

The seeding cell number was optimised further using the positive control drug, etoposide (1000nM), at different cell densities. Cells

seeded at a density of 5,000 cells/ well gave the most significant inhibition of proliferation for both the MCF7 (p=<0.0001, Figure A2.5) and OE33 (p=0.0004, Figure A2.6) cell lines, compared with untreated cells.

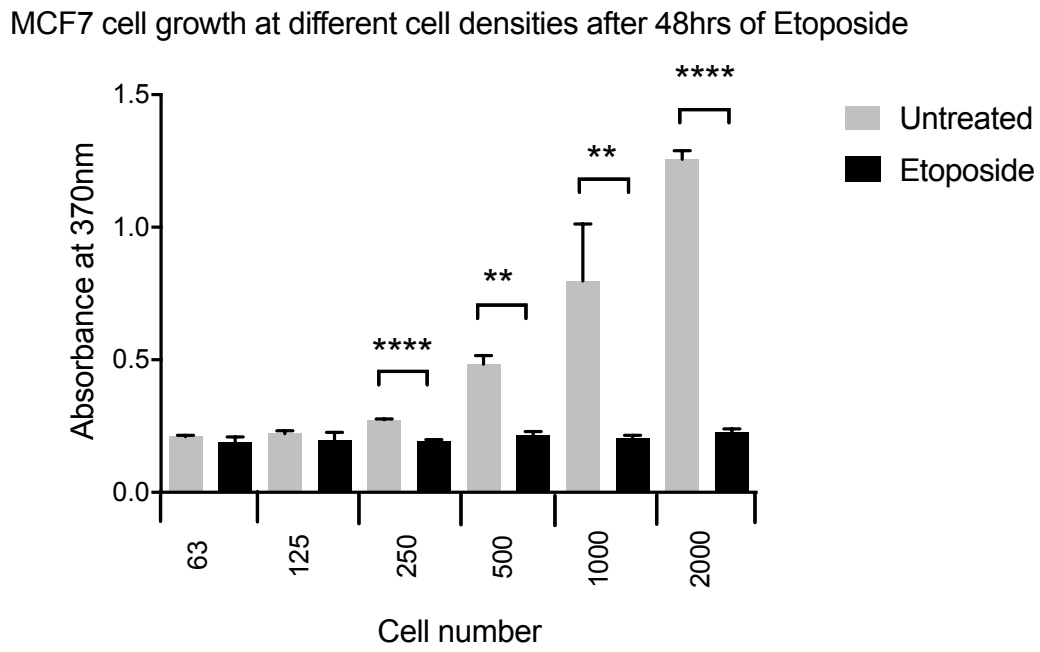


Figure A2.5. The effect of etoposide on MCF7 cells seeded at different cell densities (63 – 2000 cells). Proliferation was measured by BrdU incorporation at the absorbance of 370nm at 48 hours. There was a significant inhibition of proliferation with cells seeded at 250 (**** p=<0.0001), 500 (** p=0.0087), 1000 (** p=0.0091) and 2000 (**** p=<0.0001) cells/ well.

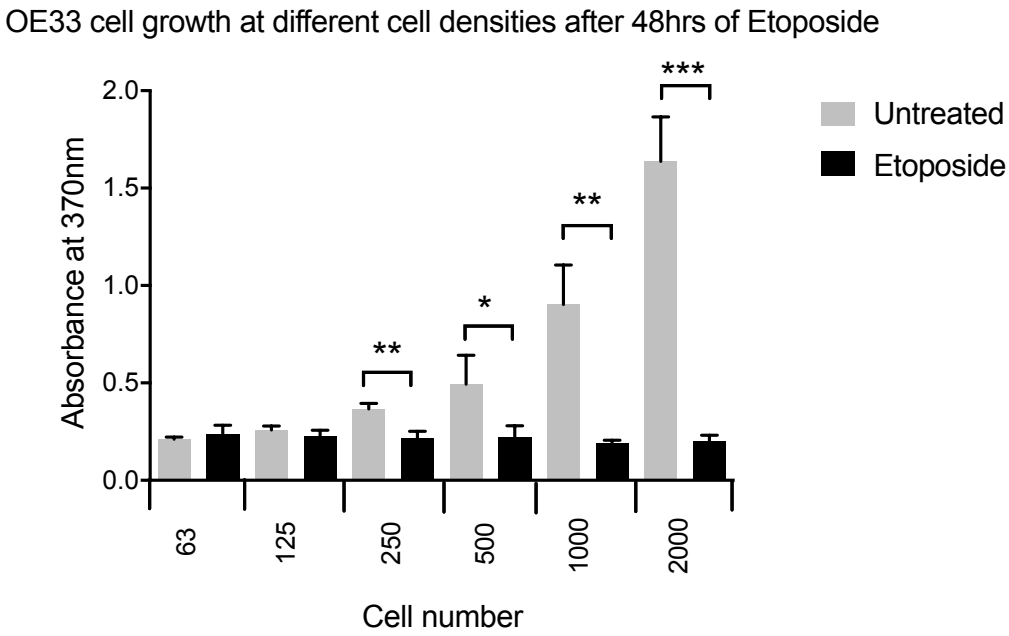


Figure A2.6. The effect of etoposide on OE33 cells seeded at different cell densities (63 – 2000 cells). Proliferation was measured by BrdU incorporation at the absorbance of 370nm at 48 hours. There was a significant inhibition of proliferation with cells seeded at 250 (** p=<0.0049), 500 (* p=0.0423), 1000 (** p=0.0037) and 2000 (*** p=0.0004) cells/ well.

Effect of different media on cell growth

The effect of DMSO and different types of media on cell proliferation was investigated. For the MCF7 cell line, there was no significant difference between cell treated with the 3 different types of media or DMSO (in PRF-RPMI, Figure A2.7, Two-way ANOVA with Tukey's post hoc analysis).

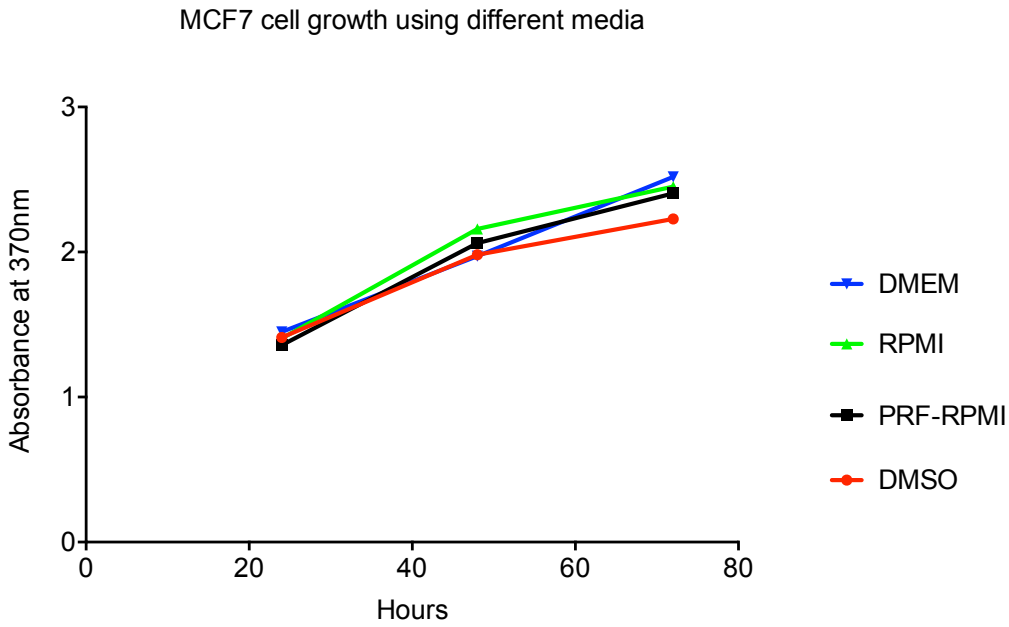


Figure A2.7. The effect of different types of cell media on MCF7 cell proliferation at 24, 48 and 72 hours, measured by BrdU incorporation at the absorbance of 370nm.

For the OE33 cell line, there was no difference between cells grown in DMSO, RPMI or PRF-RPMI. But there was a significant difference between DMEM and the other 3 growth media (p=<0.0001, Two-way ANOVA with Tukey's post-hoc analysis). RPMI was subsequently used for cell culture of both MCF7 and OE33 cell lines and PRF-RPMI was used for all siRNA and cell proliferation studies.

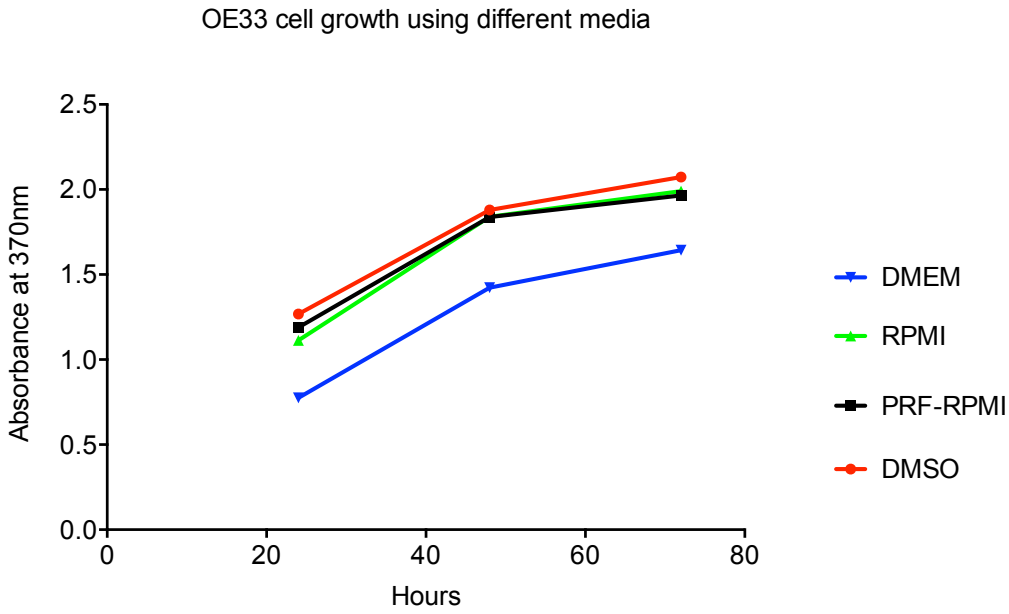


Figure A2.8. The effect of different types of cell media on OE33 cell proliferation at 24, 48 and 72 hours, measured by BrdU incorporation at the absorbance of 370nm. There was a significant difference between DMEM and the other 3 growth media (p=<0.0001, Two-way ANOVA with Tukey's post-hoc analysis).

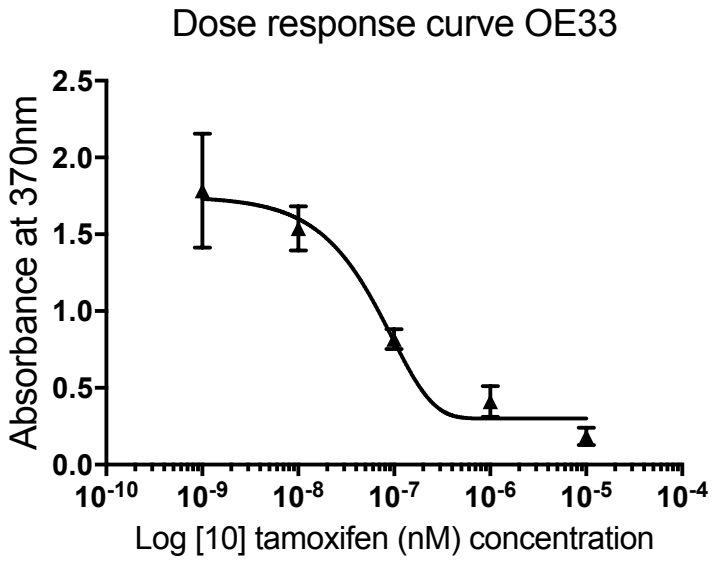


Figure A2.9. Dose response curve for OE33 cells treated with 1-10,000nM tamoxifen at 72 hours.

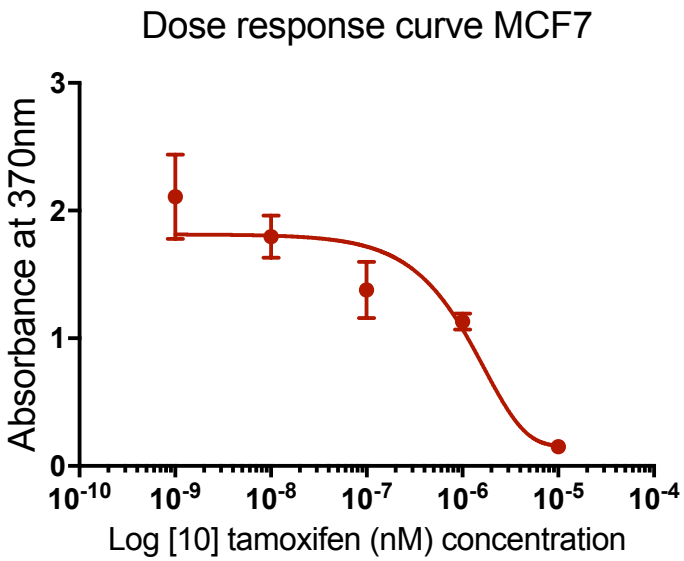


Figure A2.10 Dose response curve for MCF7 cells treated with 1-10,000nM tamoxifen at 72 hours.

8.3 Appendix 3





Participant Information Sheet

(Final version 2.1: 14th December 2015)

Title of Study: A pilot study of Tamoxifen in Oesophageal Cancer

Name of Researcher(s): Mr Syed Iftikhar, Miss Antonella De Rosa, Dr Raheela Khan, Dr Cristina Tufarelli, Mr Waleed Al-Khyatt, Mr Sherif Awad.

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

The purpose of the study is to determine if the anticancer drug, tamoxifen, which is widely used in breast cancer, impairs the growth of oesophageal cancer. Previous work by our research group, and

others, has shown that tamoxifen reduces oesophageal cancer cell growth in the laboratory, and we want to determine if it does the same in oesophageal cancer in humans.

Why have I been invited?

You are being invited to take part because you have oesophageal cancer and will not be undergoing chemotherapy or surgery. We are inviting 20 patients like you to take part.

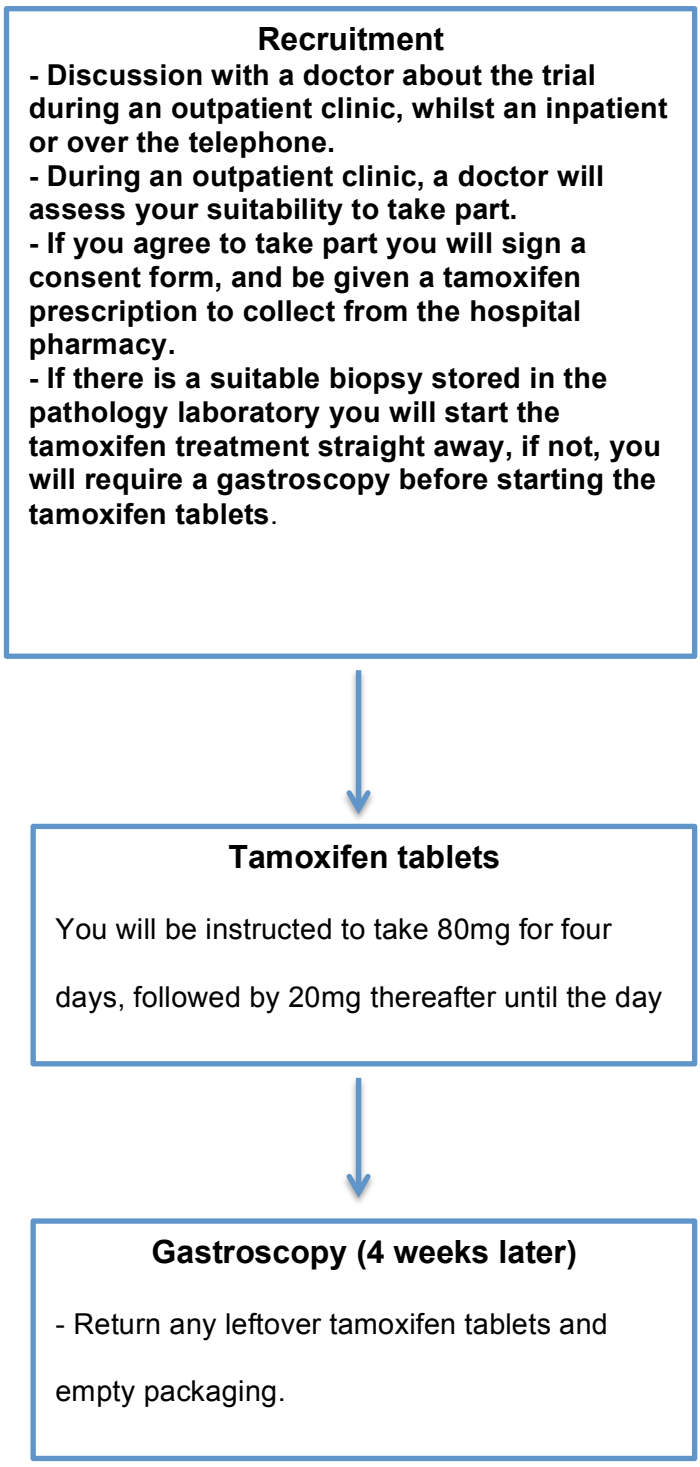
Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights.

What will happen to me if I take part?

You will be approached by one of the doctors during an outpatient appointment, **whilst an inpatient, or contacted via telephone**. The doctor will discuss the study with you, answer any questions, and ensure you are suitable to take part. If you agree to take part, it will involve you taking tamoxifen tablets every day until a gastroscopy examination (a flexible camera passed into the mouth through the gullet into the stomach), where biopsies of the cancer will be taken (removal of small tissue samples). The gastroscopy procedure will be no different to those you have previously experienced for the

diagnosis and/ or surveillance of your cancer. You will attend having fasted for 6 hours and to make the procedure more comfortable receive a spray to numb the throat with or without sedation. The gastroscopy will last approximately 5 minutes. Before being discharged, you will be given something to eat and monitored until the sedation has worn off. The gastroscopy will be arranged for approximately 4 weeks after starting tamoxifen, and you will be required to take the tamoxifen tablets every day. On the day of the gastroscopy, you will stop taking the tamoxifen and be required to return any left-over tablets and empty packaging. Your involvement in the study will end after the gastroscopy, after this, there will be no study follow-up. **If you don't have a suitable tissue sample of the cancer stored in the pathology department at the Royal Derby Hospital, you will also require a gastroscopy and biopsy before starting the tamoxifen tablets.**


Recruitment
- Discussion with a doctor about the trial during an outpatient clinic, whilst an inpatient or over the telephone.
- During an outpatient clinic, a doctor will assess your suitability to take part.
- If you agree to take part you will sign a consent form, and be given a tamoxifen prescription to collect from the hospital pharmacy.
- If there is a suitable biopsy stored in the pathology laboratory you will start the tamoxifen treatment straight away, if not, you will require a gastroscopy before starting the tamoxifen tablets.
Tamoxifen tablets
You will be instructed to take 80mg for four days, followed by 20mg thereafter until the day
Gastroscopy (4 weeks later)
- Return any leftover tamoxifen tablets and empty packaging.

Expenses and payments

You will not be paid to participate in the study, however travel expenses will be offered for attending the hospital for the purpose of the trial.

What are the possible disadvantages and risks of taking part?

Gastroscopy

The gastroscopy examination may be uncomfortable, however, normal hospital guidelines will be followed to minimise any discomfort. Gastroscopy is considered a safe procedure with a low risk of complications. The most serious complications are (1) bleeding - approximately 1 in every 1000 patients, and (2) perforation (damage to the gullet or stomach causing a hole) - approximately 1 in every 5000 patients. If you develop any of these complications you will be admitted to hospital for observation and may rarely require surgery.

Tamoxifen

Tamoxifen is the most widely prescribed anticancer drug in the world and has been used in the treatment of breast cancer for over 35 years. The most common side effect is hot flushes affecting 1 in 10 patients. Additionally, 1 in 10 women might experience vaginal bleeding, discharge, or discomfort and 1 in 10 men may experience loss of libido and impotence.

The most serious side effects of tamoxifen are blood clots in the leg or lung (venous thromboembolism or pulmonary embolism) or brain (stroke), which occur in 1 in 100 patients. You should stop taking

tamoxifen and see a doctor straight away if you develop any of the following symptoms: sudden shortness of breath, chest pain, coughing up blood, leg pain or swelling, physical weakness or slurred speech. Attached is the patient information from the drug manufacturer, which lists all of the possible risks and side effects.

What are the possible benefits of taking part?

Participation in the study will not have any benefit on the outcome of your cancer. However, if the results of the study show that tamoxifen does affect biological oesophageal cancer growth this may help patients in the future.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting the hospital Patient Advice and Liason Service (PALS) on 0800 783 7691, 01332 785156, or dhft.contactpals@nhs.net.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for 1 year after the end of the study. All other data will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot

be erased and this information may still be used in the project analysis.

Involvement of the General Practitioner/Family doctor (GP)

If you agree to take part in the study your GP will be informed by post. They will receive an information sheet informing them you will be taking tamoxifen therapy for the duration of the study and attending for a gastroscopy. The GP will have the opportunity to contact us directly should they have any further questions.

What will happen to any samples I give?

The samples you provide will be taken to the Pathology Laboratory, Royal Derby Hospital for testing.

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than current team of Mr Syed Iftikhar and Dr Raheela Khan, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not agree to this any

remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

Will any genetic tests be done?

No genetic testing will be performed on the biopsy samples provided during this study.

What will happen to the results of the research study?

Once the study has been completed the results will be written up as part of an educational qualification (thesis), and published in peer reviewed scientific journals. The results may also be presented at local and international scientific meetings. You will not be personally identified in any publication, presentation or report.

Who is organising and funding the research?

This research is being organised by the University of Nottingham and is being funded by the Upper GI Research Group, Royal Derby Hospital, Derby Hospitals NHS Trust.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by NRES East Midlands Committee – Nottingham 2, Research Ethics Committee.

Further information and contact details

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DHFT Research & Development Dept. Issued 20 NOV 2015

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Research and Development Office

TRUST APPROVAL LETTER

Mr Syed Iftikhar
Consultant Upper Gastrointestinal Surgeon
Royal Derby Hospitals
Uttoxeter New Rd
Derby
DE22 3NE

Dear Mr Syed Iftikhar

Re: A pilot study of Tamoxifen in Oesophageal Cancer

R&D Reference: DHRD/2015/031

The agreed Recruitment Target for this Study is: 20

Further to the Research Ethics Committee approval for the above study, I am pleased to confirm Trust management approval for you to proceed in accordance with the agreed protocol, the Trust's financial procedures for research and development and the Research Governance Framework (which includes the Data Protection Act 1998 and the Health & Safety at Work Act 1974).

Please supply the following information at the appropriate time points to Dr Teresa Grieve, Assistant Director of R&D via (dhft.randdadmin@nhs.net):

- The date of your first patient recruited to the study
- A report every six months if the study duration is greater than six months
- Notification of any SUSARS, amendments, urgent safety measures or if the trial is abandoned.
- Notification of end of the study and an end of study report.
- Details of any publications arising from this research project.
- As a condition of approval no samples are to be transported from the Trust to the University of Nottingham until a finalised Materials Transfer Agreement is in place.

Please note that approval for this study is dependent on full compliance with all of the above conditions.

The 70 day Target Date for Recruiting the First Patient is 3rd September 2015

The Government's Plan for Growth (March 2011) announced the transformation of incentives at local level for efficiency in initiation and delivery of research. As a result the NIHR have introduced research performance benchmarks: ***studies must recruit to time and target, and first patient must be recruited onto the study within 70 days of submission of local application.*** Trusts will be fined, otherwise penalised and funding withheld if these metrics

are not met. Please ensure you work towards recruiting the first patient by the above date, and inform us if you envisage any problems as we will endeavour to help you meet this target.

I would like to take this opportunity to wish you every success with this study.

Yours sincerely

Teresa M. Envise

pp **Dr Fran Game FRCP**
Director of Research & Development

Short Study Title: A pilot study of tamoxifen in oesophageal cancer
R&D Ref: DHRD/2015/031

In accordance with your application and subsequent R&D approval dated 20th November 2015 the following documentation was reviewed and may therefore be used on the above study with Trust approval.

List of reviewed Documents:

Document	*Version*	*Date*
Protocol	1.1	01.06.2015
IRAS R&D Application		29.04.2015
Participant information sheet	1.1	01.06.2015
Informed Consent form	1.0	10.04.2015
REC Approval Letter		05.06.2015
GP Letter	1.0	10.04.2015
CV for A de Rosa		19.06.2015
CV for S Iftikhar		
CV for R Khan		
Sponsor Indemnity		To 31.07.2016
Sponsor Letter		13.04.2015

NRES Committee East Midlands - Nottingham 2
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839436

05 June 2015

Dr Raheela Khan
Faculty of Medicine & Health Sciences, School of Medicine,
University of Nottingham, Royal Derby Hospital, Derby
DE22 3NE

Dear Dr Khan

Study title:	**A pilot study of tamoxifen in oesophageal cancer**
REC reference:	**15/EM/0225**
Protocol number:	**15021**
IRAS project ID:	**163909**

Thank you for your letter of 3 June 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Carolyn Halliwell, NRESCommittee.EastMidlands-Nottingham2@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Clinical Trials Insurance]		
GP/consultant information sheets or letters [GP Letter Tamoxifen in Patients with Oesophageal Cancer]	Final 1.0	10 April 2015

IRAS Checklist XML [Checklist_29042015]		29 April 2015
Letter from sponsor [Sponsor letter signed]		
Participant consent form [Consent Form Tamoxifen in Patients with Oesophageal Cancer]	Final 1.0	10 April 2015
Participant information sheet (PIS) [Info sheet Tamoxifen in patients with oesophageal cancer]	Final 1.1	01 June 2015
REC Application Form [REC_Form_29042015]		29 April 2015
Research protocol or project proposal [Protocol Tamoxifen in Oesophageal Cancer]	Final 1.1	01 June 2015
Summary CV for Chief Investigator (CI) [CV for CI]		
Summary CV for student [Summary CV for student]		
Summary CV for supervisor (student research) [CV for supervisor]		
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Lay Summary Tamoxifen in Oesophageal Cancer Final 1.0]	Final 1.0	03 May 2015

Statement of compliance

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

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

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

15/EM/0225	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely

pp.

Mr Ian Ross
Vice-Chair

Email:NRESCommittee.EastMidlands-Nottingham2@nhs.net

Copy to: *Angela Shone, University of Nottingham*
Teresa Grieve, Derby Hospitals NHS Foundation Trust