

STRESS HORMONE SIGNALLING
CONTRIBUTES TO TUMOURIGENESIS
THROUGH THE PRODUCTION OF ROS/RNS,
INDUCTION OF DNA DAMAGE AND
INTERFERENCE WITH CHEMOTHERAPY IN
BREAST CANCER

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A thesis submitted in partial fulfilment of
the requirements of the University of
Brighton for the degree of Doctor of
Philosophy

October 2017

Abstract

Breast cancer affects 1 in 8 women in the UK, and breast cancer patients often report increased levels of psychological stress. Psychological stress results in an increase in the circulating levels of the stress hormones glucocorticoids and catecholamines. Currently, few molecular mechanisms exist linking the actions of stress hormones and breast cancer progression. However, it has recently been suggested that stress hormones can promote DNA damage through the generation of reactive oxygen/nitrogen species (ROS/RNS). This research aims to explore the effect of stress hormone signalling on breast cancer progression and response to treatment.

The generation of ROS/RNS and induction of DNA damage was measured in breast cancer cell lines. Pharmacological inhibition of the glucocorticoid receptor (GR) and inducible nitric oxide synthase (iNOS) was used to negate the effects of stress hormone exposure. Psychological stress, using restraint stress, was induced in a syngeneic mouse model of breast cancer, alongside *in vivo* inhibition of NOS. DNA damage and repair process were examined in response to the glucocorticoid cortisol in an endocrine therapy resistant cell line, and the effect of exposure to the exogenous glucocorticoid dexamethasone on the efficacy of chemotherapy in breast cancer cells was also explored.

Stress hormones were shown to induce the generation of ROS/RNS and promote DNA damage. Specifically, exposure to cortisol produced an increase in nitric oxide (NO) through an iNOS-mediated pathway. Inhibition of both the GR and iNOS reduced cortisol-induced DNA damage. In a mouse model of breast cancer, inhibition of NOS significantly reduced primary tumour volume, angiogenic signalling in the primary tumour and metastatic spread in stressed mice. Cortisol increased ROS/RNS and DNA damage in endocrine therapy resistant breast cancer cells compared to parental cells, and deregulated DNA repair processes. The cytotoxic effect of chemotherapy agents was reduced in response to co-treatment with the glucocorticoid dexamethasone, through upregulation of the antioxidant response.

In conclusion, this research demonstrates that stress hormones impact tumourigenic progression in breast cancer through the induction of DNA damage, mediated by the release of NO. This data also shows that endocrine resistant breast cancer cells are more responsive to the actions of glucocorticoids on DNA damage and repair. Furthermore, exogenous glucocorticoids can impair the efficacy of chemotherapies, through the generation of ROS/RNS. The role of psychological stress should therefore be considered in the treatment of breast cancer patients, and stress hormone receptor signalling could provide potential therapeutic targets for the treatment of breast cancers.

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List of the main abbreviations

β -AR	Beta-adrenergic receptor
ANOVA	Analysis of Variance
ARE	Antioxidant response element
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CS-FBS	Charcoal-stripped foetal bovine serum
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DEX	Dexamethasone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECM	Extracellular matrix
EMT	Endothelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ER	Oestrogen receptor
FAS-R	Faslodex resistant
FBS	Foetal bovine serum
GR	Glucocorticoid receptor
H ₂ O ₂	Hydrogen peroxide
HER2	Human epidermal growth factor receptor 2
IL-	Interleukin-
iNOS	Inducible nitric oxide synthase
L-NAME	N(G)-Nitro-L-arginine methyl ester
MMP	Matrix metalloproteins
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NOS	Nitric oxide synthase
ONOO ⁻	Peroxynitrite
PAC	Paclitaxel
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	Progesterone receptor
qRT-PCR	Real-time reverse transcriptase polymerase chain reaction
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SEM	Standard error of the mean
TAM	Tumour associated macrophage
TBST	Tris buffered saline – tween20
TNBC	Triple negative breast cancer

Acknowledgments

Commented [RF1]: Comment 1

I would like to dedicate this thesis to the three most influential women in my life, my Mum, my Nonna and my supervisor Melanie. These women have nurtured me emotionally and professionally, and have given me the strength, motivation and determination to achieve all I set out to do. To my Mum, who inspires me daily and is my best friend and most ardent supporter, I cannot express my gratitude for everything you have done for me. To my Nonna, without whom I could not have embarked on this process. I will be forever thankful for everything she passed down, but my most treasured inheritance is the ambition she instilled in me. And to Melanie, who has afforded me every opportunity, and inspired my love of research. Her unwavering belief in me has been the driving force behind my successes. I am incredibly lucky to count her as a mentor and friend.

I would also like to extend my thanks to Dr Bhavik Patel and Dr Marcus Allen for their sound advice, guidance and support, and to our collaborator Dr Julia Gee for her generosity. Similarly, to all the academics, researchers, technicians and students in the Pharmacology lab, whom have helped and encouraged me.

To my colleagues and friends in the office, who truly understand the journey I have undertaken, and have laughed and despaired with me in equal measure. Your invaluable council and friendship I am especially grateful for. I would also like to extend heartfelt thanks to my colleagues Matt, Katie and Dave, whom not only contributed extensively to the work presented in this thesis, but also to my thorough enjoyment of my time spent in the lab.

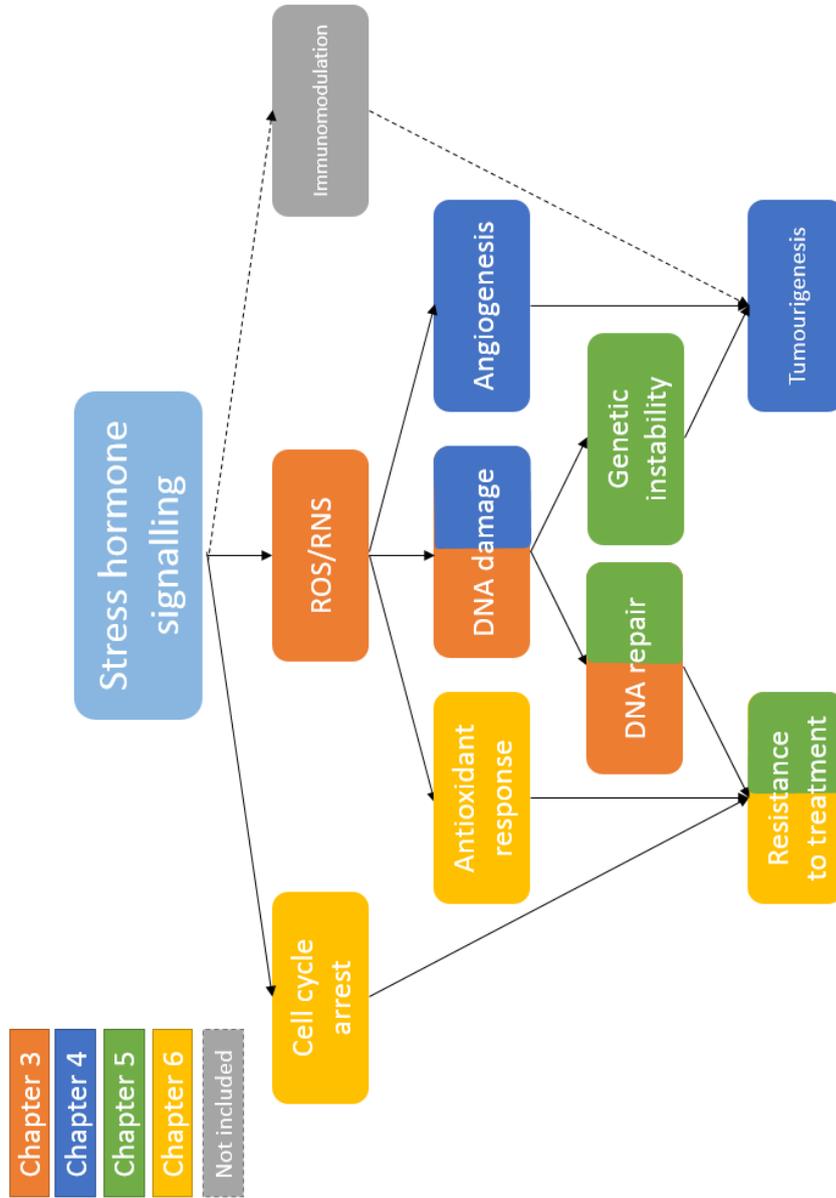
Lastly, to my family, I am eternally grateful for their unwavering love and support, and to Tom, who has kept me grounded when necessary, and lifted me up when I needed it, I owe a great deal.

Author's declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

6/10/2017



Chapter 1 – Introduction

1.1 Introduction to breast cancer

1.1.1 Breast cancer incidence and risk factors

Breast cancer is medically defined as cancer of the cells that make up the breast tissue. Breast cancer affects around 50,000 women per year in the UK, and is the leading cause of mortality in women aged 35-49 (1). In the UK, breast cancer incidence rates have increased by 72% since the 1970's, with the introduction of national screening programmes in the late 1980's causing a further increase. However, since the mid-2000's incidence rates have remained stable (2). Currently breast cancer has one of the best survival rates at 78% after 10 years, and it is accepted that approximately 27% of breast cancer cases are preventable (3).

The biological causation of breast cancer is multi-faceted and dynamic, and as such cannot be attributed to a singular factor. Several modifiable and non-modifiable risk factors have been identified that may contribute to the formation of a tumour. The lifetime risk of breast cancer is 1 in 8 for females as opposed to 1 in 1000 for males, and rates of incidence increase with age, rising steeply during a woman's reproductive lifespan then increasing at a slower rate post menopause (4). Height and weight are also considered risk factors, with taller women at a slightly increased risk, and postmenopausal women with a high BMI at a more significant risk. This is likely due to the production of oestrogen by adipose tissue (5, 6).

Reproductive hormones play an important role in breast cancer epidemiology, with oestradiol - a potent oestrogenic hormone produced by the ovaries - stimulating breast cell mitosis through oestrogen receptors (ER's) (7). As such, a positive increase in risk is associated with women who undergo early menarche and late menopause, as the period of exposure to oestradiol is extended (8). The oestrogenic fluctuations observed during pregnancy also have an effect on breast cancer risk, with a reduction seen in women who have had at least one pregnancy, compared to that of nulliparous women. Furthermore, the age at first pregnancy has an effect on incidence, with women who have their first child before 20 at a significantly lower risk, and those that have their first after 35 in the highest risk group (9). The use of oral contraceptives containing progesterone has also been linked to a slight increase in risk of breast cancer diagnosis. Women using contraceptives before the age of 20 have a higher relative risk, although since breast cancer incidence is lowest in that age group the effect on overall risk is minor. However, the risk does not increase in line with exposure and 10 years after cessation, returns to normal with no substantial increase observed in the long term (10). The use of hormone replacement therapy (HRT) has been correlated with an increased risk of breast cancer in postmenopausal women, with combined oestrogen and progesterone therapies potentially promoting pre-neoplastic lesions (11). Exposure to other carcinogens such as tobacco and alcohol have also been shown to increase risk of incidence, with active tobacco smoking

increasing risk significantly and an association with passive smoking also observed (12). Alcohol can increase the levels of circulating oestrogen in women, further stimulating mutagenic transformation (13). These risk factors are credited with the observable increase in breast cancer incidence in developed countries, as their populations are typically older, have increased obesity and alcohol consumption, and lower childbirth rates (14).

While environmental factors can be held accountable for proportion of incidences of breast cancer, mutations in specific genes can also convey susceptibility, and it is estimated that 5-10% breast cancer cases occur as result of genetic factors (15). Based on the epidemiological observation that a familial history increases the risk of developing the disease (16), exploration of the roles genetic factors may play in the formation of breast cancer is an increasingly important subject. To date, several genes have been identified as breast cancer susceptibility genes, and genetic testing is now commonplace for first generation relatives of breast cancer patients. The first gene linked to a predisposition for breast cancer was *BRCA1* in 1994 (17), and subsequently *BRCA2* was also identified. The protein products of these genes act as tumour suppressors, facilitating maintenance of genomic integrity by repairing DNA damage, amongst other functions (18). Mutations passed through Mendelian inheritance can therefore render the roles of *BRCA1* defunct, increasing the likelihood of breast cancer significantly.

1.1.2 Classification of breast cancer

In order to classify breast cancers, the structure of breast tissue must first be understood. The bulk of breast tissue comprises of adipose tissue (stroma), and within the breast there are 12-20 lobes responsible for milk production. Each lobe is made up of branching terminal duct lobular units that produce breast milk, which collects in ducts terminating in the nipple. The mammary duct is comprised of myoepithelial cells, referred to as basal cells, which are contractile in order to eject milk but do not express the hormone receptors necessary to stimulate milk production. Epithelial cells, or luminal cells, line the lumen of the duct and respond to hormonal stimulation for milk production, as they express the relevant hormone receptors. Breast stem cell populations in the breast tissue give rise to both basal and luminal cells depending on differentiation signals (19). The breast also contains a complex network of blood vessels, lymph vessels and nerves (Fig. 1.1.) (20).

Fig. 1.1 The structure of the breast

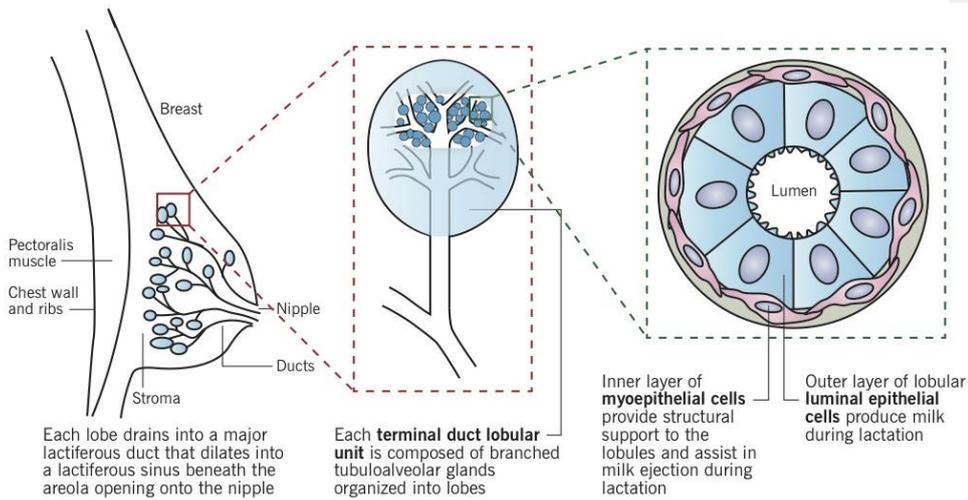


Fig 1.1 The anatomy of the breast comprises of the stroma, lobes and ducts. Each lobe is comprised of terminal duct lobular units which produce milk during lactation. Figure taken from (20).

Cancers arising in the different types of cell within the breast therefore express a different phenotype and need to be treated as distinct subtypes in order to establish a prognosis and determine the correct treatment route. Histological classification defines both the type of tissue the cancer has originated from, as well as the extent of spread. Ductal carcinomas originate from ductal tissue, and can be *in situ* (where the cells are limited to the basement membrane) or invasive (where the cells have spread beyond the basement membrane). Ductal carcinoma in situ (DCIS) accounts for 80% of the non-invasive breast cancer cases, and around 1% can become invasive. Invasive ductal carcinoma (IDC) accounts for 79% of all invasive cancers and spreads through the body (metastases) through the lymphatics and blood. Lobular carcinomas arise in the lobular tissue and lobular carcinoma in situ (LCIS) make up 20% of non-invasive cases. Invasive lobular carcinoma (ILC), which make up the remaining 10% of invasive cancers, metastasise through a distinctly different pathway and can spread through the abdominal viscera to the GI and gynaecological organs (21) (22).

Breast cancers are routinely further separated accruing to molecular subtype, specifically the expression of certain prognostic markers such as hormone receptors. The main receptors taken into consideration when classifying a tumour are the oestrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). The presence of these receptors indicate tumour cells are able to receive signals from hormones and growth factors, which may drive growth of the tumour. Furthermore, they also indicate the tumour may be responsive to hormone therapies. Other markers such as claudin (a protein responsible for regulating cell permeability and tight-junctions), KI67 (a proliferative marker) and epidermal growth factor receptor (EGFR) (a regulator of growth signalling) are also increasingly used to classify subgroups of breast cancer (21). The commonly accepted subtype classification is summarised in Table 1. Histological subtype and molecular classification are also combined with lymph node invasion and tumour size and to give an overall indication of risk according to established indices such as the TNM staging system and the Nottingham Prognostic index (23).

Table 1: Breast cancer molecular classifications

Intrinsic subtype	IHC status	Grade	Outcome	Prevalence
Luminal A	ER+ /PR+ /HER2- /KI67-	1/2	Good	23.7%
Luminal B	ER+ /PR+ /HER2- /KI67+	2/3	Intermediate	38.3%
	ER+ /PR+ /HER2+ /KI67-		Poor	14%
HER2+	ER- /PR- /HER2+	2/3	Poor	11.2%
Basal	ER- /PR- /HER2-	3	Poor	12.3%
Normal-like	ER+ /PR+ /HER2+ /KI67-	1/2/3	Intermediate	7.8%

Table 1 – data taken from (24).

These classification systems provide clinicians and researchers with the tools to allow for more informed therapeutic decision making, and provide greater accuracy in predicating the response to treatment as well as overall outcome. For example, Luminal A type ER+/HER2- cancers are likely to express similar genes to breast luminal cells, and do not express high levels of proliferation related genes, meaning that they are generally less aggressive and can be treated with therapies that target the ER (24). Conversely, cancers which have a high expression of HER2 are associated with mutations in tumour suppressor genes such as p53, and are usually of a higher grade. However, these cancers can be treated quite successfully with HER2 targeted therapies (25). Triple negative breast cancers (TNBC) are characterised by a lack of ER, PR and HER2 receptors and can be grouped into the subgroup of basal like due to their similarity in gene expression. TNBC is more likely to occur in younger women, and in women with a *BRCA1* mutation (26). The promotion of cellular proliferation and invasion as a result of the expression profile of TNBC produces a highly aggressive tumour with a high rate of metastases. As such the development of targeted treatments has been notoriously difficult, and the outcome for patients is still poor in comparison to the advancements made overall in breast cancer survival rates. Therefore patients with TNBC are most commonly treated with cytotoxic chemotherapies that indiscriminately kill cells. Metastases and resistance to treatment accounts for the majority of fatalities in patients with TNBC, with the most common sites of metastasis being the lung, brain and bone (27).

1.1.3 Breast cancer therapeutics

In the last half a century the range of treatment options for breast cancer has widened enormously, with the understanding that the term breast cancer represents a whole subset of distinct cancers that must be treated accordingly.

Classically, locoregional therapy was the first line of treatment for breast cancer, however significant advances have been made in surgical techniques as well as neoadjuvant (preoperative) therapies. Currently radical mastectomies - whereby the whole breast as well as underlying chest muscle and axillary lymph nodes are removed - are being increasingly replaced in favour of modified radical mastectomies or simple mastectomies - where breast tissue is removed but the surrounding tissue is preserved. Breast conserving surgery can also come in the form of a lumpectomy, whereby only the tumour containing tissue is removed preserving most of the breast, this is usually then followed by radiation therapy (28). Mastectomies can also be performed as a prophylactic response to genetic counselling in women who have hereditary germline mutations in *BRCA1* or *BRCA2*, genes that dramatically increase predisposition to breast cancer (29).

Significant advances have also been made in the development of targeted therapies for ER-expressing breast cancers. Anti-oestrogen therapies, through competitive binding, act to block ER signalling which is known to play a crucial role in the growth of tumours (30). The most widely used anti-oestrogen and a mainstay of endocrine therapy is Tamoxifen, with the majority of ER-positive breast cancers being treated with tamoxifen for 5 years after surgery (adjuvant therapy) (31). Tamoxifen is a selective oestrogen receptor modulator (SERM) and functions as a competitive antagonist and agonist, blocking oestrogen from binding to the receptor in breast cancer cells and promoting proliferative signalling. Selective oestrogen down regulators (SERD's) are another class of compounds capable of modulating the activity of ER's. SERD's such as fulvestrant have more potent antagonistic effects than SERM's and function to block ER activity as well as degrade the receptor. As such they are only used to treat postmenopausal women, and are used to combat Tamoxifen resistant tumours as a second line of defence in most cases (32). Post-menopausal women can also be treated with aromatase inhibitors. The enzyme aromatase catalyses the conversion of androgens to oestone and oestradiol, and its presence in breast tumours correlates with higher levels of oestrogenic signalling. As such aromatase inhibitors are most commonly used in post-menopausal women where oestrogen production is primarily a result of androgen aromatization (33). Following the success of targeted anti-oestrogenic therapies, HER2 was recognised as an appealing target due to its high rate of overexpression in breast cancer and its predictive value. The humanized recombinant antibody trastuzumab was developed, which binds to an external part of the HER2 receptor leading to inhibition of proliferative signalling, whilst having no effect on HER2-negative cells. Trastuzumab is therefore now regularly given as a first line monotherapy in patients with high expression of HER2 (34).

Endocrine therapies are the most common and effective treatments for breast cancer, however they are often limited by resistance, which can be acquired over the course of treatment, or exist intrinsically (*de novo*). Resistance to prolonged exposure to tamoxifen occurs in a high percentage of cases due to mechanisms such as loss of the steroid receptor and activation of alternate signalling pathways (35). Similarly resistance to the SERD fulvestrant can result from loss of expression of the ER, and upregulation of alternative signalling pathways such as HER2 and epidermal growth factor receptor (EGFR) (36).

For aggressive triple negative breast cancers, therapies targeting the hormonal or HER2 receptors are not an effective treatment, and often surgical intervention followed by radiotherapy is called for. Cytotoxic chemotherapy drugs can also be administered to treat TNBC alone or in combination with other therapies. Taxanes such as paclitaxel and docetaxel are widely used chemotherapeutic agents, capable of disrupting microtubule formation and arresting the cell cycle, inducing apoptosis (37). Several cancers have shown good response to

taxane treatment, however acquired resistance is common following long term exposure (38). Similarly, anthracyclines are another class of highly effective cytotoxic drugs, with doxorubicin and epirubicin used specifically in the treatment of breast cancer in a neoadjuvant and adjuvant setting. Anthracyclines work to inhibit DNA synthesis through intercalation of DNA, as well as inhibition of topoisomerase II, an enzyme involved in transcription. The metabolism of anthracyclines also promotes the generation of damaging free radicals that disrupt DNA, protein and lipid structure (39). However the use of anthracyclines in the treatment of TNBC has declined in favour of taxanes, as anthracyclines can be associated with dangerous side effects (37).

Platinum-based therapies such as cisplatin and carboplatin are also used in the treatment of breast cancer, as they cause crosslinking of DNA and subsequent inhibition of DNA repair and synthesis (40). Platinum based therapies work well in *BRCA1* deficient cancers, which are especially sensitive to inhibition of DNA repair. However there has been renewed interest in their use in the treatment of TNBC due to the newly discovered similarities between TNBC and *BRCA1*-deficient tumours (41).

Currently, new targeted treatment strategies for *BRCA*-deficient cancers are being developed in order to improve efficacy and reduce the toxicities associated with conventional chemotherapies such as platinum –based therapies. The discovery of a family of proteins, poly(ADP-ribose) polymerases (PARPs), involved in early signalling of DNA-damage repair prompted investigation into PARP inhibitors, which target the fault in DNA repair machinery caused by a *BRCA* mutation and can promote synthetic lethality in mutated cancers. PARP's function to signal the recruitment of DNA-repair complex's at sites of damage, primarily promoting base excision repair (BER) and single strand break repair (SSBR) (42). In *BRCA*-deficient cancer cells, the integrity of homologous recombination as a method of repairing DNA damage is compromised, and as such cells must rely of secondary methods of repair including BER and SSBR. The inhibition of PARP in these cells therefore prevents the repair of DNA and causes synthetic lethality, with close to no effect on *BRCA*-proficient cells (Fig. 1.2)(43). Proof-of-concept clinical trials of PARP inhibitors (PARPi) have demonstrated favourable outcomes for *BRCA*-mutated breast cancer patients (44), and larger phase 3 clinical trials are underway to investigate the efficacy of PARPi as a monotherapy or in combination with chemotherapies (45).

Fig. 1.2 PARP inhibitor mechanism of action

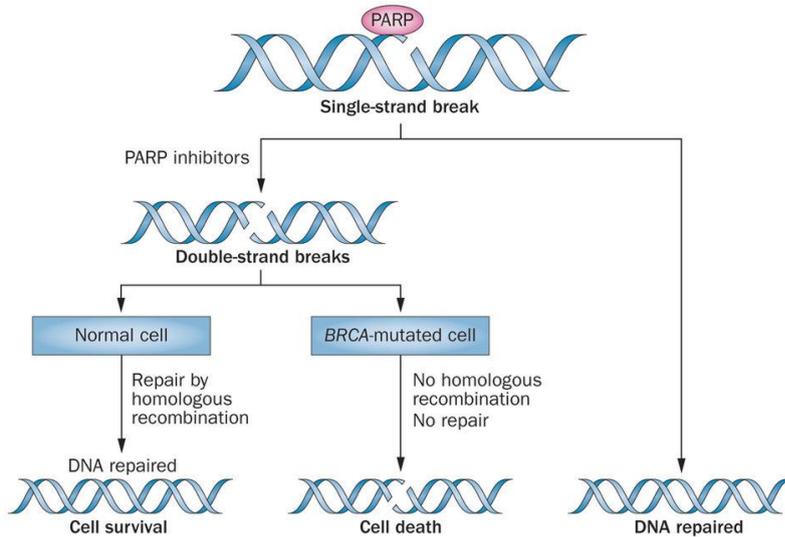


Fig. 1.2 PARP inhibitors represent a potential therapeutic strategy in *BRCA*-deficient cancers through targeting DNA-repair defects and promoting synthetic lethality. In cells with intact DNA repair machinery, inhibition of PARP signalling promotes the repair of DNA damage through homologous recombination (HR). However in *BRCA*-deficient cells where HR is compromised, inhibition of PARP prevents repair occurring and the cell is directed into apoptosis. Figure taken from (45).

1.2 Introduction to psychological stress

In order to maintain homeostasis, it is necessary that organisms are able to respond to changes in their environment. Exposure to physical and psychological stressors - which are a perceived threat to the homeostatic equilibria - calls for the ability to respond appropriately for the sake of survival, giving rise to the stress system. Thus, stress is a term that encompasses a broad range of psychological and physiological processes within the human body in response to perceived threat. Hans Selye (1907-1983) first introduced the concept of stress as a biological response in 1936 (46). His classic concept of stress detailed that the reaction to a stressor occurs in three stages – alarm, resistance and exhaustion, and that these responses constituted a general adaptation syndrome. Subsequent research built on Selye's theory has redefined the term stress and disentangled the mechanisms that underlie the stress response. It is now well established that stress does not evoke the same uniform response, instead that different types of stressors mediate different patterns of response and these vary from individual to individual. Moreover the response also differs between sexes, with the female response to stress manifesting in more biobehavioural actions thought to be associated with reproductive hormones (47).

The stress response system has evolved out of a necessity to respond to threats, however in the recent evolution of humans, what promotes the initiation of the stress response has changed dramatically, while the actual stress response has not. Modern environmental stressors identified include socioeconomic burden, social isolation and negative life events as opposed to life-threatening survival situations. As such this presents an evolutionary disparity between stimuli and response, where responses designed for a 'fight-or-flight' such as an increase in appetite to fuel increase energy expenditure, are chronically overstimulated and can lead to an increased risk of disease.

1.2.1 Biological mechanisms of stress

In humans, the stress response is controlled by fluctuations in hormonal secretions, primarily glucocorticoids and catecholamines, under the control of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) (48). Activation of these systems results in host priming responses including vasoconstriction and tachycardia, as well as metabolic catabolism and redirection of resources to stressed sites, designed to allow for a fight of flight response (49). In some instances stress exposure may exert a positive effect on performance, enhancing function on a cellular and physiological level (50). For example, short term exposure to glucocorticoids has been shown to increase mitochondrial function and improve neuroprotective effects in cells (51); a beneficial effect termed 'eustress'. However, exposure to sustained stress hormone signalling has been linked to an increased risk of diseases such as hypertension, immune dysfunction and cancer (Fig. 1.3)(52).

Fig. 1.3 The endocrine stress response

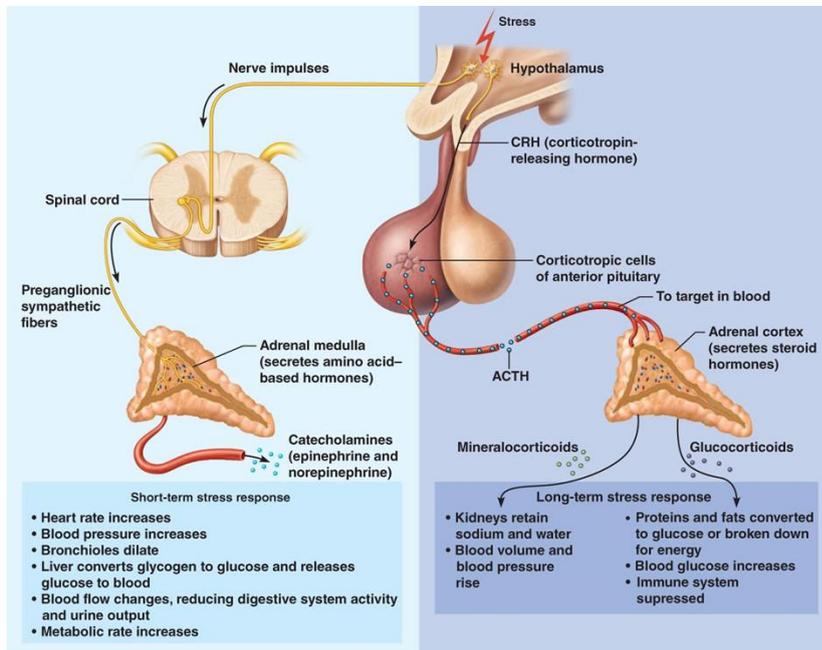


Fig. 1.3 As part of the stress response, the endocrine hormones catecholamines and glucocorticoids are released into circulation. The sympathetic nervous system signals the adrenal medulla to secrete catecholamines in response to stress signalling, which then act to induce a short term stress response including increasing blood pressure and heart rate. The hypothalamic-pituitary axis stimulates the adrenal cortex through the release of adrenocorticotrophic hormone synthesise glucocorticoids, which in turn can have longer term effects such as suppression of the immune system. Figure reproduced from (53).

1.2.2 Glucocorticoids

Glucocorticoids are regulated through the HPA axis and function in many roles, with secretions playing a part in the circadian rhythm system as well as mediating adaptive responses under the stress system (54). The glucocorticoid cortisol, an important hormone released under stressful conditions, is synthesised from cholesterol in the adrenal cortex. It is released when corticotropin-releasing hormone (CRH) in the hypothalamus triggers the anterior pituitary to release adrenocorticotropic hormone (ACTH), in response to complex stress signalling from neurons and somatic cells. This in turn stimulates the release of cortisol by the adrenal cortex. Cortisol can then act upon a number of systems to regulate homeostatic mechanisms by binding to its cytoplasmic glucocorticoid receptor (GR), present on nearly every cell in the body (55). Activation of the GR translocation to the nucleus where gene activation and transcription can be negatively or positively modulated. In this way, cortisol facilitates a priming increase in blood glucose levels by triggering the expression of enzymes necessary for gluconeogenesis. Glucocorticoids also suppresses immune responses and activates anti-inflammatory pathways by downregulating expression of certain interleukins (IL's), thus decreasing immune cell numbers and activation. This is thought to be a protective mechanism, directing the body's resources away from non-essential processes (56). Glucocorticoids are metabolised through enzymatic transformations, increasing their solubility in water allowing for urinary excretion. However the majority of serum cortisol is reduced to inactive metabolites dihydrocortisol and tetrahydrocortisone, which are cleared primarily in the liver (57).

Daily production rate of cortisol in healthy individuals ranges from 33-41 $\mu\text{mol/day/m}^2$ (58), and studies have shown that exposure to psychological stressors can increase the levels of free cortisol in both humans and animals (59-61). Monitoring of glucocorticoid levels can provide information the effects of stress on normal physiology, and importantly, the effects of stress on chronic illnesses. Measurement of cortisol as a biochemical marker of stress is challenging however, as circulating levels of cortisol fluctuate according to circadian rhythms, meaning serum and saliva sampling must be carried out at multiple time points (53). Furthermore, the half-life of cortisol in plasma is relatively short at 66 minutes, and the levels of excretion in urine are low, with 80-90% being reabsorbed. This presents further problems in the accurate correlation between perceived stress and a physical biomarker of stress (57). The measurement of cortisol in hair provides biological information regarding cortisol levels over a longer time period, as cortisol passively diffuses into the hair follicle via blood capillaries. Hair grows at around 1cm/months and can be sampled at any time to provide retrospective analysis or a baseline measurement (53).

1.2.3 Glucocorticoid receptor mechanism of action

The glucocorticoid receptor comprises of a ligand-binding domain (LBD), a DNA-binding domain (DBD) and an N-terminal domain and can be found in the cytoplasm. Unbound, it is complexed with heat shock (HSP) and chaperone proteins in order to maintain the correct ligand binding conformation. Binding of glucocorticoids induces conformational changes including dissociation from HSP's and homodimerization, allowing translocation to the nucleus. The GR is able to bind via its DBD to specific glucocorticoid response elements (GRE's) in gene promotor regions of DNA, and facilitate biphasic transcriptional activation (transactivation). GR's are also able to interact with transcription factors such as AP-1 and Nf- κ B, inhibiting their transcriptional ability, thus supressing gene expression by a mechanism known as transrepression (62). In this way glucocorticoids through the GR can regulate a number of crucial genes controlling survival, inflammation, apoptosis and tumour suppression (63).

Whilst the effects of GR activation have been classically described as genomic, GC's have been shown to mediate almost immediate non-genomic actions (i.e. not mediated through binding to gene promoters) on other signalling processes. This could be as a result of the rather more unexplored multi-protein complex that the GR dissociates from (64). The complex includes proteins such as Src, which is released rapidly after the GR activation and functions as a multifunctional protein involved in survival, proliferation and angiogenesis (65) (66). Activation of the GR has also been associated with a non-genomic upregulation of phosphatidylinositol 3-kinase (PI3K) activity, which in turn upregulates endothelial nitric oxide synthase (eNOS) and increases the production of nitric oxide (NO), an important intracellular signalling molecule (67).

Fig. 1.4 Glucocorticoid receptor mechanism of action

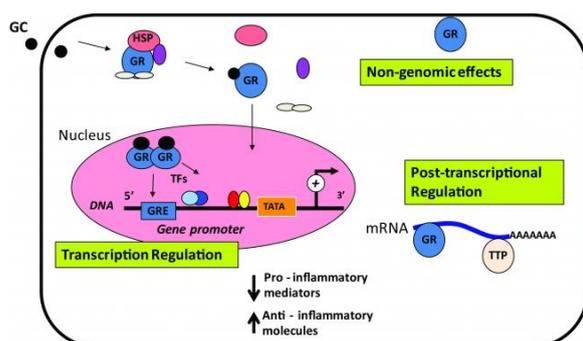


Fig. 1.4 The glucocorticoid receptor mediates transactivation and transrepression of genes through binding to gene promoters, as well as non-genomic actions mediated through dissociation of chaperone proteins. Figure taken from (68) .

1.2.4 Catecholamines

The catecholamines epinephrine (E) and norepinephrine (NE) are produced in the adrenal medulla by chromaffin cells and released into circulation when stressors activate the sympathetic nervous system (SNS). Neural fibres of the SNS are able to release these neurotransmitters into all the major organ systems within seconds, allowing for rapid physiological responses. Once released the effects of catecholamines are mediated by alpha- or beta- adrenergic receptors (AR's), which exist in subtypes (α 1-, α 2-, β 1-, β 2- and β 3-) and are distributed throughout tissues accordingly. Alpha-adrenergic receptors primarily mediate vasoconstriction and contraction of smooth muscle and are present on vascular muscle. Beta-adrenergic receptors also regulate muscle contraction with β 1-adrenergic receptors located on myocardial muscle acting to increase blood pressure and heart rate thus increasing blood flow to skeletal muscles. β 2-adrenergic receptors present on bronchial smooth muscles facilitate muscle relaxation and vasodilation and also stimulate glucose metabolism (69, 70).

1.2.5 Adrenergic receptor signalling

Activation of β -adrenergic receptors stimulates synthesis of cyclic adenosine monophosphate (cAMP) which acts as an effector in a range of cellular processes. As a result of cAMP upregulation activation of protein kinase A (PKA) can occur, which in turn allows phosphorylation of a number of PKA-receptive proteins involved in cell survival, proliferation and gene transcription. Induction of gene transcription occurs as a result of PKA-induced phosphorylation of transcription factors responsible for the transcription of genes promoting stress response elements, such as cell differentiation and metabolism (69).

1.3 Stress and breast cancer

Anecdotal evidence has long suggested a link between psychological well-being and cancer, with large movements attributing anything from increased length of survival – or in some cases complete remission - to a positive mental attitude. As such many studies in the fields of psychology, physiology and molecular biology have sought to find correlation between psychological states and cancer incidence, including factors such as stress, social isolation and depression.

1.3.1 Quantification of Psychological Stress

Much of the literature regarding the effects of psychological stress and cancer focusses on the epidemiological link between stressors, such as major life events or work stress, aiming to quantify the stress response and correlate it to cancer initiation, progression and survival (71). The quantification of psychological stress as a measurable variable is difficult as there is no single accepted definition of stress. It is largely considered to be as a result of environmental 'stressors' facilitating a biological endocrine response, however reaction to such environmental stressors varies innumably between individuals. Moreover each study is subject to confounding factors such as study design, assessment of stress and type of stress exposure. Many studies use stress questionnaires' such as the perceived stress scale (PSS) (72), a standardized self-report questionnaire used to assess a person perception of their stress levels, with higher scores indicating greater overall stress. These questionnaires include questions such as 'How often have you felt nervous or stressed?' which are rated for the past month on a five point Likert scale (0= never to 4=very often). Measures of quality of life and life events are also used to assess how traumatic life events and chronic health issues are affected (73). Questionnaires are however prone to limitations such as recall bias, and do not account for the weight of response, scoring all events with the same significance (74). Biological measurement of stress hormones are therefore a useful tool when studying the stress response. Markers of stress include blood and salivary cortisol levels and blood pressure. However these face their own limitations with levels fluctuating with circadian rhythm and the short half-life of stress hormones ranging from minutes to hours (54).

1.3.2 Epidemiological Association

The association between exposure to stress and breast cancer can be studied epidemiologically by employing different types of study design. Retrospective case-control studies compare patients in a specific population (i.e. a hospital) to a control set who are not. The exposure to an identified risk factor can then be measured and a relationship between the risk factor and disease can be established. In retrospective case-control studies aiming to identify if stress is a

risk factor for breast cancer, patients with breast cancer and their respective controls were asked to fill out questionnaires detailing their exposure to stress. Whilst analysis of the findings allows an insight into multiple potential risk factors, recall bias is a considerable limitation as patients with the disease are more motivated to recall risk factors. As such, prospective cohort studies are perhaps more useful in the context of analysis of a psychological risk factor, as they study a population over a longer period and the incidence of a particular outcome is measured after. However due to the lengthy nature of such studies they are often expensive and prone to attrition bias, meaning there is a smaller pool from which to draw conclusive evidence. A summary of studies examining the association between stress and breast cancer is presented below, and whilst not exhaustive, serves to provide a balanced representative view of current literature.

i) Evidence for an association between stress and breast cancer

The role of psychosocial factors in relation to breast carcinoma was examined in a case-control study of 514 Australian women (75). Patients requiring biopsy after routine mammographic screening were identified as either having benign breast disease, or breast carcinoma, and were assessed using self-reported measures of stressful life events, as well as social support and coping style. A significant association was observed between highly stressful life events and lack of social support. A significant increase in the risk of developing breast cancer was also observed in women who not only experienced highly stressful life events, but were also rated as having no emotional support. The study used a short time frame in which stressors were assessed to limit recall bias, however the study was limited by the homogenous population which did not vary in age, a known risk factor for breast cancer. Similarly, another case-control study examining the relationship between stress and breast cancer was carried out in 858 invasive breast cancer patients and matched controls in Poland (76). Data collected in questionnaires regarding socioeconomic status and stressful life events found that women who had breast cancer scored highest for previous stressful life events, with the death of relative or spouses increasing breast cancer risk significantly. In a smaller study of 115 Finnish women displaying breast symptoms who were referred to a hospital, questionnaires were also used to assess stressful life events and coping strategies, and women were separated into groups according to subsequent diagnosis (benign disease or breast cancer) (77). Breast cancer patients were found to have a significantly higher level of perceived stress in the 10 years prior to diagnosis than women with benign breast disease, indicating a link between stress and breast cancer incidence. However although questionnaires were taken before the patient discovered their diagnosis, this study may still be prone to recall bias. In another small study retrospective analysis of traumatic life events using screening questions from PTSD diagnosis was administered to 94 patients who

had already been diagnosed with metastatic or recurrent breast cancer (78). There were significant differences in disease-free survival found, with women reporting no traumatic life events having on average an increased survival rate compared to those who did. Therefore the effect of previous life stresses were deemed a risk factor for breast cancer. However, the participants in this particular study may also be prone to recall bias in the face of a breast cancer diagnosis, and moreover they were recruited from women interested in a stress and breast cancer study, which could also introduce bias.

However, several prospective cohort studies have also yielded results indicating that stress may be associated with an increase in breast cancer risk. In one such study baseline stress levels determined by questionnaire were measured in a cohort of 1462 middle-aged Swedish women and subsequently measured at 5 year intervals after (79). Compared to the group experiencing no stress, breast cancer incidence in those who reported stress was increased by 2-fold over a follow up time of 24 years. This effect was not attenuated when adjusted for family history of breast cancer or other variable related to socioeconomic status. Another much larger prospective study using a Finnish twin cohort also examined the role of stressful life events and breast cancer incidence over a 15 year follow up period (80). A retrospective questionnaire detailing a range of life experiences was administered to a cohort of 10,808 women, alongside assessment of psychological stress using stress scales including stress of daily activities, life satisfaction and neuroticism. In women with an accumulation of adverse life events 5 years previous to the baseline, death of a spouse, relative or close friend showed the strongest association with an increased risk of breast cancer.

To examine the effect of low level chronic stress as opposed to individual high impact stressors such as deaths of relatives, stress caused by job strain was examined in a large cohort of 36,332 Swedish women working full-time or part-time. Women were questioned on job strain using questionnaires to assess job demands, job control and social support at work. Among women working full-time (but not part-time) there was a weak correlation between low job control, high job demands and breast cancer risk. Whilst the participants in this study were representative of the population, no other types of stress were measured which may have had some effect on the outcome of the study (81).

ii) Evidence for no association

Self-reported stress was measured in 991 Scottish women over a mean follow up time of 30 years (82). A weak association between medium or high levels of stress and a higher risk of developing breast cancer was observed. However the authors noted that stress is also associated with higher BMI, smoking and alcohol consumption, and whilst adjusting for

socioeconomic status did not abolish the association, the evidence present alone is not enough to convincingly state that psychological stress increases the risk of cancer. Another prospective cohort study involving 11,467 healthy women from the UK found no evidence of an association between social stress and breast cancer incidence (83). An assessment of social adversity was completed by participants detailing stressful life events and difficult circumstances in childhood and adulthood. During a 9 year follow up there was no correlation with increased perceived social stress and breast cancer incidence, with or without adjustment for confounding factors such as smoking status or BMI. A study of the largest cohort of British women (106,000) further corroborated these findings (84). Data on stress levels and stressful life events including bereavements was gathered using questionnaires over 10 years. No association was found with perceived stress levels and breast cancer risk. An increased risk of breast cancer in women bereaved of their mother was observed, however once adjusted for familial susceptibility this was no longer significant. This indicates stress may play a role in women with existing genetic susceptibility. Furthermore, a prospective study in Denmark (n=6689) (85) actually identified a positive correlation between stress and breast cancer, with higher levels of perceived stress correlating to a lower risk of primary breast cancer. The study concluded that chronic stress may impair oestrogen synthesis which could account for the lower incidence of breast cancer in stressed women.

In the Women's Health Initiative in the US, a cohort of 84,334 post-menopausal women was examined in relation to stressful life events and social support (86). Women completed questionnaires at baseline and after 3 years follow up, detailing serious life events such as death of a family member as well as their level of social support. After adjusting for confounders there was no association between stressful life events or social support and breast cancer incidence. However it should be noted that the follow-up time was relatively short at an average of 7.6 years.

Specific stressors such as daily activities, work stress and caregiving stress have also all been studied in order to identify if a link between breast cancer and stress. Stress of daily activities, as measured by the participants of a prospective cohort study, was assessed using questionnaires regarding the toll daily activities were perceived to take on mental wellbeing (87). The incidences of breast cancer in 10519 Finnish women who answered questions was not found to be statistically higher in women identified as under severe stress from normal daily activities. With regards to work stress meta-analysis from pool participant data of 12 studies, totalling 116056 men and women was studied to ascertain if high job strain correlates to an increased risk of breast cancer (88). Work stress was defined as job strain and self-reported over 12 years. There was no association between work stress and breast cancer incidence in the cohort after adjustment for various risk factors include BMI and smoking. This indicates that job

strain alone is unlikely to be an important risk factor in breast cancer progression, however there is some suggestion that night work may affect breast cancer occurrence (89).

Caregiving related stress was examined in relation to breast cancer, with 69,886 US women from the Nurses' Health Study answering questions on informal caregiving to an ill adult or child (90). No association was found between higher levels of caregiving and breast cancer incidence, however the mean follow up time in considered relatively short at 8 years. A study on the effect of losing a child on maternal breast cancer risk in cohort of Swedish women also concluded there was no association (91). Women who had no experience of losing a child were compared to women who had lost a child, and after adjustment for age, parity, age at first birth and education, no clear association was seen between the experience of losing a child and breast cancer.

iii) Meta-analysis

Several meta-analysis have sought to extrapolate the data from the wealth of literature surround the topic of stress and cancer, and draw a firm conclusion regarding the effects of psychological stress and breast cancer. An early analysis of the epidemiological link between stress and breast cancer examined 29 studies of adverse life events and breast cancer. No link was found between breast cancer and bereavement, or any other adverse life event (92). However since 1999 the pool of literature on the subject has widened considerably. In 2003, the association between stressful life events, including death of a spouse, change in marital status and health and financial difficulties and breast cancer was investigated using meta-analysis. Statistical significance was observed in 3 categories; death of a spouse, death of a relative or friend and stressful life event. However, death of a relative or friend and stressful life event were discounted due to evidence of publication bias. A modest association was therefore observed between breast cancer risk and death of a spouse (93). In 2008, a larger study was undertaken to examine the literature on a broad range of psychosocial factors across all cancers. Meta-analysis of 165 studies indicated stress related psycho-social factors could adversely affect cancer incidence, whilst poorer survival was associated with stressful life events in 330 studies, and higher mortality in 53 studies. More specifically stressful life experiences were associated with decreased survival and increased mortality, but not with cancer incidence. Poor coping mechanisms and negative emotional responses were also associated with higher cancer mortality, as well as an increase in cancer incidence. Psychosocial stress was associated with reduced survival in breast cancer patients specifically. This review also found that studies with longer follow up times had higher hazard ratios, indicating that stress-related psychosocial factors may have a slow but cumulative effect on cancer. However publication bias was

accepted by the authors as a major limitation in this comprehensive analysis of the literature in the field (94).

A more specific meta-analysis of depression and depressive symptoms with regards to cancer risk has also been shown to further corroborate these findings. Meta-analysis of 28 studies examining mortality and 5 studies examining progression was carried out using literature that explicitly measured depressive symptoms using established measures of anxiety and depression post cancer diagnosis. A significant relationship was found between depressive symptoms and mortality among cancer patients, with major clinical depression exerting the biggest effect on mortality. There was no association found between depression and cancer progression, although the small number of studies on the subject represent a limitation of the analysis (95).

The variability in findings of meta-analysis and individual studies reflects the diversity of the literature in the field. Study design and control for confounding are major points of discrepancy in in much of the research, with information on lifestyle factors such as socioeconomic status not always available. Furthermore the type of stress exposure, timing of stress exposure and follow up all vary from study to study making direct comparisons difficult. Studies linking psychological factors and cancer incidence are also prone to publication bias, as authors are more likely to submit, and editors more likely to accept publications with positive results. Negative or inconclusive results are subsequently underrepresented in the field. Furthermore prospective studies can be affected by recall bias, since patients may interpret stressful events differently in light of a diagnosis. Participants who answer the call for volunteers for a stress study are also more likely to believe in a link between stress and breast cancer, introducing further bias. Some studies employ the use of registry records to examine breast cancer risk in relation to death of a child or spouse. Whilst important in identifying trends, methodologies that rely on records alone cannot be considered a wholly accurate measure of stress as stressors in the control population are not accounted for. Similarly, in studies that use records to study cancer mortality the primary cause of death may be attributed to another pathology or vice versa, potentially rendering the data imprecise.

1.3.3 Stress and tumorigenesis

The epidemiological association between psychological stress and cancer has prompted further investigation in the biological and molecular effects of stress hormones on tumour cells.

Stress has been shown to affect the tumour microenvironment and tumour metastasis in a number of ways.

Recently mechanistic studies have begun to explore the controversial hypothesis that stress hormones may play a role in tumour initiation in cell lines and animal models. The effects of stress hormones on mammary cells vary according to developmental stage, with cortisol signalling in the mammary gland resulting in cellular changes during puberty, pregnancy and lactation. GR activation facilitates proliferation of mammary tissues and an upregulation of endoplasmic reticulum where milk protein synthesis can occur (71). Cortisol also has an effect on oestrogen activity and this may indirectly impact tumour progression. Oestrogen is a potent growth factor and under certain conditions - such as those found in women with a genetic predisposition - binding to surface receptors can promote transcription of genes necessary for proliferation. The enzyme aromatase plays a key role in oestrogen biosynthesis converting androgens into active forms of oestrogen. It has been observed that aromatase activity can be induced by cortisol and thus excessive psychological stress may cause an upregulation in a potentially tumourigenic molecule (96).

Cortisol may also interact with tumour suppressor gene expression. In mammary cells the BRCA1 gene product plays a key role in DNA repair and is an essential part of damage-initiated cell cycle checkpoints. It also operates in an oestrogen-responsive feedback mechanism, regulating proliferation stimulated by oestrogen signalling (97). Studies have linked cortisol with a down-regulation of the BRCA1 gene in MCF10A cells (mammary epithelial), which may contribute to inappropriate proliferation in non-malignant mammary cell line, an important discovery in breast cancer development (98). This represents a conceivable link between psychological stress and tumour initiation/progression (71). Furthermore in non-malignant human mammary epithelial cells, cortisol - through activation of the GR - was able to prevent apoptosis and mediate a cell survival signal (99). In this way cortisol may also partly facilitate the multistep process of malignant transformation. Glucocorticoids and catecholamines have also been shown to exert effects on proliferation and growth of tumour cells, with activation of β -AR's producing accelerated growth in mammary tumour cells (100), and glucocorticoids also enhancing proliferation in mammary tumour cells (101).

The immune response is an important part of tumour initiation or suppression, with the host immune system targeting tumour cells for elimination and tumour cells escaping detection through immunoediting (102). Stress is known to modulate effects on the immune system and chronic exposure can blunt the immune response suppressing anti-tumourigenic activities (103). Chronic inflammation is also considered to be a contributing factor in the progression of cancer, with inflammatory mediators releasing bioactive molecules including mutagenic reactive oxygen species (ROS) (104). Higher levels of perceived stress have been associated with higher levels of pro-inflammatory markers, indicating that bio-behavioural factors can influence the tumour microenvironment in a way that enhances growth (105).

1.3.4 Stress and invasion and metastasis

Once tumour cells are established at a primary site they are able to spread to other sites through a process known as metastasis. Metastasis of tumours is dependent on complex signalling promoting cells to embolise into the bloodstream, evade immune detection and extravasate into tissues (106). The cells must then be able to stimulate the process of angiogenesis, whereby vascularization to the new site is increased. In this way, tumour growth and metastatic spread can be accelerated by the supply of oxygen and nutrients to the sites, and cells can be carried in the blood and lymphatic fluid to colonise new sites (107). As such the release of proangiogenic factors such as vascular endothelial growth factor (VEGF), IL-6, tumour growth factor alpha and beta (TGF- α , - β) and tumour necrosis factor alpha (TNF- α) is a crucial step in progression (100).

Fig. 1.5 The metastatic process

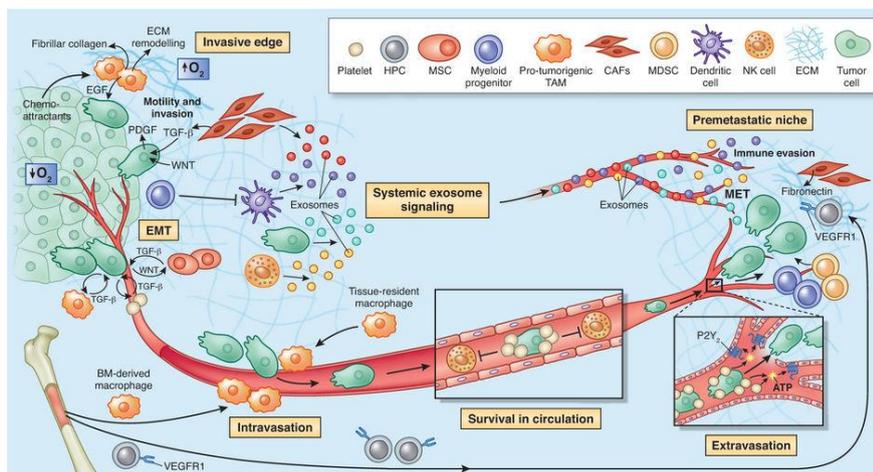


Fig. 1.5 The tumour microenvironment supports metastatic dissemination of tumour cells through immunosuppression mediated by tumour associated macrophages (TAM's) and cancer associated fibroblasts (CAF'S). Intravasation of tumour cells is facilitated by ECM remodelling, and macrophages can assist in the migration through vessel barriers. Platelets and components of the coagulation system support tumour cells in circulation, and tumour cells are able to extravasate at secondary sites made permissive to colonisation by myeloid-derived suppressor cells (MDSC's) and natural-killer T-cells (NK cells). Figure reproduced from (108).

VEGF functions as a proangiogenic mitogen eliciting a pronounced response in response to upregulatory signalling by growth factors, cytokines, and in response to hypoxia (109). Chronic stress, loneliness and depression have all been linked to an increase VEGF levels (110, 111), with experiments indicating that in ovarian cancer patients, higher levels of social support are associated with lower levels of VEGF. These results have been corroborated in cell lines, whereby catecholamines have been shown to induce VEGF expression through β -adrenergic receptor signalling mediated by the cAMP/PKA pathway (112). Interestingly, these effects can be blocked by the addition of β -adrenergic receptor antagonists, as demonstrated by other authors using a mouse xenograft model, where angiogenesis was promoted in response to chronic stress (113).

The secretion of IL-6 - another proangiogenic and pro-inflammatory factor involved in invasion and proliferation - can also be stimulated by catecholamines in ovarian cancer cells (114). The mechanism by which NE is able to regulate IL-6 is thought to be via gene transcription, with NE acting on promoter regions to upregulate transcription and mRNA synthesis (115). Similarly catecholamines have been shown to mediate effects on matrix metalloproteinases (MMP's), which function to breakdown and digest extracellular matrix in order for cells to invade and metastasize. Catecholamines are able to upregulate production of MMP's in both tumour and stromal cells enhancing the invasive potential of cancer cells into the surrounding tissue (116, 117).

The actions of glucocorticoids on metastasis are less clear, with previous studies demonstrating that glucocorticoids can be protective by inhibiting metastatic signalling and suppressing invasion in non-haematological tumours (118). Activation of the GR in *in vitro* models resulted in downregulation of MMP's, pro-angiogenic signalling molecules and proteins involved in adhesion (118). Furthermore glucocorticoids can also suppress metastasis in ovarian tumours through the induction of a metastasis suppressing microRNA (miRNA), which acts to suppress an oncogene involved in invasion (119). However, in breast cancer glucocorticoids have recently been shown to upregulate the transcription co-activator YAP which is often overexpressed in metastatic cancers, and whose dysregulation can lead to tumour initiation and progression. Depletion of the GR *in vivo* using short hairpin RNA (shRNA) was also shown to significantly reduce tumour volume and frequency of engraftment (120). Glucocorticoid signalling may further influence metastasis through immunomodulation. In breast cancer cells, glucocorticoids have been shown to be able to activate tumour associated macrophages (TAM's), which play a crucial role in tumour cell dissemination through the upregulation of MMP-9 (121). This finding was repeated in a clinical study of ovarian cancer patients, with patients scoring highly for depressive symptoms and chronic stress showing higher MMP-9 expression in TAM's within the primary tumour (122).

1.4 Stress hormones and DNA

1.4.1 DNA Damage

Stress hormones are known to play a role in DNA damage and repair. Several reviews (103, 123, 124) have sought to clarify the molecular mechanisms by which psychological factors may impact on DNA, through direct damage or interference with repair. However, the full extent of their involvement is still yet to be fully understood.

Damage to DNA induced by stress hormones has been shown to occur rapidly in fibroblast cells exposed to stress hormones for short periods of time (<30mins), with inhibition of the GR and β -AR negating the effects (125). Moreover, longer exposure to stress hormones was further shown to induce DNA damage, transformation, and tumourigenicity in fibroblast cells, as well as increasing the growth of tumours in mice (126). In both human and animal studies increases in negative psychosocial factors such as depression or the induction of psychological stress also promoted DNA damage, as measured by excreted DNA damage markers (reviewed in (123)).

It is theorised that stress hormones may induce DNA damage through the production of ROS (127). These are characterised as unstable molecules containing at least one unpaired electron and are capable of reacting with various biological compounds including nucleotides. ROS are a by-product of cellular metabolism and occur as a result of mitochondrial respiration and enzymatic reactions. Levels of ROS are controlled by antioxidants such as glutathione, levels of which are often upregulated in cancer cells, which work to neutralise them (128). However when the intracellular balance tips in favour of ROS production, oxidative stress occurs. Surrounding molecules such as lipids and proteins can also be damaged by oxidative stress and there has been several links made between an accumulation of oxidative damage, aging and disease (71) (129). Damage to DNA from ROS can result in chemical reactions such as oxidation, methylation and deamination that can indirectly cause double strand breaks (DSB's) and base pair changes. Mutagenesis can occur due to structural alterations within the DNA such as base pair mutations or insertions/deletions. In turn this may facilitate damage to tumour suppressor genes and change in expression of proto-oncogenes (130). Furthermore oxidative damage to proteins and lipids can also mediate tumourigenic effects, including alterations in cellular ability to replicate and repair DNA. DNA polymerases are especially susceptible to damage as a result of ROS generation, and can be rendered inaccurate resulting in further base mutations. In the context of breast cancer it has been shown that oxidative damage induced by hydroxyl radicals increases in metastatic tumour DNA, suggesting that the base modifications and mutations contribute to metastasis (131).

It has been suggested that through β 2-adrenergic receptor activation, catecholamines may increase ROS production, thereby damaging DNA. Activation of the receptor results a signalling cascade inducing production of cAMP and activation of PKA's that increase oxidative phosphorylation within the cell, increasing the generation of ROS. The enzymatic reactions of NADPH oxidase, which is similarly activated by β 2-adrenergic receptor activation, can further produce ROS (127). The superoxide radicals can then combine with nitric oxide to generate reactive nitrogen species (RNS), which are also highly damaging (132).

1.4.2 DNA Repair

Although DNA repair has been proven to be affected by stress hormones (127), few mechanisms exist detailing through which pathways they may exert their action. In breast cancer cells with undamaged DNA the family of CDC25 phosphatases act to facilitate entry into the cell cycle at S or M phases by activating CDK1 and CDK2 (133) (Fig. 1.6). When DNA damage is detected, the checkpoint kinases ATR and ATM are activated; ATR by single-stranded breaks, and ATM by double-stranded breaks. They function to phosphorylate, and thus activate, Chk1 and Chk2 respectively. Chk1 and Chk2 are then able to phosphorylate CDC25 phosphatases rendering them inactive and subsequently delaying progression of the cell cycle to allow time for DNA repair. ATM also phosphorylates the p53 regulator MDM2 as well as p53 itself, causing an increase in levels of p53 which in turn leads to an upregulation of the CKI p21, further halting the cell cycle (134). The addition of stress hormones has been shown to interfere with this DNA repair process, increasing the level of DNA damage, and accordingly the level of expression of Chk1 and Chk2 proteins. However they also appear to increase the level of CDC25a, a member of the CDC25 family, levels of which would be expected to decrease in response to damage as under normal circumstances they enable cell cycle continuation. As such the cell is unable to undergo delay and must replicate with the damaged DNA, increasing the potential of tumourigenic mutations (125).

Another mutation common in breast cancers occurs in the gene coding for the protein p53, a tumour suppressor that acts in many capacities to arrest growth, activate DNA repair and initiate apoptosis. A study published in Nature (135) identified a mechanism by which stress hormones may downregulate normal p53 signalling in response to DNA damage, allowing deleterious DNA damage to accumulate. Binding of catecholamines to β 2-adrenergic receptor, through PKA activation, recruits the signal transducer proteins β -arrestins. These can act directly as scaffolds, facilitating MDM2 binding to p53, or by signalling phosphorylation of MDM2, resulting in p53 ubiquitination. This means that normal p53 signalling in response to DNA damage is down regulated.

Fig. 1. 6 DNA damage signalling

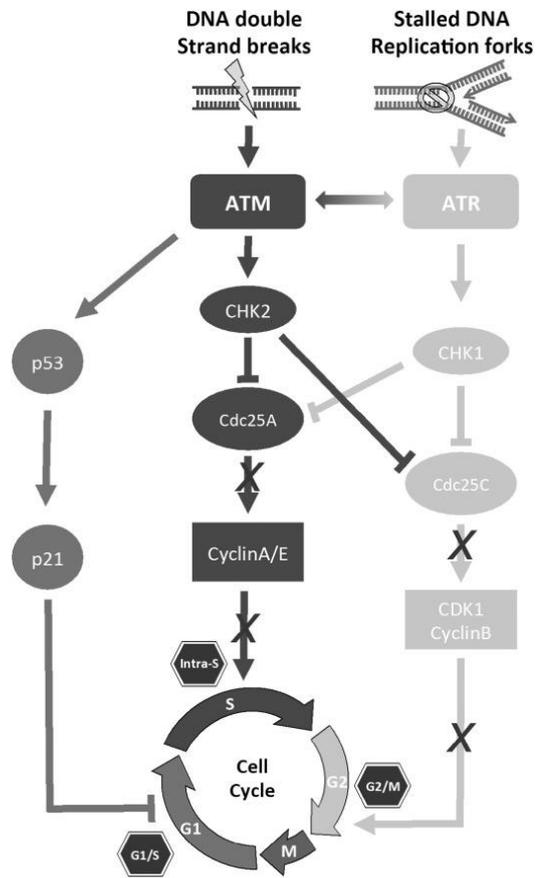


Fig. 1.6 ATM and ATR induce arrest of the cell cycle in response to DNA damage through modulation of cell cycle checkpoints. Figure taken from (136).

1.4.3 Chemotherapy

Since the establishment of a stress hormone interaction with DNA repair and cell cycle arrest processes, research has emerged linking psychological stress with reduced efficacy of chemotherapeutics. Many core chemotherapy drugs work by disrupting the cell cycle and DNA replication, thus restricting proliferation of malignant cells. Drug resistance poses a major obstacle in the successful treatment of aggressive cancers, with multiple drug resistance (MDR) affecting nearly half of metastatic breast cancer patients (137).

Taxanes such as Paclitaxel and Docetaxel are widely used chemotherapeutic agents capable of disrupting microtubule formation and arresting the cell cycle, in turn inducing apoptosis (38). Several cancers have shown good response to taxane treatment, however acquired resistance is common following long term exposure (38). Stress hormones have been shown to mediate effects on the cell cycle in breast cancer cells, upregulating cyclin-dependant-kinase 1 (CDK-1), which lead to cell cycle progression through the G2/M stage. In this way stress hormones can reduce the efficacy of paclitaxel which acts on rapidly proliferating cells by inhibiting paclitaxel-induced G2/M arrest (138). This mechanism has been further corroborated by an *in vivo* study demonstrating that the induction of psychological stress promotes tumour growth in mice treated with paclitaxel, negating its anti-tumour effect (139). It is also proposed that the induction of DNA damage can induce resistance to chemotherapies in breast cancer. In breast cancer cells treated with stress hormones the expression of DNA damage response proteins was upregulated, specifically the DNA damage sensor ataxia telangiectasia and Rad3-related (ATR) which initiates a signalling cascade activating p21 to halt the cell cycle. This allows the cell time to repair the damaged DNA. The CDK inhibitor p21 functions to inhibit progression into the S phase of the cell cycle, and it is proposed that the arrest of cells caused by the addition of stress hormones prevents the action of chemotherapies such as paclitaxel that act on dividing cells (139).

Studies have also linked glucocorticoids to the activation of survival genes in breast cancer, reducing the efficacy of some chemotherapeutics. These genes are able to decrease the susceptibility of cells to apoptosis by the downregulation of pro-apoptotic pathways (140). Moreover addition of the potent synthetic glucocorticoid dexamethasone, which is regularly administered to patients to combat hypersensitivities, has been shown to inhibit treatment induced apoptosis and promote cell viability (141).

1.5 Oxidative Stress and cancer

Oxidative stress can be generated in a number of ways within the cells, including through oxidative phosphorylation in the mitochondria as part of cellular respiration. ROS are also generated as a by-product of enzymatic reactions such as that of NADPH oxidase, increased receptor signalling and oncogene activity, however mitochondrial respiration is by far the largest producer of intracellular ROS (142). Mitochondrial respiration produces the species hydrogen peroxide (H_2O_2), by first generating superoxide (O_2^-) via the electron transport chain. Superoxide is then dismutated into H_2O_2 in the cytosol of the mitochondrial matrix by superoxide dismutases (SOD's). Under normal physiological conditions the balance of intracellular ROS is maintained by non-enzymatic molecules such as vitamin E or antioxidant enzymes such as super oxide dismutase or catalase which specifically scavenge types of ROS (143). However in the context of cancer this balance can be disrupted and deregulated, indicating that the oxidative stress/antioxidant balance is an important factor in cancer progression (144).

1.5.1 The role of oxidative stress in cancer

Increased levels of oxidative stress can promote malignant transformation and phenotypic changes in cancer cells through multiple actions within cells, including through DNA damage, survival signalling and regulation of metastasis (145).

Hydroxyl radicals generated from H_2O_2 by the Fenton reaction, where by an Iron (II) catalyses the reduction of H_2O_2 , can cause oxidised DNA bases, single strand or double strand breaks (146). The most studied type of oxidative base damage is the modification 8-oxo-7,8-dihydroguanine (8-oxoG), which promotes a high rate of mispairing during replication making this type of base damage highly mutagenic (147). An increase in the generation of 8-oxoG and the enzyme that serves to repair it, 8-oxoguanine DNA glycosylase 1 (OGG1), has therefore been shown to serve a risk factor in the development of cancer (148). Germ line mutation of OGG1 has also been identified as a predictive biomarker in breast cancer (149). Formation of 8-oxoG bases can also modify the activity of specific transcription factors, inhibiting their ability to bind to promoter regions and changing the expression of target genes (150).

Proliferation and cell survival signalling in cancer cells can be induced by oxidative stress in a number of ways. In breast cancer cells translocation of oestrogen to the mitochondria increases mitochondrial ROS, and this oestrogen-induced ROS production can drive growth through the activation of MAPK (mitogen-activated protein kinase)/Erk1/2 (extracellular-regulated kinase 1/2) signalling (151). Furthermore, ROS can also increase proliferative signalling through upregulation cyclins, mediators of progression through the cell cycle. Treatment of non-malignant mammary epithelial cells with an antioxidant was shown to delay progression through

the cell cycle, indicating that ROS can drive proliferation (152), and similarly treatment of breast cancer cells with an oxidant can increase proliferation through transcription factor upregulation (153). Cell survival can also be promoted by oxidative stress through the downregulation and avoidance of apoptotic signalling. High levels of ROS such as those generated by chemotherapy act in induce apoptosis in cancer cells, however lower levels of ROS can lead to an upregulation of protective antioxidants and anti-apoptotic proteins (143).

Many signalling pathways in cancer are sensitive to ROS which is often upregulated as a result of increased metabolic functioning amongst other reasons. As mentioned above, ROS generated by oestrogenic signalling can increase MAPK/Erk1/2 signalling which is strongly linked to cell survival, and mutations within the pathway in cancer can cause aberrant growth (154). It is thought that direct oxidative modification of a cysteine residue on Ras - the upstream regulator of Erk - is responsible for ROS-induced survival signalling. In breast cancer specifically, ROS scavengers or inhibitors of Erk can promote apoptosis and inhibit adhesion (143, 155). Another central regulator of cell function is the phosphatidylinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway, which controls cell cycle and survival process amongst many others (156). ROS generation can activate PI3K/Akt signalling in ovarian cancer cells resulting increased angiogenesis (157), and can also inhibit the activity of the tumour suppressor phosphatase and tensin homolog (PTEN) which negatively modulates the PI3K/Akt pathway (158).

The ability to induce angiogenesis in order to metastasize is an important component in cancer prognosis, with the majority of deaths occurring as a result of secondary tumours (27). In animal models, mammary carcinoma cells treated with ROS have shown enhanced metastatic capability when implanted (159). Suppression of oxidative stress by a scavenger of oxygen and nitrogen species has also attenuated the angiogenic potential in a mouse model of mammary carcinoma (160). One mechanism through which oxidative stress can act to induce angiogenesis is through enhancement of cellular motility and interference with adhesion. In non-transformed cells adhesion to the extracellular matrix (ECM) is required for growth and proliferation, with loss of this adhesion inducing anoikis, a type of apoptosis triggered by detachment of anchorage-dependant cells. In tumour cells this process is deregulated, with acquired resistance to anoikis allowing cells to survive and metastasise to distant sites. Resistance to anoikis has been closely linked to redox signalling and the induction of constitutive stimulation of proliferative signalling by oxidative stress (161). Furthermore in order to metastasise tumour cells are required to undergo epithelial-mesenchymal transition (EMT); one way in this is achieved is through the upregulation of MMP's. Treatment of mammary carcinoma cells with MMP's to induce EMT has been shown to produce an increase in oxidative stress which further stimulates EMT-related factors leading to malignant transformation (162). These mechanisms implicate oxidative stress

in almost all aspects of tumourigenic transformation and progression, providing the basis for development of therapeutic strategies that may target oxidative signalling.

1.5.2 Reactive nitrogen species in cancer

Reactive nitrogen species (RNS) is a collective term that includes nitric oxide (NO), and its by-products peroxyntirite (OONO⁻) and nitrogen dioxide (NO₂) (130). The primary RNS studied in relation to cancer is NO due to its extensive involvement in signalling across a range of tissues. NO is produced by nitric oxide synthase (NOS) which catalyses the oxidation of L-arginine to yield L-citrulline and NO in an NADPH and oxygen dependant reaction (163). Shifts in expression of NOS as well as signalling from cytokines, hormones and pro-inflammatory molecules regulates the production and release of NO, which can act as a rapid signalling molecule due to its ability to diffuse across membranes.

Fig. 1.7 The structure of nitric oxide synthase

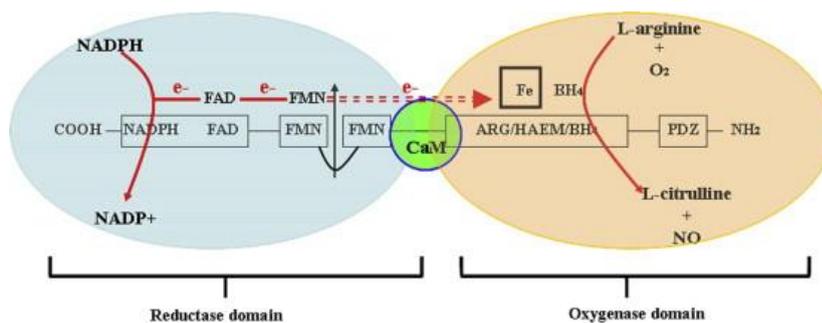


Fig. 1.7 The enzyme nitric oxide synthase (NOS) exists in monomers and when dimerized catalyses the oxidation of L-arginine to L-citrulline and NO. NOS is comprised of an oxygenase domain (N-terminal) and a reductase domain (N-terminal) separated by a calcium binding motif. The reductase domain binds NADPH and has binding sites for FNM and FAD, and the oxygenase domain binds L-arginine and tetrahydrobiopterin (BH₄). Electrons donated from NADPH are transferred from the reductase domain to the oxygenase domain via FMN and FAD, and the oxidation of L-arginine to L-citrulline produces NO. Figure taken from (164).

The role of NO in the pathophysiology of cancer is often described as complex, with low levels mediating many homeostatic processes and allowing cell proliferation, while high levels are associated with cytotoxicity and can induce apoptosis (163). Nitrosative stress caused by an imbalance of RNS, as with oxidative stress, can be associated with compromised cellular function due to damaging interactions between RNS and DNA, proteins and lipids. NO is capable of causing damage to DNA by combining with superoxide radicals forming peroxynitrite (ONOO⁻), a highly unstable and strongly oxidative agent. NO and NO-derived species can interact directly with DNA causing damage by nitrosative deamination or alkylation resulting in modifications and strand breaks (163). NO can also inactivate DNA repair proteins such as DNA ligase due to its affinity for thiol residues which could further explain why exposure to NO results in DNA damage (165). As such increases in the concentration of NO within the tumour microenvironment can be considered mutagenic, with long term exposure such as that caused by chronic inflammation known to play a role in carcinogenesis (166).

The involvement of NO in apoptotic signalling also represents another potential mechanism whereby nitrosative stress can influence cancer progression. Exposure to excess NO in mammary tumour cells expressing wild type p53 resulted in an accumulation of p53, as well as a loss of the binding activity of p53, subsequently suppressing apoptotic induction (167). Higher levels of mutation of p53 are also strongly correlated with NOS expression. The activity of the inducible isoform of NOS (iNOS), which produces the highest levels of NO of all three isoforms, is associated with specific p53 mutations in both colon and head and neck tumours (168). Interestingly wild type, but not mutant, p53 can also inhibit iNOS expression through its promoter region causing a feedback loop where high levels of NO induce DNA damage, deregulate the p53 response and promote p53 mutation. This results in strong selection pressure for p53 mutations leading to dynamic tumour development, whereby cells expressing iNOS and producing high levels of NO die via apoptosis leaving the surviving resistant cells to proliferate (169).

The evidence to suggest NO may drive malignant growth is further supported by the observation that the expression of iNOS has been found to increase in line with tumour grade and progression, particularly in breast cancer (170-173). This may be partially due to the ability of NO to cause increased vascularisation leading to more malignant carcinomas. Interestingly, whilst it has been proposed that NO may block adhesion of tumour cells to the microvasculature (163), NO has also been found to upregulate VEGF, thus promoting angiogenesis allowing for a more invasive and metastatic tumour type (174). Another pro-angiogenic mediator cyclooxygenase-2 (COX-2) is also over expressed in a high proportion of breast cancers (175). COX-2 is primarily involved in inflammatory signalling which can lead to

an upregulation of angiogenic signalling, and NO has been shown to upregulate COX-2 in multiple cancer types (176). Inhibition of NOS in order to reduce NO signalling and decrease nitrosative stress has shown considerable efficacy in reducing tumour cell migration and metastasis, indicating that NO-mediated angiogenesis represents a possible valuable therapeutic target (177).

Modulation of NO signalling through the inhibition of NOS has also proven advantageous in the treatment of some solid cancers by decreasing primary tumour growth (178). NOS inhibitors capable of inhibiting all isoforms of NOS have been shown to decrease vascular permeability (179) and blood flow to tumours (180) halting tumour growth. Co-treatment with other chemotherapies alongside inhibition of NOS has also been therapeutically beneficial, with a COX-2 inhibitor and an iNOS inhibitor showing greater chemopreventative activity in combination than either agents alone (181). NOS inhibitors have also been used in combination to enhance the effect of conventional chemotherapies such as docetaxel (172), as well as acting as a chemosensitizer to augment the antitumour effect of radiation (182). However, such results tend to be dependent on the type of inhibition as well as the type of tissue and sensitivity to NO, as other studies have shown that inhibition of NOS can promote growth (183). Tumour cells modified to express iNOS and upregulate production of NO were not shown to induce tumour formation, and cells modified with mutant iNOS proteins designed to produce defined concentrations of NO have a strong dose-dependent effect on tumour initiation (184). These studies highlight the biphasic actions of NO and RNS in cancer, with pro or anti-tumour effects strongly controlled by concentration of NO in the cellular microenvironment.

1.6 Hypothesis

Hormonal responses induced by psychological stress have a detrimental impact on DNA damage and repair processes in breast cancer, through the production of ROS/RNS. Furthermore, the stress-mediated generation of DNA damage and ROS/RNS can impact breast cancer by accelerating tumourigenesis, and reducing the efficacy of chemotherapeutic agents.

1.7 Aims

1. Identify how stress hormones affect DNA damage and repair in breast cancer cell lines.
2. Elucidate molecular mechanisms by which stress hormones generate ROS/RNS, thus contributing to DNA damage.
3. Understand how stress hormones signalling contributes to mammary carcinoma tumourigenesis *in vivo*.
4. Explore how stress hormones interact with chemotherapeutics.

1.8 Anticipation of original contribution to knowledge

Stress hormones are known to induce DNA damage and impact repair processes, however few mechanisms exist clarifying how they do so. This research intends to demonstrate that acute exposure to stress hormones induce DNA damage through the production of ROS/RNS, as well as negatively modulating the DNA repair response in breast cancer cell lines. Furthermore, it is anticipated that use of a syngeneic animal model of psychological stress will provide insight into the effect of stress on tumourigenesis in breast tumours. This research also intends to explore the interaction of stress hormones with chemotherapy agents, and study the effects of stress hormones on resistance to breast cancer therapies.

Chapter 2 – Materials and Methods

2.1 Cell Culture

2.1.1 Cell Lines

The cell line MDA-MB-231, obtained from the American Type Culture Collection (ATCC HTB-26), is an epithelial breast cancer derived originally from an adenocarcinoma (185). The cells do not express ER, PR or HER2 and therefore represent a typical triple negative basal subtype of breast cancer. The growth media used for this cell line is Dulbecco's Modified Eagle Medium (DMEM) in a 1:1 ratio with Hams F12 nutrient mixture (Gibco, UK) with 10% foetal bovine serum (FBS) (Gibco, UK).

The MCF-7 cell line was obtained from ATCC (HTB-22), is derived from an adenocarcinoma, retaining the morphological features of mammary epithelial cells (185). The cells do not express HER2 but do express ER and PR, and are as such an endocrine responsive cell line. The cell line was cultured in DMEM (Gibco) with 10% FBS. For comparative electrochemical sensing experiments MCF-7 cells were cultured in DMEM with 10% charcoal-stripped FBS (CS-FBS).

The triple negative cell line HCC38 were also purchased from ATCC (CRL-2314) and maintained in Roswell Park Memorial Institute (RPMI) medium (Gibco, UK) with 10% foetal calf serum (Gibco, UK).

The cell line MCF10A cell line was purchased from ATCC (CRL-10317), and is considered a transformed non-malignant mammary epithelial cell line (186), which do not express ER. MCF10A cells were cultured in HuMEC-ready medium (Thermo Fisher, UK) supplemented with HuMEC supplement kit (Thermo Fisher, UK).

An acquired faslodex resistant cell line (FAS-R), characterised by a loss of ER, was generated by continuous faslodex (10^{-7} M) exposure to the ER+ breast cancer cell line MCF-7 for 2 years. This work was carried out by Dr. J Gee's group at Cardiff University. Faslodex resistance was confirmed by growth curves compared to the parental MCF-7 cells. FAS-R cells were maintained in phenol red-free Roswell Park Memorial Institute medium (RPMI-1640) media, supplemented with 5% CS-FBS, 4mM L-glutamine (Gibco, UK), faslodex (10^{-7} M), penicillin/streptomycin (100iu/100mg/ml) (Gibco, UK) and Fungizone (2.5ug/ml) (Gibco, UK). The parental MCF-7 cell line (Cardiff) was maintained in parallel in RPMI with 10% FBS, penicillin/streptomycin (100iu/100mg/ml) (Gibco, UK) and Fungizone (2.5ug/ml) (Gibco, UK). For experimental conditions MCF-7 cells were culture in phenol red-free RPMI with 5% CS-FBS.

The murine mammary carcinoma cell line 4T1 was kindly donated by Dr. Hideo Okada, University of Pittsburg, USA. 4T1 cells were cultured for *in vivo* experiments in the USA by Dr. M Flint. 4T1 cells were originally isolated from a spontaneously arising mammary tumour in BALB/c mice, and are highly metastatic to the lungs as well a liver brain and bone (187). 4T1 cells were

cultured in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine and charcoal stripped bovine calf serum (10%).

The murine mammary carcinoma cell line 66CL4, a metastatic derivative of the 4T1 line, were kindly donated by Dr. Erica Sloane, Monash University, Australia. 66CL4 cells spontaneously metastasize to the lungs, and are thought to metastasize through a distinctly different route to 4T1 cells, disseminating through a lymphatic route as opposed to a haematological route (187). 66CL4 cells were therefore chosen to represent a moderately metastatic breast cancer. 66CL4 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) (Gibco, UK) supplemented with 10% FBS.

2.1.2 Cell Culture Conditions

All cell lines were maintained in humid conditions at 37°C and with 5% atmospheric CO₂. Cell culture work was performed in sterile conditions in a laminar airflow cabinet (Airstream) and standard aseptic techniques were used. Mycoplasma screening was carried out regularly to ensure no contamination was present. SNP mapping was not utilised for cell line authentication. All cell lines used were between passage 7 and passage 40.

2.1.3 Routine Cell Culture

Breast cancer cell lines were maintained in T-75cm² filtered tissue culture flasks (Nunc, UK) and passaged twice weekly when 70-90% confluency was reached. Cells were incubated with 5ml 0.05% trypsin/EDTA (Gibco, UK) for 3-5mins at 37°C and subsequently quenched with 10ml normal growth media. The suspension was centrifuged at 500G for 5mins, the pellet resuspended in growth media and an appropriate volume seeded into new T-75cm² flasks.

2.1.3 Cell Counting

Cells were manually counted using a haemocytometer for experiments requiring a specific number of cells. Cells were centrifuged and pellets resuspended in 1ml of complete media. Cell suspensions were diluted as necessary and 20µl is loaded into a haemocytometer. The number of cells in 4 grid squares is counted and averaged, then multiplied by 10⁴ to obtain cells per ml, taking into account any dilutions. The required volume of cell suspension is then reseeded into well plates.

2.1.4 Hormone Treatment

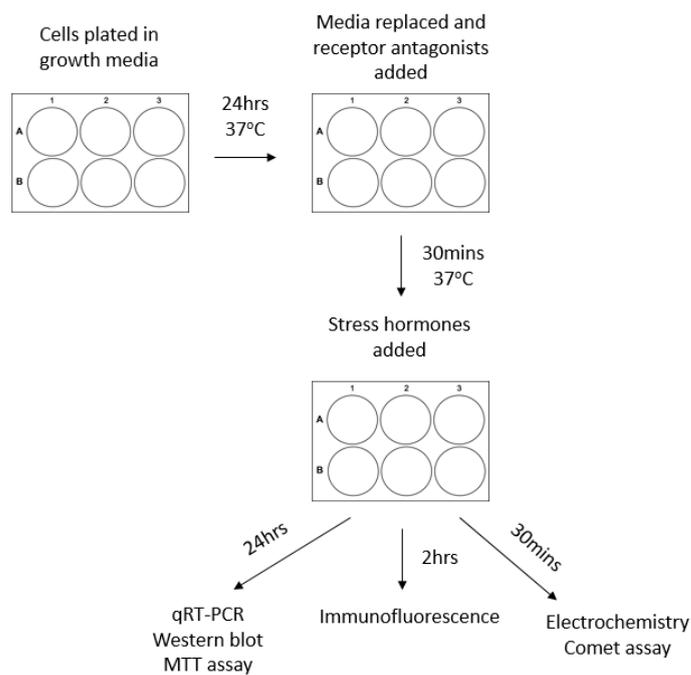
Prior to hormone treatment cells were seeded in 6 well plates at different densities depending

on the experimental procedure and incubated for 24hrs at 37°C. Cells were treated with these predetermined physiologically relevant concentrations of hormones(188) for all experiments in this way, unless stated otherwise. The growth media was removed and replaced with hydrocortisone (Sigma Aldrich, UK), purchased pre-dissolved in H₂O at a concentration of 50µM, which was then diluted in media to a final concentration 1µM. Noradrenaline (Sigma Aldrich, UK) was dissolved in ethanol to make a stock concentration 1mM, which was then diluted to a working concentration of 10µM with media, and then again in media to achieve a final concentration of 1µM. Pharmacological blocking of hormone receptors to determine specificity was achieved by incubating the cells with the glucocorticoid receptor antagonist RU486 or beta-adrenergic receptor antagonist propranolol (Sigma Aldrich, UK), dissolved in DMSO, and diluted to a final concentration of 1µM for 30mins prior to the addition of cortisol or noradrenaline respectively.

Commented [RF2]: Comment 47. changed ref to cite 'Elevated basal cortisol levels and attenuated ACTH and cortisol responses to a behavioral challenge in women with metastatic breast cancer.'

Fig. 2.1 Experimental design for stress hormone experiments

Commented [RF3]: Comment 24 – updated figure numbers



2.1. Inhibitors and chemotherapy treatments

Cells were exposed to the specific and non-specific NOS inhibitors, and a Src inhibitor for 30mins prior to hormone treatments. Cells were treated with either 1400W dihydrochloride (iNOS inhibitor; Tocris, UK) dissolved in DMSO and diluted to 10 μ M in media, L-NAME (non-specific NOS inhibitor; Tocris, UK) dissolved in H₂O and diluted to 100 μ M, L-NNA (non-specific NOS inhibitor; Tocris, UK) dissolved in H₂O and diluted to 100 μ M or PP2 (Src inhibitor; Abcam, UK) dissolved in DMSO and diluted to 10 μ M in media.

For chemotherapy studies the LD50 of chemotherapeutic agents was calculated by fitting a dose response curve to values obtained from an MTT assay. Cells were plated at a density of 1x10⁴/200 μ l in a 96-well plate and incubated overnight. Cells were then exposure to a range of concentrations (0.1-100 μ M) of doxorubicin, paclitaxel, tamoxifen or fulvestrant dissolved in DMSO and diluted in media (Sigma Aldrich, UK). To examine the effects of glucocorticoids on the efficacy of chemotherapy, cells were treated with chemotherapeutics at their LD50 alongside dexamethasone, which was dissolved in DMSO and diluted in media (Sigma Aldrich, UK) (0.1-100 μ M) for 24hrs.

Poly ADP ribose polymerase (PARP) inhibition was achieved using the selective PARP-1 inhibitor PJ34 (Caymen Chemicals, USA) dissolved in DMSO and diluted in cell culture media to a final concentration of 10 μ M. PJ34 was chosen as a selective inhibitor of PARP-1, as it has previously been used successfully inhibit PARP in triple negative and BRCA-deficient cells, alone and in combination (189) .

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2.2 Immunofluorescence

2.2.1 Principles of Immunofluorescence

Immunofluorescence is a technique used to identify the presence of proteins in tissue sections and fixed cell samples. The antigen of interest can be located in a fixed sample by the direct method of visualisation - using a primary antibody attached to a fluorophore, or indirect method - whereby an untagged primary is bound to a fluorophore tagged secondary antibody. A fluorescent microscope can then excite the fluorophore by emitting light at certain wavelengths depending on the specific fluorophore used, to obtain a fluorescent signal (190).

2.2.2 Using Immunofluorescence to Identify DNA Damage

DNA damage can be quantified using immunofluorescence to detect phosphorylated histone H2AX (191). The phosphorylation of this histone serves as a reliable marker of DNA damage, as the induction of double-stranded DNA breaks rapidly stimulates kinases involved in the DNA damage response to phosphorylate the histone. Subsequently phosphorylated γ -H2AX molecules localise to the point of the break forming foci which can be easily observed using immunofluorescence (192). In this way, analysis of γ -H2AX foci can provide semi-quantitative data indicating the percentage of cells with DNA damage.

Another protein involved in DNA repair signalling is RAD51, which can be used as a measure of induction of DNA damage. RAD51 is involved in homologous repair (HR) of DNA, and when recruited to the break forms foci, which can be semi-quantitatively assessed using immunofluorescence in the same way as phospho- γ -H2AX (193).

2.2.3 Immunofluorescence

Breast cancer cell lines were plated at a density of 2×10^5 per well of a 6 well plate onto glass coverslips (Fisher Scientific, UK) and incubated for 24 hours. Cells were subsequently exposed to cortisol and norepinephrine for 2hrs at 37°C, or treated with RU486 and 1400W dihydrochloride as outlined previously, for half an hour prior to hormone treatment, controls were left untreated. The cells were fixed in paraformaldehyde fixative 3% (Appendix 1) for 10 minutes at room temperature before washing with PBS 3 times. Permeabilization was achieved using 0.2% TritonX-100 (Sigma Aldrich, UK) in PBS for 2.5mins at room temperature. Blocking was achieved using 2% BSA in PBS for 20mins. Incubation with the primary antibody; anti-phospho-Histone H2AX mouse monoclonal (1:800 in 2% BSAPBS) (Cell Signalling, UK) or anti-RAD51 (1:200 in 2% BSAPBS) (Cell signalling, UK) lasted for 45mins at 37°C, and was followed by 3 washes with PBS and incubation with the secondary antibody; anti-rabbit IgG FITC (1:200 in 2% PBSBSA) (Sigma Aldrich, UK) at 37°C for 20mins. The slides were stained and mounted with Vectashield plus 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, UK) and visualised using fluorescent microscopy (Leica, Germany). Fluorescent foci in 100+ cells were detected using

confocal microscopy and positive cells (>5 foci) expressed as a percentage of total cells counted. Total fluorescent intensity was not used as the foci formation was not as prominent as in other studies e.g. in radiation studies, where the level DNA damage induced is much higher. Furthermore both manual and automated quantification have been shown to produce replicable results (194). Each experiment was carried out with a minimum of 3 biological replicates.

The GR and ER were also immunofluorescently identified in MCF-7 and 66CL4 cells. Cells were processed as above and stained with anti-GR (1:200 in 2% BSAPBS) (Santa Cruz Biotech, UK) or anti-ER (1:200 in 2% BSAPBS) (Santa Cruz Biotech, UK). Cells were counterstained with DAPI, mounted and visualised using confocal microscopy at x40 magnification.

2.3 Comet Assay

2.3.1 Principles of the Comet Assay

The single cell gel electrophoresis assay, or comet assay, is a simple and fast technique that can be used to visually identify DNA damage signified by strand breaks in cells (195). The method followed here closely follows the alkaline method developed in 1988 by Singh et al. (196), whereby cells are embedded in agarose gel and electrophoresed under alkaline conditions. The migration of single stranded DNA produces 'comet tails' which can be visualised thereby providing a method to quantify single strand DNA breaks. The comet assay can also function as an indirect measure of DNA repair. Cells that have been treated with DNA damaging agents and then returned to normal conditions in order to facilitate DNA repair processes can give an indicative measure of whether or not repair processes have been compromised when compared to treated samples that have not been allowed to repair.

2.3.2 Detecting DNA Damage

In order to measure DNA damage and repair, 2×10^5 cells were plated and incubated overnight. Cells were exposed to H_2O_2 ($50 \mu M$) (Sigma Aldrich, UK), cortisol and noradrenaline for 20mins. Treatment groups were removed immediately and repair samples washed in PBS and incubated at $37^\circ C$ in growth media for a further 20mins to allow for DNA repair. Cells were then mixed with 1.2% low melting point agarose (Sigma Aldrich, UK) and pipetted onto slides previously coated with 0.6% ultrapure agarose (Invitrogen, UK). The gels were allowed to set at $4^\circ C$ and lysed in comet lysis buffer (Appendix 1) before immersion in electrophoresis buffer (Appendix 1) for 45mins. Electrophoresis was carried out at 25V for 25mins and the slides neutralised in 0.4M Tris pH7.

Cells were stained with ethidium bromide ($0.1 mg/ml$) (Sigma Aldrich, UK) and the 'comet tails' scored visually using a fluorescent microscope (Nikon) at $\times 20$ magnification. A minimum of 100 comet tails were scored using a 0-4 scoring system based on the length of the tails. DNA damage was then expressed as a proportion of the maximum damage score i.e. 400.

Fig. 2.2 Comet scoring guide

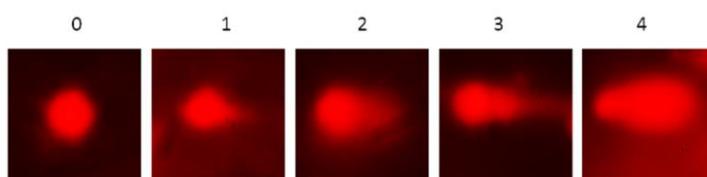


Fig. 2.2 Comet scoring guide (0-4)

2.4 Electrochemistry

2.4.1 Principles of electroanalytical detection

Reliable measurement of chemically reactive and unstable ROS/RNS has classically proved difficult, with methods such as the chemical assays or chemiluminescent probes not always delivering the sensitivity or specificity required for biological systems (197, 198). The development of electroanalytical techniques offers the sensitivity required for such measurements, as well as the ability to measure multiple ROS/RNS simultaneously (199). In this study, the electrochemical detection employed uses microelectrodes to measure ROS/RNS through multi-step amperometry. This technique uses a fixed voltage and measures the change in the flow of electrons, i.e. current. The change in current resulting from the oxidation of an electroactive species can provide information regarding the composition of a solution, whereby the change in current is proportional to the concentration of analyte (200). The voltage at which each ROS/RNS reaches peak oxidation can be characterised, and in this way selective real-time measurements of the release of ROS/RNS can be made.

2.4.2 Fabrication of ROS/RNS electrodes

Electrodes were fabricated by B.A Patel, using a modification of a previously published approach (201). The conductive composite material was made by mixing 15 % of 30-50 nm (inner diameter of 5-10 nm and specific surface area of 60 m²/g) multiwall-carbon nanotubes (MWCNTs; Cheap Tubes Inc., VT, USA) with 85 % epoxy resin (10 parts of RX771C/NC Resin to 1 part of HY1300GB hardener, Robnor Resins, Wiltshire, UK). This composite material was packed into the tip of a plastic pipette tip (200 μ m internal diameter). The tip was placed flat onto a smooth glass surface and a copper wire was pushed firmly from the end of the plastic pipette tip until it was approximately 2 mm from the end of the plastic pipette tip. The electrodes were left for 48 hrs at >20 °C to allow the composite electrodes to set.

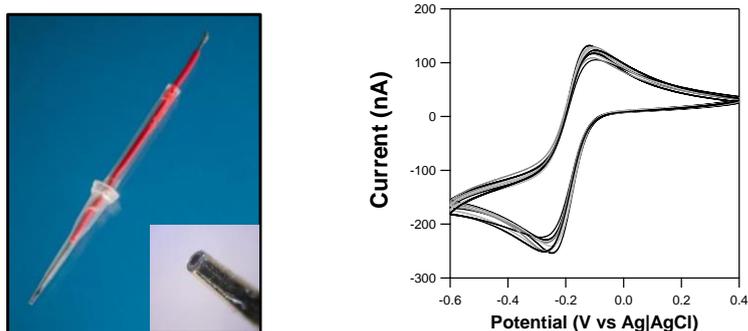


Fig 2.3 Platinum-black composite electrode and calibration voltammogram.

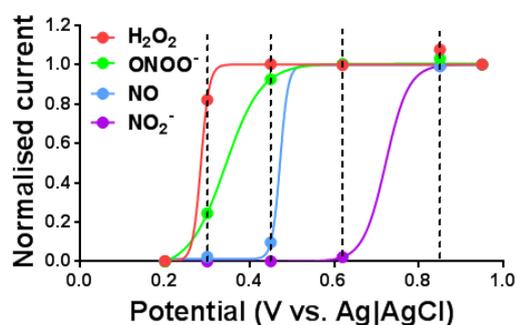
Following this, to provide support to the electrode for easy handling, a glass capillary was inserted over the copper wire and adhered to the back to pipette tip with superglue, adhering the capillary and sealing the electrode. The electrode surface was smoothed and polished with 0.3 and 0.05 micron alumina powder in succession for 30 seconds. To achieve a platinum black coating the electrode was gently roughened with 0.3 micron alumina powder and then placed into a solution composed of 1 ml of hydrogen hexachloroplatinate(IV) solution (8% wt in water; Sigma-Aldrich) and 1.6 mg lead(II) acetate trihydrate (99.8%, Sigma-Aldrich) added to 6.36 ml PBS. The deposited layer of platinum black was achieved by placing the electrode to be coated as the working electrode with a Pt counter electrode and Ag|AgCl reference electrode to complete the three electrode system. A potential of -80 mV was applied until the total charge passed was 3 mC. The Pt-black composite electrode was then rinsed three times with PBS and then DI water before being used. Optimisation and calibration of sensors was achieved by producing a voltammogram measuring a 1mM ferrocyanide solution (Fig 2.2).

2.4.3 Solutions

Peroxynitrite solutions were prepared from dilution of 10 mM peroxynitrite solution (Cayman Chemicals) with a mixture of 0.01M PBS and 0.1 M sodium hydroxide so that the pH was between 8.4 and 11.1. The stability of peroxynitrite was significantly reduced in solutions that were not alkaline. For nitric oxide solutions, 10 mM stock solutions of DEA-NONOate were prepared in 0.01 M sodium hydroxide. Both peroxynitrite and DEA-NONOate solutions will decompose over time, therefore measurements were conducted within 1 minute of solution preparation. Stock solutions were stored at -20 °C for 24 hours prior use. To initiate release of NO, specific volumes of the stock solution were placed in aerated PBS buffer (pH 7.4). For hydrogen peroxide and nitrite (Sigma Aldrich, UK), 10 mM stock solutions were prepared in PBS buffer.

2.4.4 Characterisation of sensors

Electrochemical measurements were performed using a Ag|AgCl reference electrode and Pt counter electrode (CH Instruments, UK) at room temperature using a potentiostat (CH760E, CH Instruments, UK) controlled by CH Instruments software. Multiple step amperometry was utilised for detection of the various ROS/RNS species. Recordings were carried out in a stirred solution of PBS buffer, where a baseline was achieved and after 10 s a volume of stock solution was added to make the final concentration of the 10 µM of either peroxynitrite, DEA-NONOate, hydrogen peroxide and nitrite. For characterisation studies, the oxidation peak potential was measured for the various ROS/RNS species at +0.3 V, +0.45 V, +0.62 V and +0.85 V. The current differences between these voltages were shown to provide selective detection of the four species as illustrated in Fig 2.3. Characterisation of the sensors was carried out by BA Patel.



$$I^{0.85V} = I_{H_2O_2} + I_{ONOO^-} + I_{NO} + I_{NO_2^-}$$

$$I^{0.62V} = I_{H_2O_2} + I_{ONOO^-} + I_{NO}$$

$$I^{0.45V} = 0.99 I_{H_2O_2} + 0.90 I_{ONOO^-}$$

$$I^{0.3V} = 0.85 I_{H_2O_2} + 0.29 I_{ONOO^-}$$

Fig. 2.4 Voltammogram for ROS/RNS. Dash lines define the potential offering the best sensitivity and selectivity for the detection of each species.

2.4.5 Detection of ROS/RNS from cancer cell lines

MDA-MB-231 and MCF-7 cells were plated at a density of 5×10^4 per well of a 6 well plate and incubated for 24 hrs. In order to understand the time course during which ROS/RNS generation occurred cells were exposed to cortisol and noradrenaline for 15, 30, and 90 mins. Control wells were left untreated. Following this period, the media was removed and cells lysed using 500 μ l lysis buffer (Trevigen, UK). Control wells were measured at the beginning and end time points to ensure routine handling of the cell culture plates and the replacement of cell culture media did not affect the generation of ROS/RNS (Appendix 4 Fig. 1.1A-B) Lysates were then collected and ROS/RNS levels were quantified using multiple-step amperometry using a stainless steel counter electrode and non-leak Ag|AgCl reference electrode. Measurements of the current were obtained at +0.3 V, +0.45 V, +0.62 V and +0.85 V for a duration of 30 s.

Additional measurements were also carried out to understand how ROS/RNS levels were altered with the addition of RU486, propranolol, 1400W dihydrochloride and L-NAME for 30mins prior to hormone treatment for 30mins. Furthermore, measurements were carried out in media supplemented with 10% charcoal-stripped FBS (CS-FBS) in place of FBS, to determine if the low levels of steroid hormones, including hydrocortisone and oestrogen present in FBS, had any significant effect on the generation of ROS/RNS (Appendix 4 Fig. 1.1C-D).

2.4.5 Data analysis

The baseline current values were obtained at +0.3 V, +0.45 V, +0.62 V and +0.85 V from lysis buffer alone. The last 10 seconds of the trace were monitored and the average was utilised as the current obtained at the specific voltage. Responses from cellular measurements were obtained and the current was subtracted from that obtained at the same voltage from the lysis buffer response. This current was then analysed using the equations above (Fig. 2.4) to obtain the current for the four different analytes. Once obtained, then the current was converted to concentration using calibration response. Data were shown as mean \pm standard error.

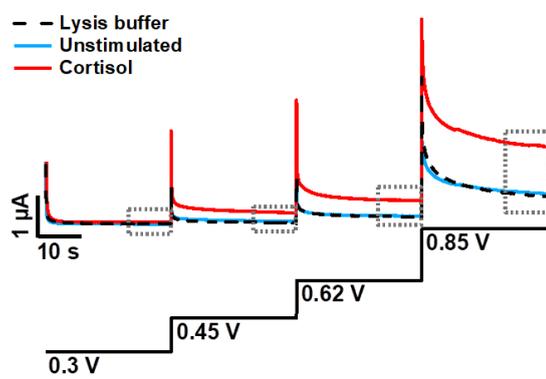


Fig. 2.5 Example voltammogram. Grey boxes indicate where measurements were taken in the last 10s.

2.5 Griess Assay

2.5.1 Principles of the Griess assay

The Griess test is used to detect nitrite (NO_2^-) ions in solution. The presence of NO_2^- infers the presence of NO, as it represents a stable end product. The Griess test utilised the reaction first described by Peter Griess in 1858, whereby NO_2^- reacts with sulphanilamide under acidic conditions, and can be measured spectrophotometrically by the formation of an azo dye, with the addition of N-(1-naphthyl)ethylenediamine (NED) (202). This test provides a reliable method for the measurement of nitrogen species in biological liquid matrices such as urine, plasma and cell culture media (203).

2.5.2 Griess assay

Cells were plated at a density of 1×10^5 in a 12-well plate and incubated for 24hrs. Cells were treated with cortisol with or without RU486 and L-NAME for 30mins, or doxorubicin ($1 \mu\text{M}$) and dexamethasone ($1 \mu\text{M}$) alone or in combination for 24hrs. Cell culture media was removed and aliquoted into a 96 well plate and the Griess assay was carried out using the Griess reagent system (Promega, UK). A standard curve was constructed using fresh cell culture media to dilute a stock nitrite solution, and to the standards and samples sulphanilamide solution was added. The plate was covered in foil and incubated at RT for 10mins. NED solution was added and the plate incubated for a further 10mins protected from the light. The plate was then read at an absorbance of 520nm, and the standard curve used to calculate the concentration of nitrite present. A reduction step (nitrate to nitrite) was not included as the levels of nitrate determined previously by electrochemical sensing were undetectable.

2.6 qRT-PCR

2.6.1 Principles of qRT-PCR

The expression of genes of interest can be measured through quantification of messenger RNA (mRNA). Transcription of a gene produces mRNA which leaves the nucleus and is translated into a protein (204). The expression of a gene and its protein product can therefore be considered to be proportional to the levels of mRNA, with higher levels of gene-specific mRNA correlating to an increase in protein levels. Real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) can be used to accurately detect low levels of mRNA and measure gene expression, by first converting template mRNA to complementary DNA (cDNA) using reverse transcription. The cDNA is then used as a template for exponential amplification by PCR in the presence of gene-specific primers. Binding of the primers to the complementary cDNA sequence produces double-stranded DNA, which can be recognised by fluorescent dyes and provide a relative signal depending on the levels of PCR products (205).

2.6.2 RNA Extraction and Quantification

RNA was extracted using an RNeasy Kit (Qiagen, UK) as per the manufacturer's instructions. Briefly, cells were harvested using trypsin-EDTA (0.025%) and centrifuged, the pellet was resuspended in 350 µl buffer RLT. Alternatively 350 µL RLT buffer was added directly to the well and lysates scraped. Lysates were centrifuged for 3 mins and supernatant mixed in a 1:1 ratio with 70% ethanol. The solution was transferred to an RNeasy spin column and centrifuged for 15s at 8000 x g. Buffers RW1 and RPE were subsequently added to the spin column and centrifuged and the pure RNA elute collected. The quality and quantity of the RNA was assessed using spectrophotometric analysis to provide ratios for purity and concentration. Samples were considered pure if their A260/280 value fell between 1.8-2.2, and forwarded into the work-flow.

2.6.2 cDNA Synthesis

cDNA was synthesised from template RNA using a Quantitect Reverse Transcription kit (Qiagen, UK) as per the manufacturer's instructions. Briefly, genomic DNA was eliminated from the RNA template by combining gDNA wipeout buffer with 1 µg of the template RNA and heating to 42°C for 2 mins. Reverse transcription was achieved by combining reverse transcriptase with the gDNA reaction, RT primer mix and RT buffer and heating to 42°C for 15 mins and 95°C for 3 mins.

2.6.3 qRT-PCR Amplification

A master mix using a Rotor-Gene SYBR Green was prepared according to Table 2 as per the

manufacturer's instructions. Gene-specific primers used are outlined in Table 3. For primers not purchased as pre-designed Quantitect Primer Assays (Qiagen, UK), standard curves to assess efficiency and melt curves to assess presence of nonspecific products were carried out. Primers were used if their amplification efficiency fell between 0.85-1.15 and displayed on peak on the melt curve. Reactions were amplified using a Rotor-Gene qRT-PCR thermocycler as per the cycling conditions outlined in Table 4.

Table 2: qRT-PCR Reaction

	VOLUME (UL)	FINAL CONC.
2X ROTOR-GENE SYBR GREEN PCR	12.5	1x
MASTERMIX		
PRIMER A	2.5	1µM
PRIME B	2.5	1µM
RNASE FREE WATER	Variable	
TEMPLATE CDNA	Variable	≤100ng
FINAL VOLUME	25	

Table 3: Primer sequences

PRIMER	SEQUENCE (5' → 3')	BASE PAIRS	MANUFACTURER
<i>ACTB</i> (HUMAN)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>ACTB</i> (MOUSE)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>NOS2</i> (HUMAN)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>NOS2</i> (MOUSE)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>TWIST1</i> (MOUSE)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>VEGFA</i> (MOUSE)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>NR3C1</i> (HUMAN)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>NR3C1</i> (MOUSE)	F:CCCGTTGGTCCGAAAATTG R:AGCTTACATCTGGTCTCATGC	20 21	Eurofins, UK
<i>TP53</i> (HUMAN)	F:GCCATCTACAAGCAGTCACAG R:TCATCCAATACTCCACACGC	21 21	Eurofins, UK
<i>CDKN1A</i> (HUMAN)	F:GTGTCTGAGCGGCTGAAGA R:GGCACTTCAGGGTTTTCTCTTG	20 21	Eurofins, UK
<i>RAD51</i> (HUMAN)	F:GAGAAGTGGAGCGTAAGCCAG R:AGCTGCATCTGCATTGCCATT	21 20	Eurofins, UK
<i>NFE2L2</i> (HUMAN)	Quantitect Primer Assay	N/A	Qiagen, UK

<i>NQO1</i> (HUMAN)	Quantitect Primer Assay	N/A	Qiagen, UK
<i>HMOX1</i> (HUMAN)	F:TTCAAGCAGCTCTACCGCTC R:CTTGGTGCATGGGTCAGCA	20 20	Eurofins, UK

Table 4: Cycling conditions

STEP	TIME	TEMPERATURE °C
ACTIVATION	5 mins	95
DENATURATION	5 secs	95
ANNEALING/EXTENSION	10 secs	60
NUMBER OF CYCLES	40	

2.6.4 Analysis

Ct values were obtained using Rotor-Gene Q software. Relative fold change in expression was measured using the $\Delta\Delta C_t$ method (comparative method) (206), and expressed as fold change \pm SEM. Data was normalised to the gene *ACTB*, which is known to be stably expressed in cancer cells (207). Example of raw Ct values shown in Appendix 7 Fig. 1.2.

2.7 Extraction, Quantification and Electrophoresis of Protein

2.7.1 Principles of Western Blotting

Western blotting is a technique that was developed 1975 to detect specific proteins from cell lysates or tissue samples and observe changes in expression. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), whereby a current is applied to proteins loaded onto sodium dodecyl sulphate polyacrylamide gel, is first used to separate out proteins according to size. The proteins can then be blotted onto membranes and probed with a specific antibody against the desired protein (208).

2.7.2 Protein Extraction for Western Blotting

On three separate passages, MDA-MB-321 and MCF-7 cells plated at a density of 2×10^5 cells per well of a six well plate were treated with hormones or a combination of receptor blockers and hormones and incubated for 2hrs at 37°C. Cells were then washed twice with cold PBS and incubated with ice cold RIPA buffer (Appendix 1) for 1-2 mins. Cells were then scraped using a cell scraper and lysates were collected and kept on ice. The lysates were subsequently spun at 13,000g for 14mins at 4°C and the supernatant collected, to be stored at -20°C until further use.

For immunoprecipitation MCF-7 and MDA-MB-231 cells were incubated with PP2 (10 μ M) for 30 minutes prior to 30 minutes with cortisol. Samples were immunoprecipitated for heat shock protein 90 (HSP90) using Dynabeads Protein A precipitation kit as per the manufacturer's instructions (Thermo Scientific, UK) and anti-HSP90 antibody (Santa Cruz, USA). A Bradford assay (Sigma Aldrich, UK) was used to measure total protein concentration and 10 μ g of protein loaded per sample. Membranes were incubated with the primary antibody for SRC 1:2000 in 10% milk (Biosource, UK) and anti-rabbit secondary 1:5000 in 5% milk.

2.7.3 Protein Quantification

The Bradford assay was used to measure total protein concentration quickly and accurately against a standard curve of known concentrations. Bovine serum albumin (BSA) (Sigma Aldrich, UK) was diluted from a stock concentration of 2mg/ml into a series of standards (0.25-2mg/ml) and 10 μ l added in triplicate to a 96 well plate (Thermo Fisher, UK). The same volume of sample was also added to the plate alongside water to blank, 200 μ l of Bradford reagent (Sigma Aldrich, UK) was then added and the plate left to stand for 5mins. The plate was read at 595nm (Digiread, Biotech, UK) and the standard curve plotted to calculate the total protein concentration.

2.7.4 SDS-PAGE Electrophoresis

This method of protein analysis is widely used and considered a standard technique in cell biology, following closely the Laemmli method developed in 1970 (209). A resolving gel (see Table 5 for composition) used to separate proteins was poured and set and a stacking gel to concentrate the proteins set on top. The protein sample was combined with 2x Laemmli sample buffer (Biorad, UK) and heated at 95°C for 10mins before being loaded into the wells at a final concentration of 20µg of total protein. The gels were placed in a Biorad Protean tank filled with running buffer (Appendix 1) and electrophoresed at 100V for 90mins. After electrophoresis the gels were removed and placed in transfer buffer (Appendix 1) ready for Western transfer.

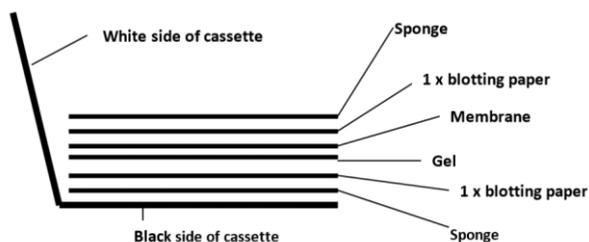
Table 5: SDS-PAGE gel composition

	10% resolving gel		4.5% stacking gel
ddH₂O	3 ml	ddH₂O	2.915 ml
30% Bis-Acrylamide	2.5 ml	30% Bis-Acrylamide	750 µl
1.5M Tris pH 8.8	1.875 ml	0.5M Tris pH 6.8	1.25 ml
10% SDS	100 µl	10% SDS	50 µl
10% APS	100 µl	10% APS	100 µl
TEMED	10 µl	TEMED	10 µl

2.7.5 Transfer of proteins to PVDF membrane using wet transfer

The transfer process can be achieved using either the wet or semi-dry method, both of which have advantages or disadvantages depending on the membrane chosen, the molecular weight of the protein and the timescale. In this instance wet transfer was chosen to blot proteins onto a polyvinylidene difluoride (PVDF) membrane. The membranes were first soaked in methanol before being placed in transfer buffer (Appendix 1) alongside blotting paper and sponges necessary to assemble the transfer 'sandwich' (Fig 2.1). Once the cassettes were assembled containing the PVDF membrane and protein gel they were placed in a Biorad transfer tank, submerged in running buffer and run at 100V for 1hr. When the transfer was complete the membranes were removed and stained with Ponceau S (Appendix 1) to check the transfer was successful. The membranes were then destained with TBST wash buffer (Appendix 1).

Fig. 2.6 Set-up for western blot transfer.



2.7.6 Antibody Probing

The membranes were incubated with an appropriate blocking buffer depending on the protein of interest (either 5% BSA or 10% skimmed milk powder (Marvel)) for 1hr at room temperature whilst shaking to block the nonspecific binding sites. Primary antibodies (see Table 6) were diluted to their recommended concentration in the blocking buffer and the membranes incubated overnight at 4°C whilst shaking, after they were then washed up to 5 times in TBST. The membranes were subsequently incubated with the appropriate horseradish peroxidase (HRP)- conjugated secondary antibody (Table 7) diluted in the blocking buffer and incubated at room temperature for 1hr whilst shaking. The washes were repeated and the membranes either stored at 4°C in TBST or developed immediately.

Table 6: Primary Antibodies

	Dilution	Source	Incubation
β-actin mouse monoclonal	1:5000	Santa-Cruz, UK	4°C O/N
iNOS rabbit polyclonal	1:2000	Thermo Scientific, UK	4°C O/N
SRC rabbit polyclonal	1:2000	Biosource, UK	4°C O/N
ATR rabbit monoclonal	1:1000	Cell Signalling, UK	4°C O/N
pATR rabbit monoclonal	1:1000	Cell Signalling, UK	4°C O/N
ATM rabbit monoclonal	1:1000	Cell Signalling, UK	4°C O/N
p21 rabbit monoclonal	1:1000	Cell Signalling, UK	4°C O/N

Table 7: Secondary Antibodies

	Dilution	Source	Incubation
Anti-Rabbit IgG HRP conjugate	1:2000	Cell Signalling	RT 1hr
Anti-Mouse IgG HRP conjugate	1:5000	Cell Signalling	RT 1hr

2.7.7 Membrane Development and Imaging.

The membranes were developed using Amersham ECL Prime detection kit which was prepared as per the manufacturer's instructions. Briefly, a luminol and peroxide solution were mixed in a ratio of 1:1 and applied to the membranes for 3mins at roomtemperature. They were placed in a film cassette and exposed to Amersham Hyperfilm, the film was the processed by immersion developing solution and fixing solution in a developing system (Xograph Compact X4). The films were imaged in a Chemi Imager (Alpha Inotech) and images analysed using ImageJ software to determine the optical density of the bands. Protein expression was normalised again expression of β -actin on the same blot, a cytoskeletal protein found in high concentrations in mammalian cells. β -actin was chosen as a housekeeping gene due to its consistent expression across cancer cells (207).

2.8 Image cytometry

2.8.1 Cell cycle analysis

Automated DNA content measurements can be used to quantify the proportions of cells in each phase of the cell cycle within a population. DNA-selective fluorescent stains allow for stratification of cells based on staining intensity, which can provide information on whether cells are undergoing DNA replication or are in growth arrest (210).

2.8.2 Two-step cell cycle analysis

Cells were plated at a density of 2×10^5 per well of a 6 well plate and incubated overnight. Cells were treated with doxorubicin ($1 \mu\text{m}$) and dexamethasone ($1 \mu\text{m}$) for 24hrs and cell cycle analysis was performed according to the NucleoCounter NC-300 two-step cell cycle analysis protocol. Cells were washed with PBS and lysed in the wells with Solution 10 supplemented with DAPI $10 \mu\text{g}/\text{ml}$. cells were incubated at 37°C for 5 mins, resuspended with lysis neutralisation buffer Solution 11, and loaded in 8-chamber cell counting slides. The slides were loaded into the NucleoCounter NC-3000 and cellular fluorescence quantified. Using the Chemometec 3000 software, gates were placed on the control sample histograms to determine the intensity representing specific stages of the cell cycle.

Fig. 2.7 Cell cycle analysis using DAPI

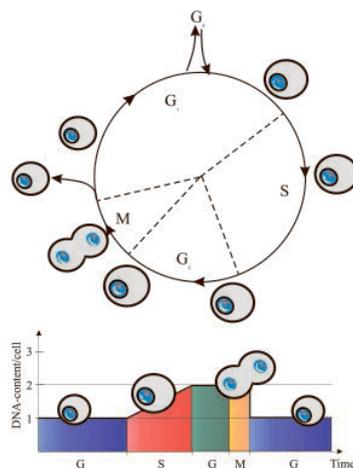


Fig 2.7 During the cell cycle the quantity of DNA content can be measured using nuclear stains such as DAPI. The intensity of DAPI staining represents the amount of DNA content and can provide quantitative information regarding the proportion of cell in each phase of the cell cycle. Figure reproduced from Chemometec 3000 software.

Commented [RF5]: Comment 37 explanation updated

2.9 Cell viability

2.9.1 Principles of the MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is a colourimetric assay, and has become the gold standard for reproducible and reliable assessment of cell viability. The assay measures viability in terms of mitochondrial activity, as the tetrazolium compound is reduced by dehydrogenases in the mitochondria to insoluble formazan crystals (211, 212).

2.9.2 Dose response curves

The LD50 for doxorubicin, paclitaxel, fulvestrant and tamoxifen was determined using dose response curves. Cells were plated at a density of 1×10^4 /200 μ l in 96-well plates and incubated for 24hr before a further 24hr incubation in the presence of chemotherapeutic agents (0.1-100 μ M). Dose response curves were plotted using non-linear regression (Graphpad Prism) and the LD50 determined. The LD50 for doxorubicin in MCF-7 cells was 1.543 μ M and for MDA-MB-231 cells was 3.51 μ M (Appendix 6 Fig. 1.1). It was not possible to obtain LD50 values for tamoxifen and fulvestrant (Appendix 6 Fig. 1.2).

2.9.3 MTT assay

Cells were plated at a density of 1×10^4 /200 μ l in 96-well plates and incubated for 24hr before a further 24hr incubation in the presence of chemotherapeutic agents. Cell culture media was subsequently removed and replaced with 200 μ L of 0.2mg/ml MTT powder dissolved in complete cell culture media. Plates were protected from the light and incubated for a further 2hrs at 37°C. The MTT solution is then removed and replaced with 200 μ L dimethyl sulfoxide (DMSO), the plate is placed on a shake plate for 5mins or until the crystals have fully dissolved. The plates are then read spectrophotometrically at 495nm (Digiread), and the viability expressed as a percentage of the control.

2.10 *In vivo* breast cancer models

2.10.1 Mouse models of breast cancer

Breast cancer models can be initiated in mice through the transplantation of breast cancer cells. Tumour cells can be transplanted through orthotopic injection into the mammary fat pad, or ectopic injection into the subcutis. Both methods induce the formation of palpable tumours which can then be measured. Cells can also be injected via tail vein to mimic metastatic disease (213). Depending on the genetic background of the implanted cells, breast cancer models are considered syngeneic, whereby the cells are of the same genetic background as the host, or xenograft, where the cells are from another species such as human. Syngeneic transplantation prevents host-graft reactions and allows study of an intact immune system, whereas xenografts have to be carried out in immunocompromised mice (214).

2.10.2 Retrospective analysis

A retrospective study was carried out using samples from the Department of Pharmacology and Chemical Biology, Pittsburgh, USA. Samples were obtained using the following protocol. The data regarding this study have been published previously (215).

MS Flint carried out the following protocols. Female BALB/c mice (6 weeks old; 20 ± 2 g) were used. Mice were injected with 1×10^5 4T1 cells/0.2mL of PBS into the left mammary fat pad. The tumours took 2 weeks to become established, with tumour volumes approx. 100 mm^3 . Tumours were measured twice weekly using a digital calliper and the tumour volumes calculated using formula, $\text{vol} (\text{mm}^3) = L \times W^2 / 2$; length (L, mm) and width (W, mm). Mice were randomized into one of the stress groups 3 days before treatment (day -3). At day 0, groups of mice were either placed individually in adequately ventilated tubes for 1 hour 3 times a week (RRS acute) or experienced no stress (NS). All mice were sacrificed at 4 weeks. All primary tumours were harvested at necropsy. All tumours were histologically confirmed by H & E staining.

2.10.3 Syngeneic mouse model

The *in vivo* study was carried out according to protocols approved by the home office (license number: PPI 70-8361), with MS Flint as the PI and RL Flaherty as the project license holder. In line with guidelines by Workman et al (216) on the welfare and use of animals in cancer research, MS Flint injected the minimum number of cells in the smallest volume e.g. 1–5 million cells in $100 \mu\text{l}$ into the mammary fat pad. All further handling, restraint stress and drug administration work was performed by the author.

Female BALB/c mice were purchased at 6 weeks old from Envigo, UK. They were housed 5 per cage with food and water *ad libitum* in a 12 hour light/dark cycle. Mice were handled daily. Tumours were induced by the subcutaneous injection of 1×10^5 66CL4 cells were injected into

the 4th mammary fat pad. Tumours were measured using digital callipers until they reached 150-200mm³, mice were then randomized into n=5. Groups were treated with intraperitoneal (IP) injections of saline or L-NAME (80mg/kg dissolved in saline) (Sigma Aldrich, UK), 30 mins prior to restraint stress. Restraint stress was carried out at the same time each day (10am-12pm). Tumour volumes were measured twice a week using digital callipers and calculated using the formula for an ellipsoid sphere; volume (mm³) = shortest (S)² x longest (L) x 0.52. Mice were also weighed once a week. Mice were sacrificed after 2 weeks of treatment or when the tumour size reached 1.2cm as per the protocol. Mice were monitored for pain, distress and weight loss. Signs of pain and distress included weight loss, ungroomed appearance, inability to ambulate, respiratory distress and/or heart rate. Any animal demonstrating any of these signs was immediately euthanised. In some instances, mice were sacrificed prior to the end of the 3 weeks study if the animal appeared unwell – and as such the n numbers reflect this.

To induce psychological stress, a well characterised restraint stress model was used. Restraint stress has been shown to induce an elevation of stress hormones levels (cortisol/corticosterone), and repetitive restraint stress has been shown to result in a sustained elevation (61). As such, restraint stress is considered a representative model of chronic stress, inducing behavioural and biochemical changes in a laboratory setting (217). Restraint stress was achieved by placing individual mice in adequately ventilated 50ml conical tubes for 2hrs 6 days a week for 2 weeks (Fig. 2.8).

2.10.4 Necropsy and Tissue harvest

Necropsies were performed to observe metastatic sites, and organs with metastases were resected for histological confirmation. Primary tumours were weighed, dissected and cut in half. Half was flash frozen in liquid nitrogen and half fixed in 10% neutral buffered formalin. Liver and lungs were also removed and fixed in formalin.

Fig. 2.8 Schematic diagram illustrating timescale of the *in vivo* model

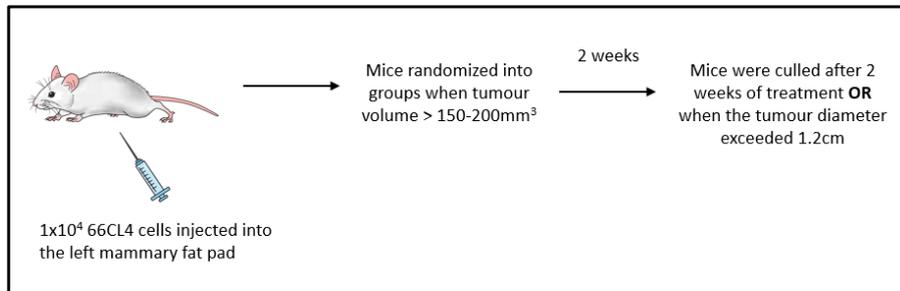
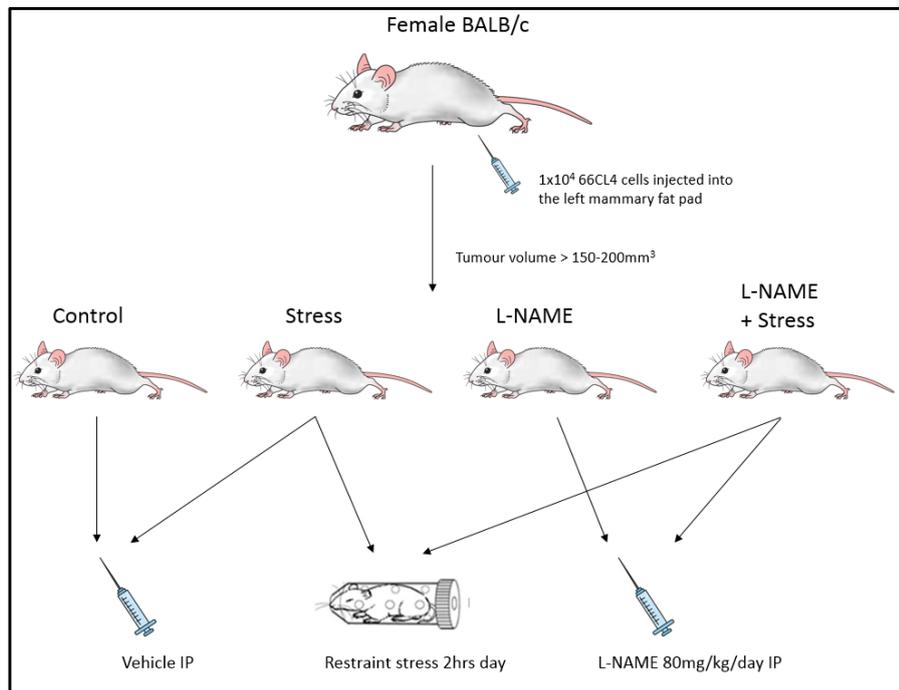


Fig. 2.9 Schematic diagram illustrating treatment groups.



2.11 Immunohistochemistry

2.11.1 Principles of Immunohistochemistry

Immunohistochemistry (IHC) is used to demonstrate the presence and location of proteins within a fixed tissue sample. This technique allows the sample to remain intact facilitating the observation of proteins within the histological context of the tissue. It relies on the principles of the immunological reaction, whereby antigen-antibody binding takes place and can be detected using a secondary antibody and signal amplification (218).

2.11.2 Tissue Sample Preparation

Tissue was fixed in 10% formalin for 24hrs, and placed into 70% ethanol before being placed into cassettes for paraffin embedding. The samples were dehydrated and coated in paraffin wax before being embedded in paraffin blocks which could be sectioned. Paraffin-embedded breast tumours were then sectioned into 5µm thick sections using a microtome and the placed in a water bath at 40°C to expand. Sections were placed onto Superfrost Ultra Plus slides (ThermoScientific, UK) and baked at 60°C overnight.

2.11.3 Deparaffinisation, Rehydration and Antigen Retrieval

Sections were deparaffinised and rehydrated in serial ethanol solutions. Briefly, sections were immersed in xylene (Sigma Aldrich, UK) twice for 5mins, 100% ethanol twice for 5mins, 90% ethanol once for 5 mins, and 70% ethanol once for 5mins and then rinsed with deionised water. Antigen retrieval was performed with Tris-EDTA buffer (Appendix 1) at 95 degrees for 10mins. Once the samples were cooled down to room temperature they were washed twice with PBS and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity.

2.11.4 Haematoxylin and eosin staining

Sections were deparaffinised as above and stained with Mayer's Haematoxylin (Fisher, UK) for 4 mins. Sections were washed with tap water for 5 mins and stained with 1% eosin (Sigma Aldrich, UK) for 2 mins. Sections were then dehydrated by immersion in 70% ethanol for 2mins followed by 100% ethanol and 100% xylene for 2 mins. Slides were mounted with DPX mounting solution (Sigma Aldrich, UK).

2.11.5 Immunostaining

Immunostaining was carried out using IHC Select HRP Detection Kit (Millipore, UK) per the manufacturer's instructions. Non-specific binding to endogenous immunoglobulins was prevented by incubating with blocking solution (IHC Select, Millipore) for 1hr at room temperature. Samples were further incubated with the primary antibody against iNOS (1:200) (Thermo Scientific, UK) diluted in 2% PBSBSA and subsequently washed with 0.2% PBS-

Tween20. Secondary antibody and streptavidin-HRP solutions were then applied (IHC Select, Millipore). To produce chromogenic staining DAB peroxidase substrate kit solution was dropped onto the samples and rinsed off in running tap water for 5 mins. Haematoxylin (Sigma Aldrich, UK) was applied for a nuclear counterstain and the samples rinsed again in running tap water for 5 mins. The samples were dehydrated by immersion in 70% ethanol for 3mins, 90% ethanol for 3mins and 100% ethanol twice for 3 mins, then cleared in xylene. Finally, the slides were mounted with DPX mounting solution (Sigma Aldrich, UK) and left to dry overnight. The slides were visualised using light microscopy (Nikon) at x20 magnification and the images were scored from 0-3, where 0=no staining, 1=mild, 2=moderate, 3=strong as described in (219).

Microvessel density (as evidenced by the presence of platelet endothelial cell adhesion molecule – PECAM-1) is used as a prognostic factor in breast cancer to identify areas of angiogenesis (220) (221). To quantify microvessel density in the primary tumours, sections were incubated with the primary antibody anti-CD31 (Abcam, UK) and secondary anti-rabbit FITC conjugated (Sigma Aldrich, UK) for 1 hour and 30 minutes at room temperature respectively. Slides were mounted in vectorshield plus DAPI (Sigma Aldrich, UK) and stored in the dark at 4°C, before imaging (x400) using confocal microscopy (Leica). Areas of high microvessel density were identified at low magnification (x20), and at (x40) the number of CD31-positive vessels were counted per field.

2.12 Bioinformatic analysis

2.12.1 Use of bioinformatics in breast cancer research

The advances made in whole genome, transcriptome and proteome sequencing, and the increasing availability of high-throughput gene expression analysis in recent years has made bioinformatic analysis a staple of modern biology (222). In cancer research, several databases are now available which allow the analysis of large patient datasets in relation to gene expression or mutation frequency. For example, The Cancer Genome Atlas (TCGA) has completed genomic characterisation of 33 cancers in over 11,000 patient samples, using various techniques to profile genomes and genomic alterations involved in tumourigenesis (223). Specifically, in breast cancer research the use of bioinformatic analysis is now considered integral to high quality research, in order to better understand disease progression at a molecular level (224).

2.12.2 Gene expression analysis

The expression of genes of interest in human breast carcinomas was examined using the publicly accessible OncoPrint Cancer Microarray database. Analysis of The Cancer Genome Atlas (TCGA) Breast database (n = 137) and Curtis Breast database (n = 1600) was used to compare expression between normal breast tissue and invasive breast carcinoma.

2.12.3 Kaplan-Meier survival analysis

Analysis of gene expression microarray data from the European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA) was used to ascertain the impact of expression of a gene on survival proportions, using Kaplan-Meier analysis. Breast cancer patients were separated according to receptor status (ER/PR/HER2), grade (1-3) or molecular subtypes (basal (ER-/HER2-), luminal A (ER+/HER2-/KI67 low), luminal B (ER+/HER2-/KI67 high and ER+/HER+) and HER2+ (ER-/HER2+)), before being stratified according to their expression status (high vs low).

2.13 Statistical Analysis

Graphpad Prism v5.0 was used for all statistical analysis. For continuous data assuming normal variance student's t-test's or one way analysis of variance (ANOVA) was used with Tukey's multiple comparisons tests between groups. For grouped analysis two-way ANOVA was used with Bonferroni's post-tests, and for discrete data Mann-Whitney tests were used. Statistical significance was determined where $p < 0.05$. All the results are representative of the mean of three independent experiments ($n=3$) \pm SEM unless otherwise stated. * indicates a significant difference. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Chapter 3 - Glucocorticoids induce reactive nitrogen species (RNS) production and DNA damage through an inducible nitric oxide synthase (iNOS) mediated pathway

3.1 Introduction

3.1.1 Psychological stress and tumourigenesis

There are numerous genetic and environmental factors that contribute to the formation and metastasis of tumours (225), however epidemiological and bio-behavioural evidence is accumulating to suggest that psychological stress may play a role in tumour biology. Exposure to hormones released as part of the stress response has been linked to an increased risk of diseases such as hypertension, immune dysfunction and cancer (52, 105). The neuroendocrine hormones, glucocorticoids and catecholamines (stress hormones), mediate a wide range of effects throughout the body. Stress hormones are able to influence tumour biology through activation of hormone receptors and initiation of signalling cascades, deregulation of which are hypothesised to modulate tumourigenesis.

Cortisol, is a glucocorticoid secreted by the adrenal cortex in response to stressors. Cortisol binds to the glucocorticoid receptor (GR) through which its biological function is primarily determined. The GR, located in the cytoplasm, is bound to chaperone proteins forming a complex which, following activation, translocates to the nucleus where it binds to response elements on GR-controlled target genes. In this way cortisol regulates processes including metabolism, growth and apoptosis. The release of glucocorticoids and their effect on tumours can be modulated through manipulation of the stress response *in vivo* using models of psychological stress. Several models of psychological stress exist, including social isolation in normally social animals such as rodents, stress induced by noise or temperature, forced swim tests or physical restraint stress. These stressors have all been shown to induce an endocrine stress response, and can be administered in an acute setting i.e. for a short period of time, or repetitively to model chronic stress (61, 217, 226). Restraint stress is the most well defined model of stress, and has been shown to induce behavioural, biochemical and physiological changes in laboratory animals repeatedly (61, 227). In studies of the effects of stress on cancer, chronic restraint stress has been shown to induce metastatic signalling and suppress immune responses (113, 215), as well as remodel lymph vasculature which promotes tumour cell dissemination (228). Similarly, social isolation has been shown to increase the malignant burden of mammary tumours (229), and also alter the gene expression profile in transgenic mice, upregulating glucocorticoid-controlled metabolic pathways and eventually increasing spontaneous mammary tumour formation (230). Social confrontation in male rats stimulated adrenergic activation and increased metastatic development, further implicating a role for both arms of the stress response in tumour formation and development (231). Exogenous administration of glucocorticoids and catecholamines has also exerted similar effects, with dexamethasone, a potent synthetic glucocorticoid promoting resistance to apoptosis in

tumour models (232), and NE treatment upregulating metastatic markers *in vivo* (233).

Clinical studies utilising transdisciplinary approaches to psychology and biology have previously focused on negative emotions, lack of social support, social isolation and depression in relation to already diagnosed cancers (234). Stress reduction interventions have therefore been employed with varying success to improve outcome in breast cancer patients, although there is still a level of controversy regarding the effects of stress in the progression of cancer. Psychological interventions intended to promote coping strategies and as well as alter health behaviour, have been able to reduce the risk of recurrence and breast cancer-related death in patients (235). Furthermore, whilst supportive-expressive group therapy (SEGT) was shown to have no effect on the outcome of ER-positive patients, in ER-negative patients SEGT resulted in a reduction in tumour progression (236), however this data was not replicated in a further study of SEGT and breast cancer (237).

3.1.2 Glucocorticoids and breast cancer

The proposed mechanisms behind the apparent effects of stress hormones on cancer are diverse, ranging from immune modulation, increased metabolic capabilities, angiogenic signalling, growth factor signalling and oxidative stress (238). This places the role of stress hormone signalling at the centre of a complex web, affecting nearly all of the processes identified as hallmarks of cancer (239).

High levels of the GR have been linked an increase in the progression of ER-negative breast cancers, indicating that increased glucocorticoid signalling contributes to tumour progression (240). Cortisol has been shown to promote growth of mammary tumour cells and exert cell survival effects in the absence of serum in culture (99), as well as inhibiting apoptotic signalling through transactivation of anti-apoptotic genes (140). Binding of growth factors can further enhance proliferation in breast cancer, and glucocorticoids many increase epidermal growth factor (EGF) binding, as well as promoting expression of the receptor (EGFR) (241). Oncogene activation and tumour suppressor downregulation are also important markers of a switch to an aggressive invasive phenotype in breast cancer. Upregulation of the proto-oncogene *c-fms* through a GR-mediated mechanism (242), and crosstalk between the GR and tumour suppressor p53 may therefore be partly responsible for a glucocorticoid mediated shift to a pro-survival phenotype (243). In TNBC glucocorticoids were found to upregulate genes associated with drug resistance (244), and can block cell death induced by chemotherapies, promoting chemotherapy resistant transformation (140).

Psychological stress also supresses and dysregulates immune function, and glucocorticoids are

well known to directly modulate immune cell function. Glucocorticoids can act as immunosuppressive by inducing apoptosis in immune cells, changing differentiation fate and inhibiting cytokine release, mostly controlled through transrepression of GR-controlled genes (118). It's thought that immunosuppression caused by glucocorticoids may exacerbate tumour development through the downregulation of cellular immunity and particularly natural killer (NK) cells, which can protect against malignant cells (103). In a clinical setting, higher levels of stress have been shown to correlate with a decrease in NK cell activity in post-operative breast cancer patients (245).

Fig. 3.1 Effects of stress hormone signalling in cancer

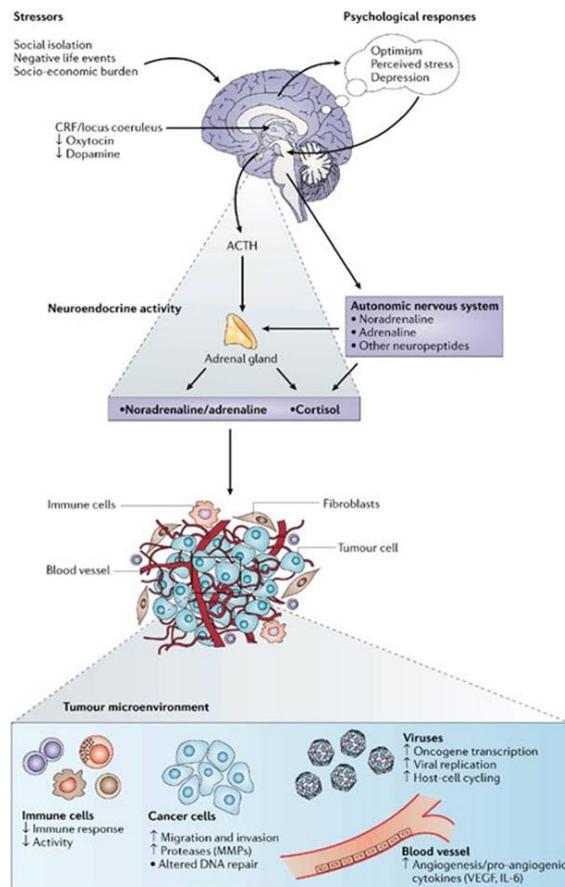


Fig. 3.1 Stress hormone signalling can exert a range of effects on malignant cells as well as stromal tissue. Figure taken from (238).

3.1.2 Stress and DNA damage/ repair

Stress hormones are now known to play a role in DNA damage and repair (135), potentially affecting oncogenic transformation. Several studies have correlated psychological stress with increased DNA damage, with the addition of stress hormones to immortalized mouse fibroblasts resulting in significant DNA damage as measured by the comet assay (125, 127). In animal models, psychological stressors have been shown to promote DNA damage in a range of tissues including brain, GI tract and liver, although some of these studies may be confounded by interference with physical pain signalling as they often employ methods such as foot shocks (123). The literature regarding DNA damage caused by stress in humans is also complex, with the measures of stressors varying from biological measures (e.g. serum and salivary cortisol) to more subjective methods such as questionnaires (e.g. the perceived stress scale (72)). There have been numerous studies on caregiving, with particular emphasis on the stress of parent with sick children. For example, perceived stress in parents of ill children versus healthy children correlated with an increase in telomere shortening and oxidative stress (246), as did measures of DNA strand breaks in stressed students (247). Patients suffering from depression also had higher levels of an excreted marker of oxidative DNA damage (248).

One mechanism through which the induction of DNA damage by stress hormones has been proposed is via an interaction with p53, a crucial gatekeeper of DNA damage and repair responses. Stimulation of the β -AR by catecholamines induced phosphorylation of γ -H2AX, an early marker of DNA damage, through p53 degradation. Pharmacological inhibition revealed that this was as result of murine double minute 2 (MDM2) phosphorylation by PI3K/AKT signalling (135). DNA damage may also be caused through activation of the immune system; more specifically through pro-inflammatory cytokine signalling. Acute stressors have been associated with increased pro-inflammatory cytokine production (249), and increased pro-inflammatory cytokine signalling can induce DNA damage through generation of RNS (250).

In light of increased DNA damage cause by psychological stress, functional DNA repair processes are crucial in order to maintain the genetic integrity of the cell and prevent transformation. Previous work suggests that stress hormones may interact with some DNA repair pathways, and that this interaction in malignant cells slows or halts the rate of repair (251). In particular, the addition of stress hormones allows circumnavigation of DNA damage cell cycle checkpoints (125). If the cell is unable to halt the cell cycle, and replicates with the damaged DNA, this increases the potential of tumourigenic mutations. Furthermore, Hara et al. have shown that binding of catecholamines to β 2-adrenergic receptors recruits the signal transducer proteins β - arrestins. These are able to interfere with the DNA damage response of

p53 resulting in down-regulation of normal p53 signalling, another potential mechanism by which deleterious DNA damage is allowed to accumulate (135). Cortisol can also downregulate the expression of the DNA repair protein BRCA1, which functions to signal DNA repair cascades. BRCA1 is heavily involved in the induction of tumourigenesis in breast cancer, with mutations in the gene dramatically increasing the risk of breast cancer (252). As such decreased efficacy of repair caused by glucocorticoids and catecholamines may represent a link between stress and breast cancer risk.

3.1.3 Stress and ROS/RNS

The aforementioned induction of stress hormone mediated DNA damage has been suggested to occur as a result of the production ROS/RNS, capable of interacting with DNA causing base changes and strand breaks (253). Both acute and chronic exposure to stress in rats produced an increase in ROS in the GI mucosa (254), and in the liver of rats oxidative damage was significantly increased in response to restraint and water stress (255).

Cancers with a propensity to become metastatic have a progressive increase in ROS, contributing to tumour angiogenesis and metastasis (256). Studies have shown that specific ROS/RNS can sensitise cancer cells to ROS-inducing chemotherapy agents (257, 258). It is thought that catecholamines have the potential to increase the production of ROS through β -AR activation, upregulating PKA activation and levels of ROS, generating oxidative phosphorylation within the cell (132). Additionally, isoforms of the enzyme nitric oxide synthase (NOS) which produce nitric oxide (NO), a potentially damaging RNS, are upregulated in certain cancers including breast (259, 260). Overexpression of inducible NOS (iNOS) and the subsequent increase in NO has wide reaching implications in the context of malignancy, as NO is involved in several central signalling pathways regulating survival and proliferation (261). Recently, the inhibition of iNOS as a potential treatment in breast cancer has been gathering momentum, with studies showing that iNOS inhibition can reduce the growth of tumours *in vivo* (172).

3.1.4 Aims

The aims of this research are to determine if stress hormones generate oxidative stress in breast cancer cells. Furthermore, to establish a mechanism for the induction of DNA damage by stress hormones, as well as to explore the effect of stress hormones on the efficacy of DNA repair.

3.2 Results

3.2.1 Stress hormones induce ROS/RNS production in breast cancer cell lines

Electrochemical sensors were used to measure the production of ROS/RNS from breast cancer cells treated with stress hormones. The sensors were run using amperometry, whereby a change in current is proportional to the oxidation or reduction of an electroactive species in a biochemical reaction. By applying different voltages to a microelectrode the measurement of distinct reactive oxygen/ nitrogen species according to their oxidation peak potential can be achieved, providing a sensitive and reliable method to analyse endogenously produced species (201).

In order to establish a relationship between stress hormones and the generation of ROS/RNS, breast cancer cells were exposed to physiological concentrations of the stress hormones cortisol and noradrenaline (262). Breast cancer cell lines representing different subtypes were chosen based on their GR expression, to investigate the role of ROS/RNS in DNA damage in TNBC versus non-TNBC. The TNBC lines MDA-MB-231 and HCC38 were selected based on their differing GR status, and MCF-7, an ER-positive cell line based on its similar level of GR expression to MD-MB-231. These cell lines also possess β 2-AR's and are aggressively tumourigenic

Electrochemical analysis of cell lysates post hormone treatment revealed that ROS/RNS are generated in a time dependant manner, with both MDA-MB-231, MCF-7 and HCC38 cell lines responding to treatment at 15, 30 and 90 minutes. In response to cortisol, levels of nitric oxide (NO) increase significantly and peak after 30mins in MCF-7 ($p < 0.05$), MDA-MB-231 ($P < 0.001$) and HCC38 ($p < 0.001$) (Fig. 3.2.1A-C). Levels of nitrite (NO_2^-) also peak at 30mins and remain significantly elevated at 90mins in MCF-7 ($p < 0.01$), MDA-MB-231 ($P < 0.001$) and HCC38 ($p < 0.001$) (Fig. 3.2.1A-C). Levels of peroxynitrite (OONO^-) were significantly elevated after 30mins in MDA-MB231 and HCC38 cells, but not MCF-7 (Fig. 3.2.1B-C).

After exposure to NE hydrogen peroxide (H_2O_2) levels in MCF-7 cells were significantly higher after 30mins ($p < 0.05$) but did not remain elevated (Fig. 3.2D). In MDA-MB-231 cells there was a significant increase in levels of H_2O_2 after 15 mins ($p < 0.01$) and levels continued to rise for 90mins ($p < 0.001$) (Fig. 3.2.1E), as was the case in the second TNBC line HCC38 ($p < 0.001$) (Fig. 3.2.1F).

This data shows that cortisol increases the generation of RNS and NE the generation of ROS in TNBC and ER+ breast cancer cell lines.

Fig. 3.2.1 Stress hormones induce ROS/RNS production in breast cancer cell lines

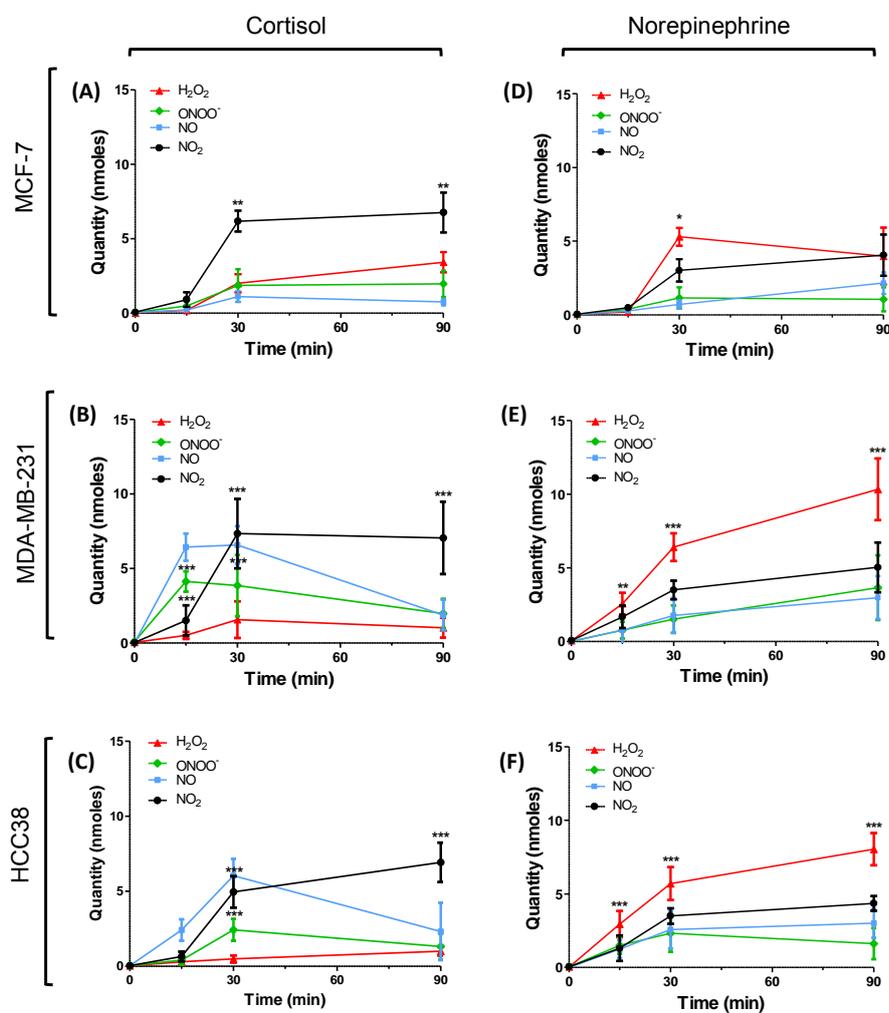


Fig. 3.2.1 MCF-7, MDA-MB-231 and HCC38 cells were incubated with Cort (1 μ M) (A-C) and NE (1 μ M) (D-F) for 15, 30 and 90 mins. Cell lysates were collected and electrochemical sensors used to measure levels of hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), nitric oxide (NO) and nitrite (NO₂). Data are shown as mean \pm SEM and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant increase compared to baseline. * = p<0.05, ** = p<0.01, *** = p<0.001.

3.2.2 Stress hormones induce production of ROS/RNS through a GR or β -AR mediated mechanism

To determine if the effects of cortisol and NE were mediated through a receptor-based mechanism, levels of NO_2^- , the stable byproduct of NO, and H_2O_2 were measured in cells treated with receptor antagonists RU486 (GR) and propranolol (β -AR) prior to hormone treatment. Cells were also treated with inhibitors of nitric oxide synthase (NOS), L-NAME which inhibits all isoforms and 1400W dihydrochloride which inhibits iNOS selectively prior to hormone treatment.

In MCF-7 cells, levels of NO_2^- are significantly increased after cortisol exposure ($p < 0.001$) and decreased in response to incubation with RU486 ($p < 0.001$). There was also a significant reduction in NO_2^- in cells inhibited with both L-NAME and 1400W prior to cortisol exposure compared to cortisol alone ($p < 0.001$) (Fig. 3.2.2A). In MDA-MB-231 cells there was also a significant increase in NO_2^- in response to cortisol ($p < 0.01$), however this was not significantly reduced by RU486 treatment. Selective and non-selective inhibition of NOS did significantly reduce cortisol induced NO_2^- levels compared to cortisol alone ($p < 0.01$) (Fig. 3.2.2B).

In response to NE, levels of H_2O_2 increase significantly in both cell lines (MCF-7 ($p < 0.05$), MDA-MB-231 ($p < 0.001$)), and this effect is negated in the presence of propranolol, with significant reductions compared to NE alone (MCF-7 ($p < 0.05$), MDA-MB-231 ($p < 0.001$)). Levels of H_2O_2 induced by NE remained unaffected by exposure to L-NAME and 1400W (Fig. 3.2.2C-D).

This data demonstrates that the generation of NO_2^- by cortisol, and H_2O_2 by NE is receptor mediated, and that inhibition of NOS can also mediate the generation of RNS by cortisol, but not ROS by NE.

Fig. 3.2.2 Stress hormones induce production of ROS/RNS through GR or β -AR mediated mechanism

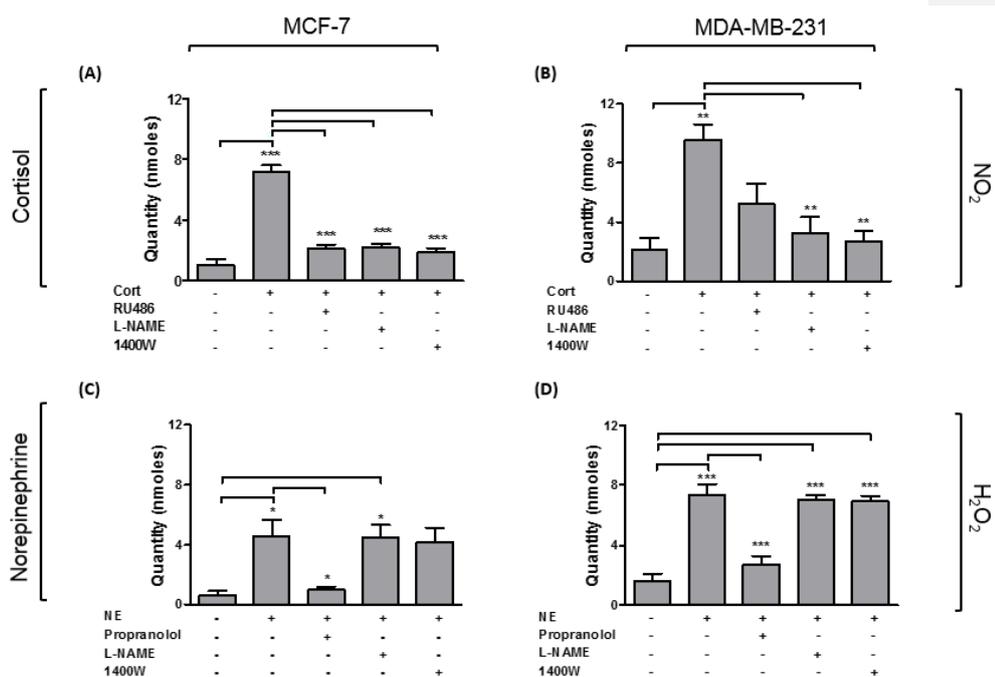


Fig. 3.2.2 MCF-7 (A+C) and MDA-MB-231 (B+D) cells were incubated with Cort (1 μ M) and RU486 (1 μ M). Cells were also incubated with NE (1 μ M) and Propranolol (1 μ M), as well as non-specific and specific NOS blockers (L-NAME (100 μ M) and 1400W (10 μ M)) for 30 mins prior to 30 mins stress hormone exposure. Levels of NO₂⁻ (A-B) and H₂O₂ (C-D) were measured using electrochemical sensors. Mean \pm SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference. * = p<0.05, ** = p<0.01, *** = p<0.001.

3.2.3 Stress hormones induce DNA damage in breast cancer cells

Stress hormones are known to mediate DNA damage in breast cancer and it suggested that this may be as a result of the production of ROS/RNS. DNA damage can be quantified using the formation of phosphorylated γ -H2AX foci which form in response to DNA damage signalling. Breast cancer cell lines MDA-MB-231 and MCF-7 were incubated with cortisol and NE at physiologically relevant concentrations for 2hrs, alongside incubation with the GR antagonist RU486 and the selective iNOS inhibitor 1400W for half an hour prior to hormone treatment.

Analysis of fluorescent foci expressed as a percentage of total cells counted, indicates that there is a statistically significant increase in DNA damage in response to cortisol in MCF-7 cells ($p < 0.001$) (Fig. 3.2.3A) and MDA- MB-231 cells ($p < 0.001$) (Fig. 3.2.3B), compared to the unstimulated control. The addition of NE also produced a significant increase in DNA damage in MDA-MB-231 ($p < 0.001$) and in MCF-7 cells compared to the control ($p < 0.001$). In MCF-7 cells the effect of cortisol on DNA damage was significantly reduced in in the presence of a receptor antagonist ($p < 0.01$), as was the case with MDA-MB-231 ($p < 0.001$). Furthermore, in both cell lines the effect of cortisol on DNA damage was reduced by treatment with the iNOS inhibitor 1400W prior to cortisol treatment ($p < 0.001$). Incubation with 1400W prior to treatment with NE did not reduce the DNA damage promoted by NE.

Fig. 3.2.3 Stress hormones induce DNA damage in breast cancer cells

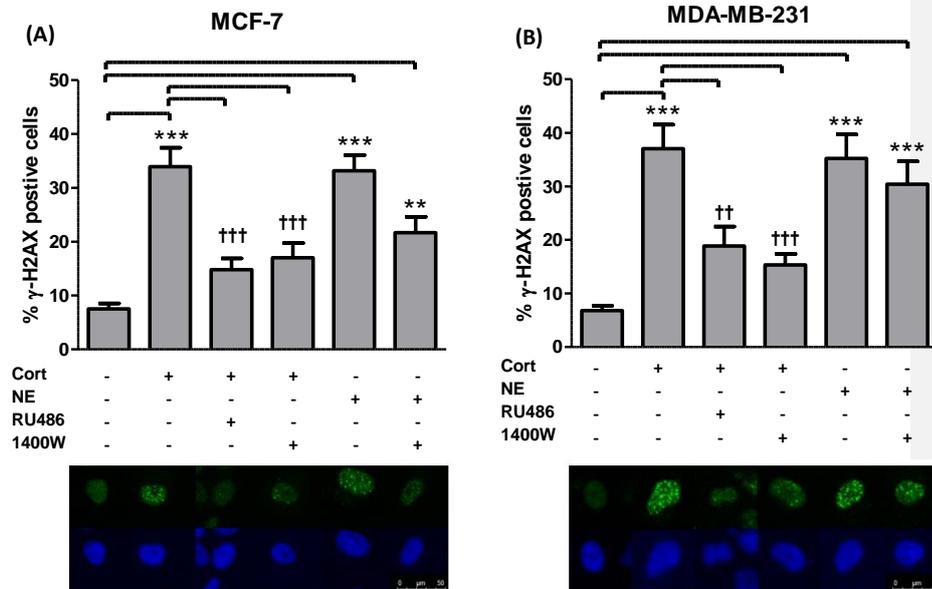


Fig. 3.2.3 MCF-7 cells (A) and MDA-MB-231 cells (B) were incubated with cortisol (Cort) and NE for 2hrs in the presence and absence of RU486 and 1400W. Cells were immunofluorescently labelled and cells with >5 foci (in green) were scored as positive. Representative images shown. Mean \pm SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant increase compared to unstimulated cells, † indicates a significant decrease compared to cortisol. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2.4 DNA repair is adversely affected by stress hormones

Stress hormones have been shown previously to adversely affect repair of damaged DNA (125). To examine how stress hormone exposure affects repair in breast cancers, single cell electrophoresis was used in the form of the comet assay. DNA repair was monitored by incubating MDA-MB-231 with stress hormones, and concurrently incubating cells with stress hormones and then allowing a period of repair (20mins) in hormone-free media. Comet tails indicating DNA strand breaks were visually scored according to intensity (0–4).

A significant increase in DNA damage was immediately observed compared to the control after 30 minutes treatment with cortisol and NE ($p < 0.01$) (Fig. 3.3.4A). Damage was also observed in the H_2O_2 control ($p < 0.01$), and this was reduced after a 20-minute repair period. However, the levels of DNA damage in the cells that were allowed to repair post cortisol treatment remained significant compared to the H_2O_2 repair control ($p < 0.05$). Representative images are shown.

Fig. 3.2.4 DNA repair is adversely affected by stress hormones

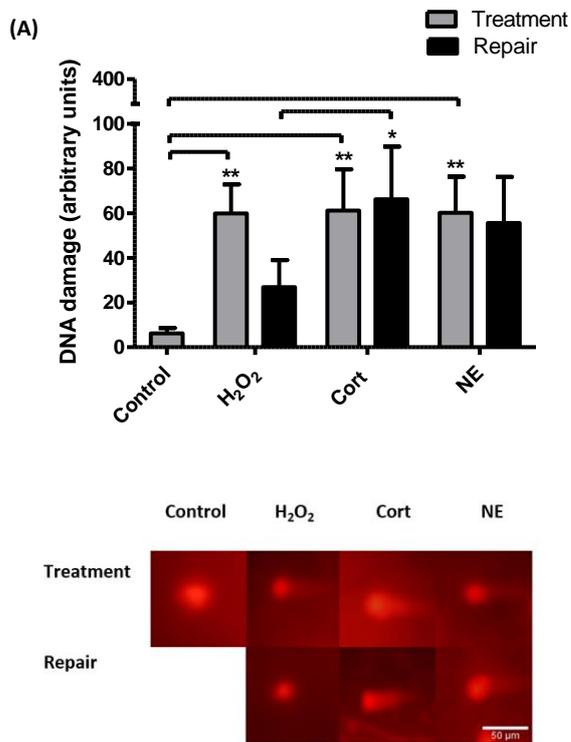


Fig. 3.2.4 (A) MDA-MB-231 cells were incubated with cortisol (Cort) and NE for 30mins and assessed for DNA damage using the comet assay. Comet tails indicating DNA strand breaks were visually scored according to intensity (0–4). Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey’s multiple comparisons). * indicates a significant difference. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2.5 iNOS expression is upregulated in response to cortisol

In order to probe the mechanism underlying the glucocorticoid mediated effect on RNS, the expression of iNOS at an mRNA and protein level in response to glucocorticoids was examined. MCF-7 cells were exposed to cortisol for 30mins and 24hrs and mRNA was extracted. This was converted to cDNA and amplified with gene specific primers for iNOS and β -actin using q-PCR. Ct values for iNOS were normalised against β -actin and the fold change in mRNA levels expressed. A significant 60-fold increase in levels of iNOS mRNA was observed at 24hrs compared to 30mins and the control ($p < 0.05$) (Fig 3.2.5A).

Protein also extracted from MCF-7 cells exposed to cortisol for 30mins and 24hrs was resolved onto membranes and probed with antibodies against iNOS and β -actin. Optical densitometry values for iNOS were normalised against the β -actin loading control. Western blot analysis of protein levels of iNOS after acute and 24hr exposure show that cortisol does not increase expression in an acute setting, however after 24hr exposure protein expression increases although not significantly (Fig 3.2.5B).

Fig. 3.2.5 iNOS expression is upregulated in response to cortisol

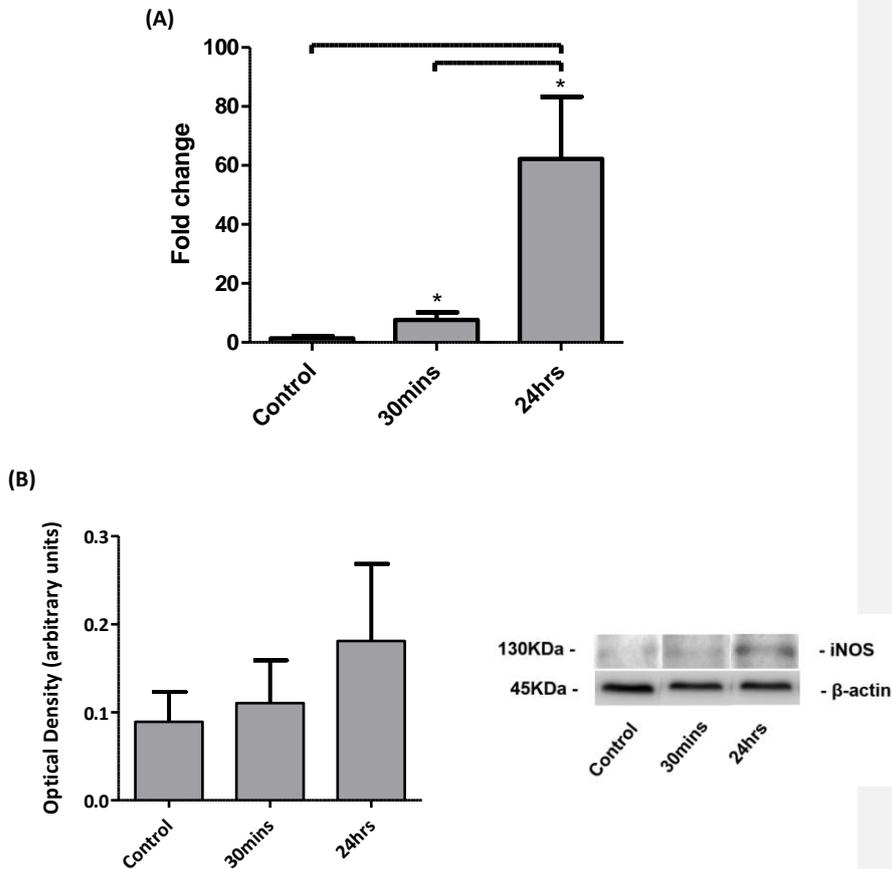


Fig. 3.2.5 (A) MCF-7 cells were exposed to cortisol for 30mins and 24 h and mRNA was extracted. cDNA was synthesised and amplified in the presence of gene-specific primers for *NOS2* and *ACTB* using q-PCR. Cycle threshold (Ct) values for *NOS2* were normalised against *ACTB* (β -actin) and fold change was calculated using the delta-Ct method. **(B)** Protein was extracted from MCF-7 cells exposed to cortisol for 30mins and 24hrs. iNOS expression was analysed using western blotting and expressed as optical density values normalised against β -actin. Data are mean \pm SEM and significance was determined using one-way analysis of variance (post hoc Tukey multiple comparisons). * indicates a significant difference. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2.6 iNOS expression is upregulated in mammary tumours in mice exposed to stress

The effect of restraint stress in a model of aggressive breast cancer on iNOS was determined using paraffin embedded primary tumour sections from previously published study (263). This work was carried out by MF Flint in the US.

Immunohistochemical analysis of tumours harvested from syngeneic mouse models exposed to acute psychological stress (1hr/3 times a week for 28 days) confirmed the presence of iNOS in the tissue. Paraffin embedded sections were incubated with a primary antibody against iNOS and intensity of staining was scored from 0-3, where 0=0% staining, 1= 15%, 2= 15-50%, 3= 50-100%. Both groups scored positively for iNOS within the tumours, however samples from psychologically stressed animals displayed a statistically significant ($p<0.01$) higher average IHC score than non-stressed (Fig 3.2.6A). Representative staining shown (Fig 3.2.6B).

Fig. 3.2.6 iNOS expression is upregulated in mammary tumours in mice exposed to stress

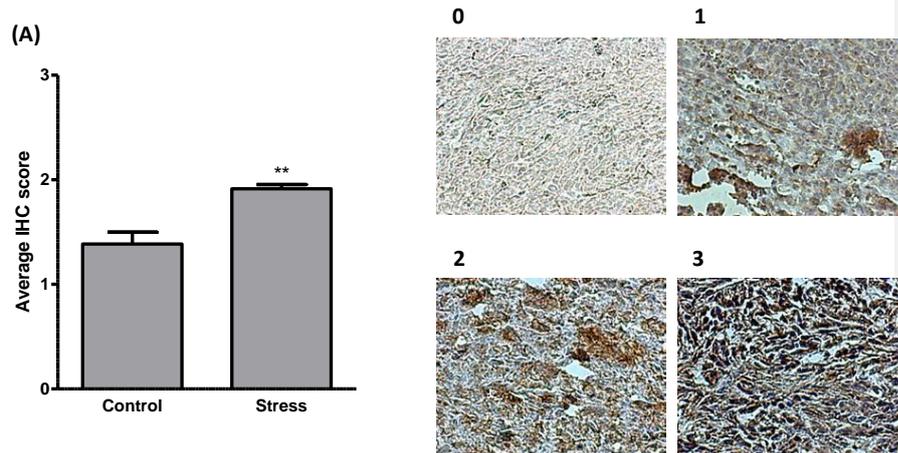


Fig. 3.2.6 (A) The 4T1 mouse mammary tumour cells were transplanted into the fourth mammary fat pad of female BALB/C mice and the animals randomised into groups exposed to either acute restraint stress (n=5) or no stress (n=5). Tumours were harvested, fixed in paraffin and sectioned subsequent to immunohistochemical detection (IHC) of iNOS. Labelling was scored (0–3) according to intensity; representative panels are shown. The Mann-Whitney test was used to ascertain statistical significance. *Significant increase compared to control: *p < 0.05, **p < 0.01, ***p < 0.001

3.2.7 Src dissociates from the GR complex in response to cortisol

Associated to the GR in its ligand-unbound state is a multi-complex of proteins including heat shock protein 90 (HSP90) and Src kinase (264). The association of Src to the GR complex was examined to determine if the GR induces rapid non-genomic effects on iNOS through Src to produced RNS.

The cell lines MCF-7 and MDA-MB-231 were treated with cortisol for 30mins and 24hrs and the selective Src inhibitor PP2 for 30 mins and the expression of Src was assessed using western blotting. There was no significant increase in Src protein expression levels in MCF-7 or MDA-MB-231 cells treated with cortisol (Fig. 3.2.7A) but the expression of Src was blocked with PP2 (Fig. 3.2.7B). Subsequently MCF-7 and MDA-MB-231 cells were treated with cortisol for 30 mins alone or in the presence of Src inhibitor PP2 and immuno-precipitated for HSP90. Western blot analysis shows that in unstimulated cells Src remains bound to HSP90 whereas in cells expose to cortisol for 30 mins Src levels decrease indicating dissociation from the complex. The dissociation was not blocked by the addition of PP2 in MDA-MB-231 or MCF-7 cells (Fig. 3.2.7A).

MCF-7 cells were also incubated with cortisol alongside the Src inhibitor PP2 and GR-antagonist RU486. Immunofluorescent analysis shows cortisol induced translocation of the GR to the nucleus, and this was blocked by incubation with RU486 and reduced with PP2 (Fig. 3.2.7C).

Fig 3.2.7 Src dissociates from the GR complex in response to cortisol

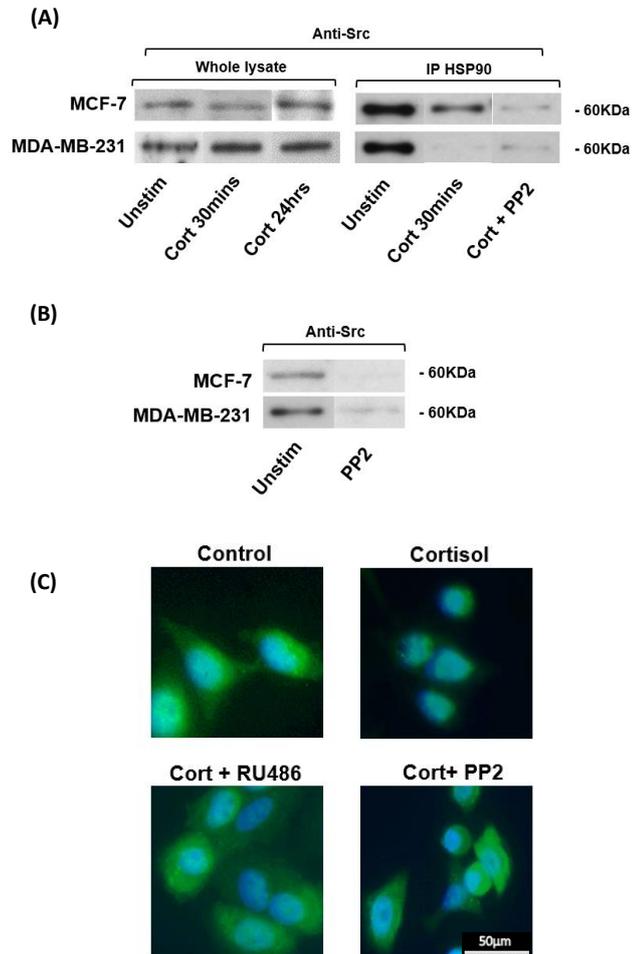


Fig. 3.2.7 (A) MCF-7 and MDA-MB-231 cells were exposed to cortisol for 30 mins or 24 hrs. **(B)** Cells were also exposed to selective Src inhibitor PP2 for 30 mins. Samples treated with cortisol and PP2 were immuno-precipitated for HSP90 (right panel). Equal amounts of protein were immunoblotted and Src protein expression was visualised. **(C)** MCF-7 cells were exposed to cortisol for 30 minutes in the presence or absence of RU486 or PP2. Cells were immunofluorescently labelled for the GR and counterstained with DAPI. Representative images are shown.

3.2.8 Glucocorticoid-induced production of RNS is reduced by Src kinase inhibition

To determine if GR associated Src is involved in the generation of RNS; electrochemical sensors were used to detect ROS/RNS in cell lysates in MCF-7 cells. A significant decrease in NO_2^- was observed in response to cortisol and Src inhibitor PP2 compared to cortisol alone ($p < 0.001$) (Fig. 3.2.8A). Similarly a significant decrease in NO_2^- was also observed in response to NE and PP2 compared to NE alone ($p < 0.01$) (Fig. 3.2.8B). Cortisol and NE in combination have an additive effect on the generation of NO_2^- increasing it significantly compared to the control ($p < 0.001$), and this effect is reduced by PP2 ($P < 0.001$) (Fig. 3.2.8C). Levels of H_2O_2 in response to cortisol increase and remain elevated with the addition of PP2 (Fig. 3.2.8D). NE mediated levels of H_2O_2 were unaffected by the addition of PP2 with both increasing compared to the control ($p < 0.001$) (Fig. 3.2.8E). Cells treated with cortisol and NE in combination produce higher levels of H_2O_2 when incubated with PP2 than alone ($p < 0.001$) (Fig. 3.2.8F).

Fig. 3.2.8 Glucocorticoid-induced production of RNS is reduced by Src kinase inhibition

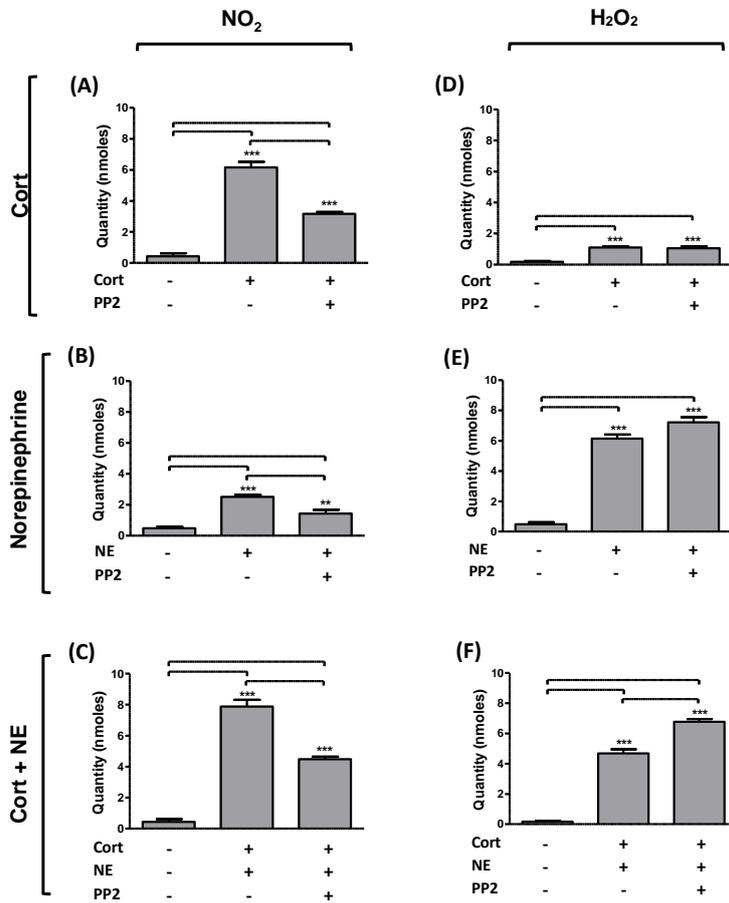


Fig. 3.2.8 MCF-7 cells were incubated with Cort (A, D), NE (B, E) and in combination (C, F) in the presence or absence of PP2. Cell lysates were collected and electrochemical sensors used to measure levels of hydrogen peroxide (H₂O₂) and nitrite (NO₂). Data are shown as mean ± SEM and statistical significance was determined using one-way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference. * = p<0.05, ** = p<0.01, ***= p<0.001.

3.2.9 Expression of iNOS and Src is upregulated in invasive breast carcinomas

Bioinformatic analysis using microarray databases of patient samples provides useful biological insight into the prevalence and expression of genes in a real-world clinical setting.

Expression of the gene encoding iNOS (*NOS2*) was analysed using Oncomine Cancer Microarray databases. In invasive breast carcinoma (n = 53) *NOS2* expression was significantly increased ($p = 7.19 \times 10^{-9}$) compared to normal stromal breast tissue (n = 6) (Fig. 3.2.9A). Expression of Src was also analysed using Oncomine Cancer Microarray databases. In invasive breast carcinoma (Curtis Breast database, n = 1456), Src expression was significantly increased ($p = 1.46 \times 10^{-51}$) compared to normal breast tissue (n = 144) (Fig. 3.2.9B)

Fig. 3.2.9 Expression of iNOS and Src is upregulated in invasive breast carcinomas

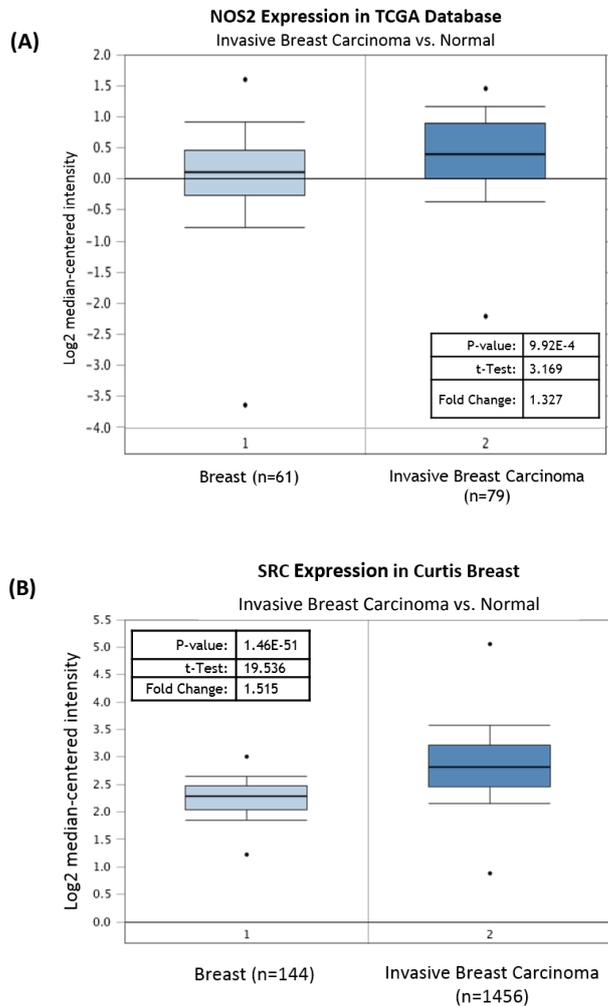


Fig. 3.2.9 (A) Oncomine Cancer Microarray databases were used to analyse expression of NOS2 in the The Cancer Genome Atlas (TCGA) Breast database (n = 137). Expression was compared between normal breast tissue (n = 61) and invasive breast carcinoma (n = 79). **(B)** Oncomine Cancer Microarray databases were used to analyse expression of SRC in the Curtis Breast database (n = 1600). Expression was compared between normal breast tissue (n = 144) and invasive breast carcinoma (n = 1456).

3.2.10 Stress hormones mediate a reduced effect on non-tumorigenic breast epithelial cells

To ascertain if the effects of stress hormones on ROS/RNS production, DNA damage and iNOS were cancer cell specific, the immortalised non-tumorigenic breast epithelial cell line MCF10A was used.

MCF10A's mammary epithelial cells were exposed to stress hormones and their respective receptor antagonists, and ROS/RNS levels were measured using multi-step amperometry. In response to cortisol, NO_2^- levels were significantly increased ($p < 0.001$), although this was almost 4-fold less than previously observed in MCF-7 and MDA-MB-231 cells. The effect was still reversed with addition of the GR inhibitor RU486 ($p < 0.001$) and neither NE alone or in combination with propranolol had any significant effect on NO_2^- levels (Fig. 3.2.10A). Levels of H_2O_2 were also generated in a similar pattern to breast cancer cell lines, with NE increasing levels ($p < 0.01$) and incubation with propranolol blocking NE mediated generation ($p < 0.001$), although levels were also ≈ 4 -fold lower (Fig. 3.2.10B).

Levels of DNA damage in MCF10A cells were significantly increased in response to the positive control, exogenous H_2O_2 treatment ($p < 0.05$), however they were not significantly increased by exposure to cortisol or NE (Fig. 3.2.10C). Exposure to cortisol did increase the expression of iNOS as measured by qPCR however not significantly so compared to the control (Fig. 3.2.10D).

Fig. 3.2.10 Stress hormones have a reduced effect on non-tumourigenic breast epithelial cells

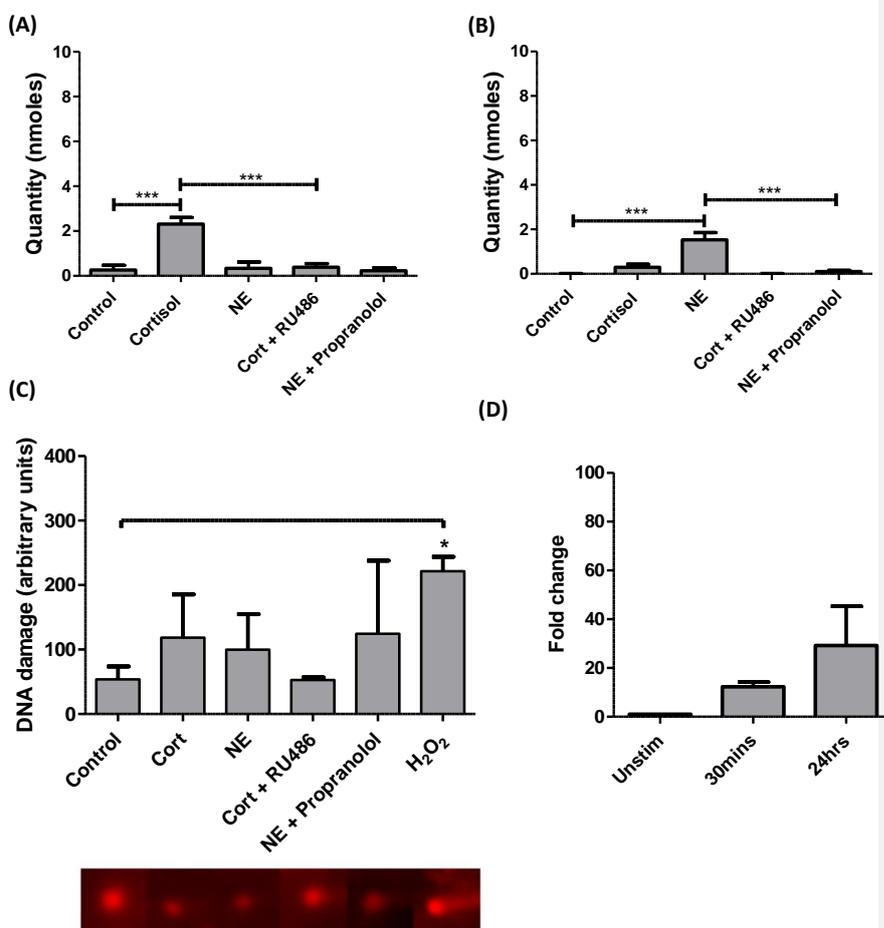


Fig. 3.2.10 MCF10A cells were incubated with Cort and NE in the presence and absence of the receptor antagonists RU486 and propranolol. Cell lysates were collected and electrochemical sensors used to measure levels of (A) nitrite (NO₂) and (B) hydrogen peroxide (H₂O₂). (C) Comet tails indicating DNA strand breaks were visually scored according to intensity (0–4). (D) MCF10A cells were exposed to cortisol for 30mins and 24hrs. mRNA was extracted and levels of iNOS expression were quantified using qPCR. Cycle threshold (Ct) values for NOS2 were normalised against *ACTB* and fold change was calculated using the delta-Ct method. Data are shown as mean ± SEM and statistical significance was determined using one-way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference. * = p<0.05, ** = p<0.01, *** = p<0.001.

Fig 3.2.11 Potential pathway through which stress hormones may stimulate the production of ROS/RNS

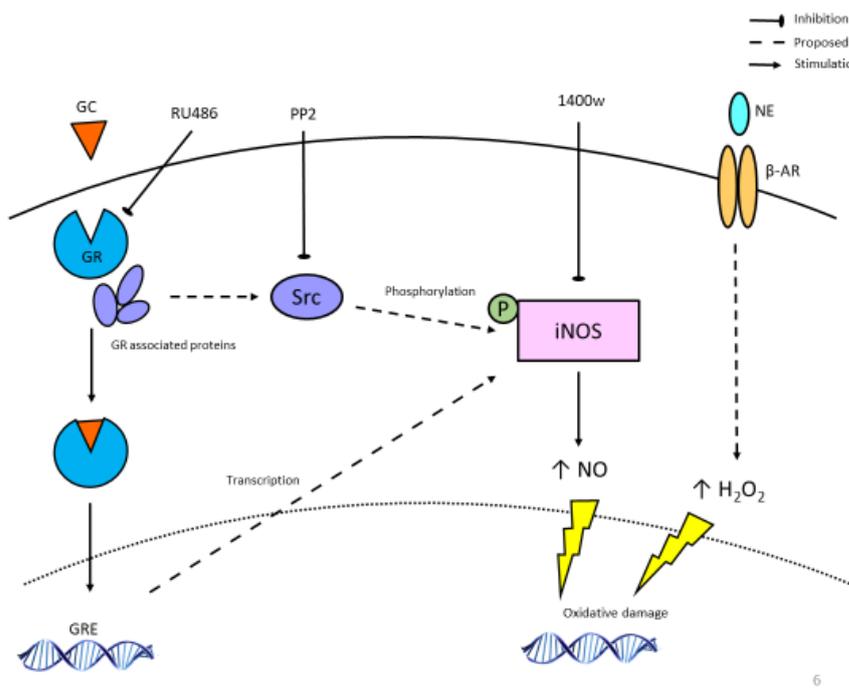


Fig 3.2.11. Glucocorticoid (GC) and norepinephrine (NE) induce DNA damage through the production of RNS/ROS. Activation of the glucocorticoid receptor (GR) may facilitate non-genomic effects on iNOS through a post-translational modification. Binding of glucocorticoids to the GR promotes translocation to the nucleus to act on GR response elements (GRE), facilitating transactivation or transrepression of genes. Binding also induces conformational changes including dissociation of a multiprotein complex comprising HSP's and Src. Src may then mediate phosphorylation of iNOS extending its half-life and promoting the generation of NO.

3.3 Discussion

These results show that acute exposure to stress hormones can induce DNA damage and that the efficacy of the subsequent repair was also negatively affected. Using electrochemical methods levels of ROS/RNS were detected and were shown to increase as a result of either cortisol or NE incubation. The ability of chronic exposure of stress hormones to induce DNA damage in breast cancer has recently been shown by our group (139). This data corroborates the assertion that both catecholamines and glucocorticoids, at physiological levels, can increase DNA damage through receptor mediated signalling. The use of electrochemical analyses to quantify production of ROS/RNS in response to ferrocifens in breast cancer has also been explored previously (265), indicating that TNBC cells produce ROS/RNS in response to stimuli.

3.3.1 ROS/RNS promote DNA damage

The main findings presented here indicate that the effect of cortisol on both the production of RNS and DNA damage is abrogated in the presence of NOS and iNOS inhibitors (L-NAME and 1400W). This not only suggests that glucocorticoids induce ROS mediated DNA damage, but that they also have a previously unobserved effect on the activity of iNOS. This is a particularly relevant finding in regards to breast cancer as the expression of iNOS has been found to increase in line with tumour grade and progression (260), indicating that NO activity may under certain circumstances drive malignant growth (170-172). Bioinformatic analysis of breast cancer patient microarray data undertaken here, confirms that iNOS expression is increased in invasive breast cancers compared to normal tissue. As such, NOS inhibitors are emerging as an area of investigation for potential treatments to combat the tumorigenic effects of NO (266). However, the role of NO in the pathophysiology of cancer is complex, with low levels mediating many homeostatic processes and allowing cell proliferation, while high levels are associated with cytotoxicity and can induce apoptosis (163). It is unclear as to whether or not the rise in NO in response to stress hormones is a protective mechanism – or as a result of increased cellular activity caused by DNA damage at this time. Although it is possible to conclude that the generation of RNS must in part be influenced by the GR, as the presence of a receptor antagonist, RU486 reduces both the DNA damage and production of RNS. Within the cells lines there is some variation in the effect of the glucocorticoid receptor antagonist RU486, with a more prominent reduction seen in MCF-7 cells. This may be attributed to the cell line MCF-7 expressing a higher percentage of total GR's compared to MDA-MB-231 cells (139), as well as the inhibitory effect of RU484 on progesterone receptors, also present on MCF-7, but not MDA-MB-231 cells (25).

Interestingly, DNA damage was not significantly reduced in cells exposed to the selective iNOS inhibitor prior to treatment with NE, indicating that, unlike cortisol, NE does not interact with or

induce iNOS. Moreover the composition of levels of ROS/RNS produced by NE treated samples differs greatly from that of the samples treated with cortisol. In NE treated cells a significant increase is seen in the generation of H₂O₂ compared to other ROS/RNS, and this effect is reversed through inhibition of the β -adrenergic receptor using propranolol. The mechanism through which β -adrenergic receptor induces the generation of ROS is still unclear, however it is thought that this may be as a result of Gs-PKA signalling (135). Incubation with both hormones produces the biggest effect on the production of RNS indicating that the mechanisms of DNA damage and ROS/RNS generation for these two stress hormones are distinct from each other, however in combination they may have an additive effect.

The effect of stress hormones on the generation of ROS/RNS and DNA damage also appears to be much greater in breast carcinoma cell lines as opposed to non-tumorigenic mammary cells. In MCF10A cells, stress hormones produced similar patterns of ROS/RNS but at much lower levels. There was also no significant increase in DNA damage or iNOS expression in response to cortisol, indicating that glucocorticoids promote oxidative stress through NO signalling specifically in transformed breast cancer cells. The difference may also be attributed to a lower expression of the GR in MCF10A cells, or a lower basal expression of iNOS, although comparative levels of either were not explored in this study. In regards to tumour initiation, there is little work to support a role for stress hormones in the accumulation of mutagenic properties. However, the data presented here demonstrates that stress hormones are capable of promoting oxidative/nitrosative stress in non-transformed mammary epithelial cells, which may have implications for tumour initiation. Oxidative stress is known to contribute to the formation of a malignant phenotype in breast cancer (267), although it is unclear if the quantities of ROS/RNS generated in response to stress hormones in this case are of significance.

3.3.2 GR-mediated effects on iNOS activity

The expression of iNOS protein was increased in tumours from mice experiencing chronic psychological stress, compared to tumours from non-stressed mice. Previously, exposure to the oestrogenic signalling hormone oestradiol has also been shown to increase iNOS expression in mammary carcinomas, indicating the activity of NO may be linked to hormonal regulation (268). However, levels of iNOS mRNA were elevated after 24hr exposure to cortisol, but had no corresponding increase in protein levels. This indicates that cortisol does not act to upregulate transcription of iNOS in response to acute exposure. The actions of glucocorticoids have classically been described as genomic, mediated through the glucocorticoid receptor; however glucocorticoids have been shown to induce almost immediate non-genomic actions on other signalling processes as a result of proteins dissociating from the GR complex (64, 66, 269). The complex includes proteins such as HSP90 and Src, a multifunctional protein involved in survival,

proliferation and angiogenesis (65). Src kinases are overexpressed in many cancers including breast cancer and can be used as metastatic markers (270, 271). The activation of Src via phosphorylation as a result of downstream adrenergic signalling has also been identified as a key switch in tumour metastases, with Src implicated in NE mediated VEGF and IL-6 production, ultimately promoting invasion and metastases (272). Furthermore, Src is capable of phosphorylating iNOS in breast cancer cells, prolonging their half-life and promoting NO generation (273).

Immunoprecipitation of the GR-complex protein HSP90 demonstrates dissociation of Src from the complex in response to cortisol. Furthermore inhibition of Src using PP2 attenuated the glucocorticoid induced production of RNS, however it should be noted that PP2 has also been found to inhibit other members of the Src family of protein kinases (274). As such this data may suggest a potential mechanism through which glucocorticoid binding to the GR may indirectly exert a non-genomic effect on iNOS to produce damaging levels of RNS, a previously unexplored action of glucocorticoids (Fig. 3.2.11).

3.3.3 Future perspectives

Further exploration of the mechanisms proposed here is needed to corroborate the hypothesis that GR mediates non genomic effects through the release of Src through complex protein-protein interactions. In order to do so, work is ongoing in our laboratory using immunoprecipitation combined with mass spectrometry approaches to dissect the interactions of Src and GR

This work also raises questions regarding the role of stress induced iNOS in tumour initiation, since the effects of stress hormones were much more prominent in cancer cells versus non-tumourigenic. Previous work has detailed how genetic modification of mice to knockout iNOS expression can result in delayed formation of mammary malignancies, indicating that iNOS is involved in early tumourigenesis (275). As such a potential future directive for this work could be to monitor oxidative stress and iNOS in MCF10A cells undergoing induced transformation. Furthermore iNOS knockout in breast cancer and mammary epithelial cells could be used to study tumour initiation and progression. To explore the role of stress-induced oxidative stress *in vivo*, mice exposed to psychological stress could be treated with a radical scavenger to reduced oxidative stress, and the burden of mammary tumours measured.

Similarly, the effect of stress hormones on DNA repair needs further clarification. Since cortisol is known to downregulate BRCA1 it follows that DNA repair by homologous recombination (HR) may be affected. In order to probe the specific DNA repair processes that may be compromised,

reporter construct assays will be used. BRCA1 mutated or BRCA1 knockout strains could also aid in the identification of the repair pathways affected.

Lastly while the role of NE, ROS production and DNA damage has been partially explored here, and convincing evidence exists linking β -adrenergic signalling to oxidative stress, a specific pathway has yet to be elucidated. The catabolism of NE by monoamine oxidases (MAO's) may be a target for research regarding the NE-mediated generation of H_2O_2 , as this process has been shown induce ROS and oxidative stress (276, 277). Pharmacological inhibition of MAO's would therefore be a logical step in further exploration.

**Chapter 4 - Inhibition of nitric oxide synthase (NOS)
reduces the effect of stress hormone signalling on
metastasis in breast cancer**

4.1 Introduction

4.1.1 Nitric oxide

Nitric oxide (NO) is a heteronuclear diatomic small molecule consisting of one nitrogen and one oxygen. Its bonding structure contains an unpaired electron, thereby classifying it as a free radical. However unlike other free radicals whose unpaired electron renders them highly chemically reactive, NO is less so, and has no significant tendency to dimerize under biological conditions (278). Moreover, due to its structure and lack of charge NO is able to diffuse freely across membranes without the need for a transporter making it an ideal messenger. As such these properties allow NO to interact with all biological molecules and function as an important biological signalling molecule. NO acts directly by forming nitrogen-oxygen radicals, as well as indirectly through post translational protein modifications via S-nitrosylation and tyrosine nitration (279). S-nitrosylation describes the formation of S-nitrosothiols, formed by translocation of an NO⁺ group to a sulphur atom of a thiol. The bioactivation of proteins by S-nitrosylation allows them to subsequently act as NO donors, as well as changing the protein activity, interactions or sub cellular location (163). Such interactions have important implications in cancer cell biology. For example, the S-nitrosylation of the transcription factor NF- κ B, prevents its DNA binding activity and subsequently promotes cell death in cancer cells (280). S-nitrosylation of the Fas death receptor also induces apoptosis in cancer cells (281). Conversely, S-nitrosylation can prevent apoptosis via the s-nitrosylation of caspase 3 and 9 (282). NO also regulates several physiological processes through the soluble-guanylyl-cyclase-cGMP pathway. NO released from generator cells signals soluble guanylate cyclase (sGC) in target cells to catalyse the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn stimulates cGMP-dependant proteins (PKG's) initiating signalling cascades that results in smooth muscle cell relaxation amongst many others (283).

NO is capable of modulating responses in a range of organs, and the physiological function of NO is more often than not determined by its concentration. Low and high concentrations can mediate pleiotropic effects, with low concentrations of NO produced by the NOS isoforms endothelial NOS (eNOS) and neuronal NOS (nNOS) acting in a signalling capacity to regulate vasodilation and neurotransmission. Whereas high concentrations of NO such as those produced by the inducible form of NOS (iNOS) are involved in cytotoxic defence mechanisms, and are thought to affect immunomodulation, with chronic production of NO associated with several inflammatory pathologies (284). In the context of cancer, this dichotomy poses several interesting questions regarding how best to manipulate NO in order achieve a favourable outcome. On the one hand NO can suppress growth via activation of anti-tumour immune responses, however NO can also drive growth through pro-tumourigenic signalling (177).

Fig 4.1 NOS isoforms and their role in cancer

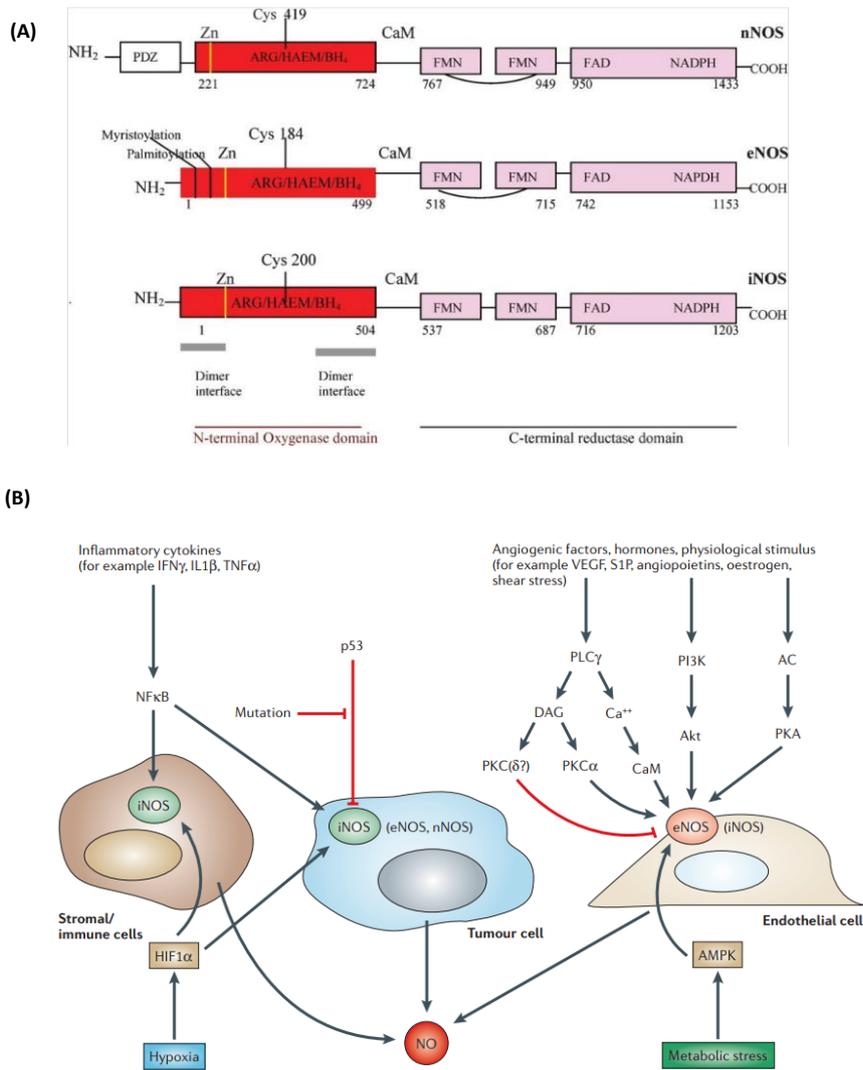


Fig. 4.1 (A) NOS isoforms neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Reproduced from (285). **(B)** The role of NOS isoforms varies according to tissue type and distribution. Fig reproduced from (261).

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4.1.2 iNOS/NO and metastasis

Transition from a normal cell to a mutagenic cell occurs usually as a result of genetic alterations, involving the silencing of tumour suppressor genes or the promotion of oncogenes. Tumourigenic transformation is likely induced in some cases by prolonged inflammation and exposure to NO. Relatively high concentrations of NO are produced by iNOS, compared to other isoforms, and it is thought that exposure to this nitrosative stress can drive aspects of transformation (168). However, the role of iNOS in cancer is generally accepted to be somewhat ambiguous, with large bodies of work demonstrating its ability to suppress tumour growth through NO-mediated cell killing (284, 286, 287).

Despite the complicated and complex nature of the role of NO in cancer, a compelling relationship does seem to exist between iNOS and cancer progression. Expression of iNOS has been correlated with poor outcome in multiple cancers including breast (288), melanoma (289), prostate (290) and head and neck sarcoma (291) and has been further implicated in early tumour formation (275) as well as the metastatic potential of some tumours (172). It is therefore proposed that NO can act to drive malignant growth and metastasis through the induction of angiogenesis, migration and invasion both in tumour cells and the surrounding host cells (261). An example of one such way NO can drive invasion is through the NO-mediated activation of protein kinase G (PKG), which interacts with Ras-Raf-ERK pathway inducing increased cell proliferation and migration through MMP13 (292). PKG's can also activate PI3K/Akt signalling which stimulates migration and NO-induced angiogenesis (293).

Increased angiogenic signalling in tumours is also thought to be stimulated by NO in tumour cells through NO-mediated upregulation of vascular endothelial growth factor (VEGF). However, VEGF can also act to upregulate iNOS and eNOS expression (294). These mechanisms may exist to signal angiogenesis in response to hypoxia, since both proteins are upregulated in response to hypoxic conditions such as those experienced in the tumour microenvironment. This is supported by the finding that in cancer cells that constitutively expressed iNOS and had a p53 mutation, accelerated tumour growth was associated with increased VEGF expression (295). Stromal cells may also over express iNOS, and NO produced by stromal cells can induce VEGF expression (261). However, the transfection of iNOS-deficient tumour cells into wild type (+/+) and iNOS-deficient mice (-/-), showed that expression of host iNOS can suppress tumour growth, as wild type mice that expressed stromal iNOS did not develop tumours as quickly as iNOS-deficient mice. (296). These conflicting findings may be as a result of different sensitivity and responsiveness to NO in the host tissue type or tumour cells as well as p53 status.

Several studies using animal models genetically engineered to suppress iNOS expression have indicated that iNOS can promote tumour initiation and metastasis in cancer. One such study

employed a genetically enhanced mouse model of spontaneous mammary tumour development (MMTV-PyV-mT). These transgenic mice were crossed with iNOS^{-/-} mice and the development of hyperplasia was delayed, indicating a role for iNOS in tumourigenesis (275). In another study, iNOS^{-/-} mice had a substantial decrease lung primary tumour formation, and significantly less expression of VEGF implying a reduction in angiogenesis (297). The regulation of VEGF by iNOS was further examined in a melanoma mouse model induced in iNOS^{-/-} mice. Expression of VEGF was significantly decreased in iNOS^{-/-} mice, and while all wild type mice formed tumours, the rate of successful engraftment (i.e. the successful formation of a tumour after injection with tumour cells) was much less in iNOS^{-/-} mice. This suggests a role for host derived iNOS in tumour growth, whereby NO generated from iNOS in normal tissue is needed for the promotion of tumourigenic cell growth (298). However more recently iNOS gene therapy using nanoparticle delivery increased iNOS expression in a breast cancer xenograft model, and prolonged survival of metastases-bearing mice through direct cytotoxicity (299).

4.1.3 NOS inhibitors in breast cancer

Selective or non-selective inhibition of NOS has been used as a tool to better understand the role of NO in cancer, as well as a potential therapy to alleviate tumourigenic NO signalling (300).

All three NOS isoforms catalyse the conversion of L-arginine to L-citrulline and NO, using oxygen and NADPH. Electrons are donated by NADPH and proceed via FAD and FMN redox carriers to the cytochrome p450-like hemoprotein at the active site to catalyse the reduction of oxygen with L-arginine. Calcium dependent NOS isoforms (eNOS and nNOS) require a bound calcium to facilitate electron flow (301). Reflecting their complex biochemistry, NOS inhibitors come in many forms, including L-arginine analogues, haem binding imidazole, calmodium antagonists and flavoprotein inhibitors. However, by far the most specific and widely used inhibitors are the L-arginine derivatives, which compete with the substrate at the binding site. NG-methyl-L-arginine (L-NMMA) was identified in 1987 as a competitive reversible inhibitor, and since then several simple derivatives have followed (302). NG-nitro-L-arginine methyl ester (L-NAME) is a potent non-specific NOS inhibitor, which remains inactive until its hydrolysis to active NG-nitro-L-arginine (L-NOARG) (303).

Several studies have sought to use inhibition of NOS *in vivo*. In mammary tumours the pan-NOS inhibitor L-NAME was able to decrease tumour angiogenesis (304), as well as to reduce the growth and metastasis to the lung of LPS/IFN- stimulated tumour cells when implanted (305). Selective inhibition of iNOS using 1400W dihydrochloride, which also competes with L-arginine, reduced primary mammary tumour size in a syngeneic mouse model as well as in a human colon cancer xenograft, confirming that iNOS specifically mediated a pro-tumour effect (306). In a TNBC model, iNOS inhibition alongside a conventional chemotherapy regime improved the

survival prospects and significantly reduced primary tumour growth compared to chemotherapy alone (172). Selective targeting of eNOS was also able to delay tumour progression in mice by reducing tumour vasculature hyperpermeability which is necessary for angiogenesis (307).

However inhibition of NOS has also been shown to block host NO-mediated cytotoxicity in metastasising melanoma and hepatoma (261). The efficacy of NOS inhibitors is therefore dependant on localization, expression and activity of NOS isoforms as well as the concentration and length of exposure to NO.

4.1.4 Stress and NO signalling

Stress hormones have been linked to oxidative stress and NO signalling as shown previously, with glucocorticoids and catecholamine's each mediating distinct pathways involving ROS and RNS (135). These pathways have been the focus of research seeking to disseminate the role of stress in cancer progression, and the relationship between stress and oxidative/nitrosative in tumorigenesis. One way in which stress hormones may interact with NO and affect tumourigenic signalling is through the upregulation of VEGF in tumour cells by NE (308). The effects of glucocorticoids on NO are less well studied, however cortisol has also been shown to have a dose dependent effect on VEGF in ovarian carcinoma cell line, with higher concentrations of cortisol stimulating increased VEGF expression (309). This induction of VEGF by stress hormones could promote the release of NO through VEGF-mediated upregulation of eNOS and iNOS in host stromal tissue (294). Furthermore, previously published data has shown that glucocorticoids mediate a non-genomic effect on iNOS and increase NO signalling in breast cancer cells (310).

4.1.5 Aims

This work aims to identify the effects of psychological stress on breast cancer progression, and understand the mechanism through which this is mediated. The effects of cortisol will be examined in a tumourigenic mouse cell line as a proof of principle, which will then be used in a syngeneic *in vivo* model of breast cancer. Psychological stress will be induced, and non-selective inhibition of NOS will be used in an attempt to abrogate stress hormone-NO mediated metastatic signalling.

4.2 Results

4.2.1 Cortisol activates the GR in mouse mammary tumour cells

The primary glucocorticoid secreted from the adrenal glands in mice is corticosterone which, although similar to cortisol, has some structural differences. However, cortisol is still able to bind to the GR in mice, but with less affinity than corticosterone (311).

In order to assess the capability of cortisol to bind to the GR and induce translocation to the nucleus in mouse mammary tumour cells, 66CL4 cells were treated with cortisol in the presence or absence of the GR inhibitor RU486. The GR was then immunofluorescently labelled and visualised under a high magnification. Cortisol treatment stimulates the translocation of the GR from the cytoplasm to the nucleus, and this is inhibited by RU486 (Fig. 4.2.1A).

Fig. 4.2.1 Cortisol activates the GR in mouse mammary tumour cells

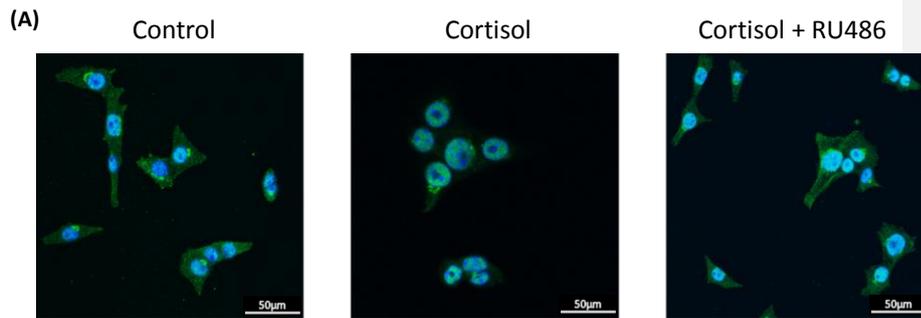


Fig. 4.2.1 (A) 66CL4 cells were exposed to cortisol (1 μ M) for 30 mins in the presence of absence of the GR-antagonist RU486 (1 μ M). Cells were immunofluorescently labelled for the glucocorticoid receptor (GR) (green) and counterstained with DAPI (blue). Representative images are shown.

4.2.2 Cortisol increases levels of intra- and extracellular nitrite

Previous work has demonstrated the ability of glucocorticoids to induce the production of RNS, specifically NO_2^- - the stable by product of NO, in human breast cancer cell lines. Multi-step amperometry using electrochemical sensors can be used to detect specific reactive species in the lysates of cells, providing information on the generation of ROS/RNS in response to stimuli.

The mouse mammary tumour cell line 66CL4 was treated with cortisol in the presence and absence of RU486, as well as the specific and nonspecific NOS inhibitors 1400W dihydrochloride and L-NNA. A significant increase in NO_2^- was observed in response to acute (30 min) cortisol treatment ($p < 0.001$) which was blocked by pre-incubation with RU486 ($p < 0.001$). Cortisol induced production of NO_2^- was also reversed with GR inhibition using RU486 ($P < 0.001$), pan-NOS inhibition by L-NNA ($p < 0.001$), as well as with specific inhibition of iNOS by 1400W ($p < 0.001$) prior to cortisol treatment (Fig. 4.2.2A).

Extracellular NO_2^- secreted into the cell culture media was measured using the colourimetric Griess assay, which is considered a less sensitive measure (312). Cortisol induced a significant increase in NO_2^- ($p < 0.05$), which was abrogated by inhibition of the GR using RU486 ($p < 0.05$). Inhibition of NOS prior to cortisol produced a decrease in NO_2^- levels however this was not statistically significant compared to cortisol alone, although it was also not significantly higher compared to the control (Fig 4.2.2B).

For the purposes of comparison, the human mammary carcinoma cell line MCF-7 was also treated with the same concentration of cortisol in the presence or absence of RU486 and L-NAME, and NO_2^- measured by the Griess assay. Cortisol did produce an increase in extracellular NO_2^- levels and whilst this was higher than the average levels produced by 66CL4 cells it was not significantly different from the control (Fig. 4.2.2C).

Fig 4.2.2 Cortisol increases levels of intra- and extracellular nitrite

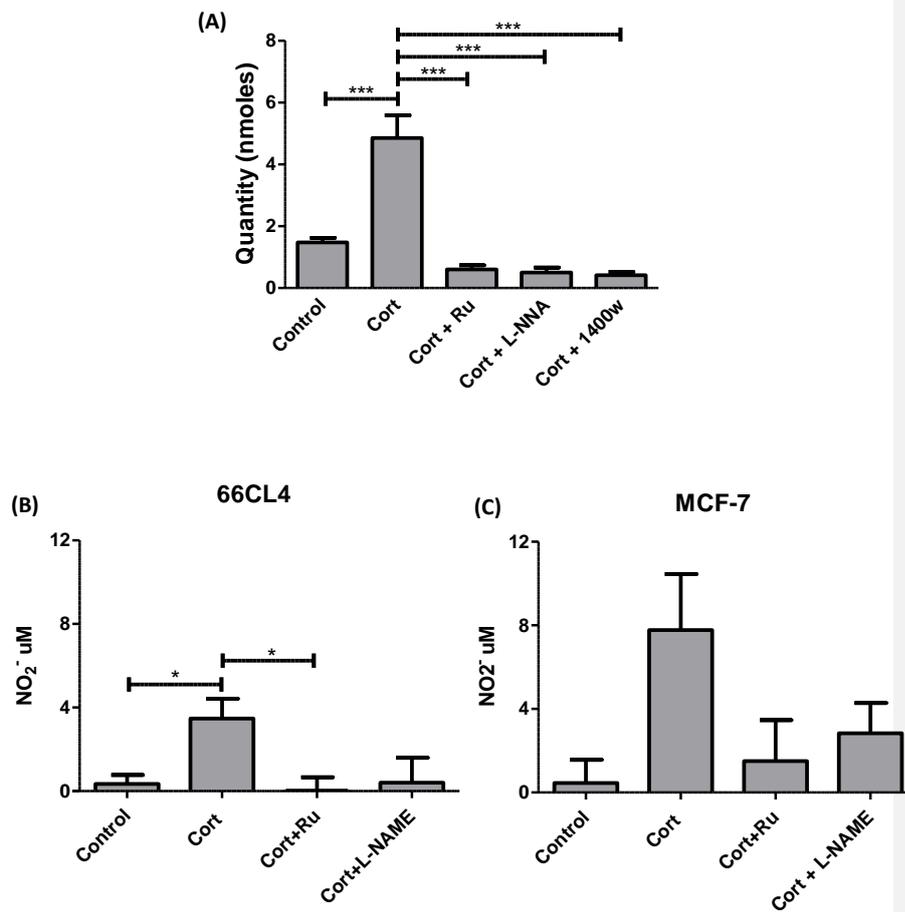


Fig. 4.2.2 (A) 66CL4 cells were incubated with Cort for 30 mins in the presence and absence of RU486, L-NNA and 1400w. Intracellular levels of nitrite (NO₂⁻) were measured using electrochemical sensors. (B-C) 66CL4 and MCF-7 cells were incubated with cortisol +/- RU486 and L-NAME. Extracellular NO₂⁻ levels were quantified using the Griess assay. Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant increase where * = p<0.05, ** = p<0.01, *** = p<0.001.

4.2.3 Cortisol induces phospho- γ -H2AX foci formation in 66CL4 cells through a NOS-mediated mechanism

Previously, glucocorticoids have been shown to induce DNA damage in human breast cancer cell lines which could be reduced with pharmacological inhibition of NOS. To assess cortisol induced damage in a mouse cell line, the marker of DNA damage, phosphorylated γ -H2AX foci, were visualised immunofluorescently in 66CL4 cells.

In response to acute exposure to cortisol (30 mins) the percentage of foci positive cells was significantly increased compared to control levels ($p < 0.001$). This effect was inhibited by incubation with RU486 beforehand ($p < 0.01$). Incubation with L-NAME, the pan-NOS inhibitor, prior to cortisol also significantly reduced the percentage of foci positive cells compared cortisol alone ($p < 0.01$) (Fig. 4.2.3A). Representative DAPI and phospho- γ -H2AX staining is shown (Fig 4.2.3B)

Fig. 4.2.3 Cortisol induces phospho- γ -H2AX foci formation in 66CL4 cells through a NOS-mediated mechanism

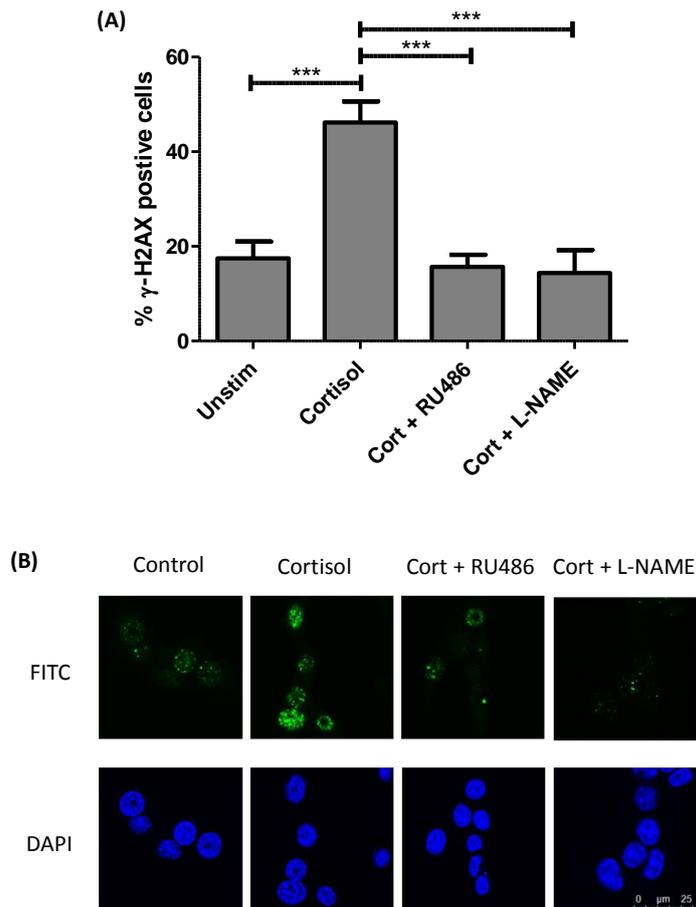


Fig. 4.2.3 (A) 66CL4 cells were incubated with cortisol (Cort) for 30 mins in the presence and absence of RU486 and L-NAME. Cells were immunofluorescently stained for phosphorylated γ -H2AX (green) counterstained with DAPI (blue). Cells with >5 foci were scored as positive. Representative images shown. Mean \pm SEM expressed and statistical significance was determined using one-way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.4 Cortisol induces RAD51 foci formation in 66CL4 cells through a NOS-mediated mechanism

The formation of RAD51 foci can be used indirectly assess the extent of DNA damage in a cell. Damage to DNA recruits damage sensors that can promote RAD51 binding to the site of damage to facilitate repair through homologous recombination (HR). Formation of foci can therefore be used as a reliable marker of DNA damage and repair, and cells can be scored according to the number of foci.

In 66CL4 cells exposed to cortisol for 30 mins, a significant increase in the percentage of RAD51 foci positive cells was observed ($p < 0.01$). Pre-incubation with RU486 and L-NAME significantly reduced the effect of cortisol of RAD51 formation in a similar manner to the phospho- γ -H2AX foci formation ($p < 0.01$) (Fig.4.2.4A).

Fig. 4.2.4 Cortisol induces RAD51 foci formation in 66CL4 cells through a NOS-mediated mechanism

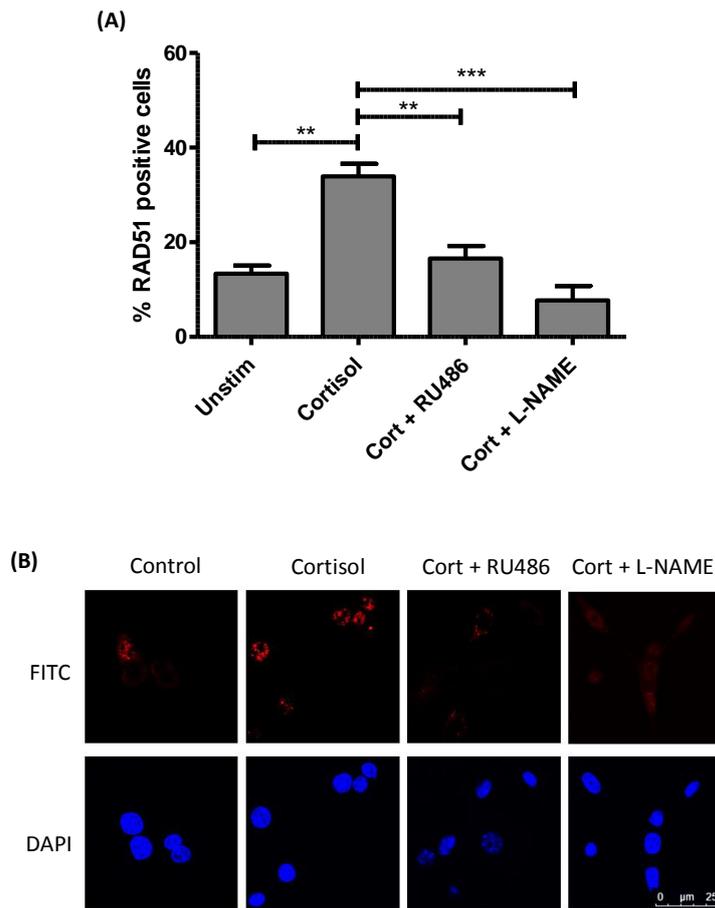


Fig. 4.2.4 (A) 66CL4 cells were incubated with cortisol (cort) for 30 mins in the presence and absence of RU486 and L-NAME. Cells were immunofluorescently stained for RAD51 (red) and counterstained with DAPI (blue). Cells with >5 foci were scored as positive. Representative images shown. Mean \pm SEM expressed and statistical significance was determined using one-way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.5 Cortisol increases the expression of iNOS and metastatic markers in mouse mammary tumour cells

The expression of VEGF is known to correlate to an increase in metastatic potential in breast cancer through an increase in angiogenic signalling (313). The transcription factor TWIST also plays a role in cancer metastasis through regulation of epithelial-mesenchymal transition (EMT) and suppression of cell adhesion molecules, thereby increasing the capacity for migration and invasion (314). Furthermore, iNOS has also been shown to be over expressed in more aggressive metastatic breast cancers, with a large body of research detailing the effects of iNOS in relation to metastasis (284). The expression of iNOS has also been shown to be upregulated by cortisol in human breast cancer cells as detailed previously in this work.

To study the potential of cortisol to induce metastatic signalling in breast cancer, the expression of iNOS, TWIST and VEGF-A was examined in 66CL4 cells using qPCR. Relative levels of *NOS2* mRNA were seen to increase after 24hrs cortisol exposure by over 2-fold ($p < 0.05$) (Fig. 4.2.5A), as were levels of *TWIST1* ($p < 0.001$) (Fig. 4.2.5B). A significant increase in *VEGFA* mRNA was also observed in response to cortisol, although it was less than 2-fold ($p < 0.05$) (Fig. 4.2.5C).

Fig. 4.2.5 Cortisol increases the expression of iNOS and metastatic markers in mouse mammary tumour cells

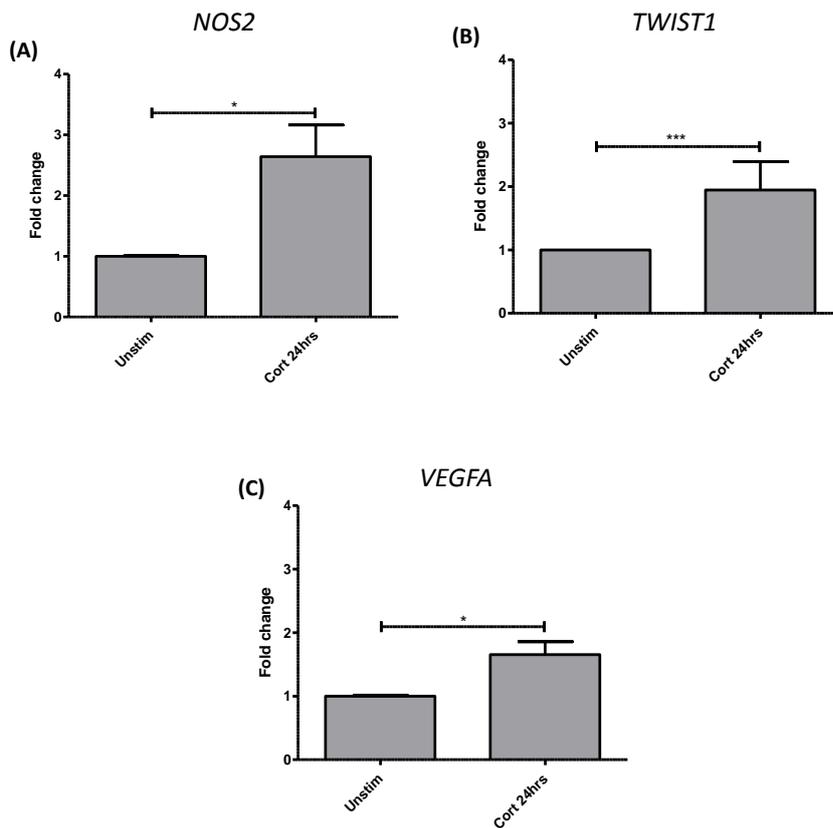


Fig. 4.2.5 66CL4 cells were incubated with cortisol (Cort) for 24hrs and RNA extracted. cDNA was synthesised and amplified in the presence of gene specific primers for **(A) NOS2**, **(B) TWIST1**, **(C) VEGFA** and *ACTB* using q PCR. Ct values were normalised against β -actin and fold change calculated using the delta-Ct method. Mean \pm SEM expressed and statistical significance was determined using students t-test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.6 Inhibition of NOS reduces primary tumour growth and affects potential metastatic spread in stressed mice

The NOS inhibitor L-NAME has been used widely in the exploration of NO activity as a target for cancer both *in vitro* and *in vivo* (177, 300). L-NAME is water soluble and therefore easily administered, it is the readily hydrolysed to the active compound L-NNA in biological systems which is a strong inhibitor of all NOS isoforms. For these reasons L-NAME has also been used in animal studies and clinical trials as a treatment for septic shock (315, 316), as well as asthma and to regulate cardiac physiology (317).

In order to build upon the hypothesis that's glucocorticoids increase NO signalling in tumour cells, and *in vivo* mouse model of aggressive mammary carcinoma was used alongside a model for chronic psychological stress. Cells from the 66CL4 line, a derivative of 4T1 cells with a highly metastatic potential, were cultured and injected into the 4th mammary fat pad of female BALB/c mice. Once tumours reach a sufficient volume (200mm³) mice were divided into four groups and treated as follows; daily IP injection of the vehicle saline, daily vehicle IP prior to 2hrs restraint stress, daily L-NAME IP injection or daily L-NAME IP injection plus 2hrs restraint stress (Fig. 2.5).

Primary tumour volumes were measured using callipers twice a week. There was no significant difference in primary tumour volume observed between the control and stress groups over the course of 14 days. There was also no significant difference between the control and L-NAME treated group over 14 days. However, a significant difference was observed between stress and L-NAME + stress groups, with the addition of L-NAME prior to restraint stress reducing tumour volume compared to stress alone ($p < 0.001$) (Fig. 4.2.6A). After sacrifice the weight of the excised primary tumours was also measured. There was no difference in the average weight of the primary tumour of the control group compared to the stress group, however the primary tumours in the L-NAME and L-NAME + stress groups were on average lower, although not significantly (Fig. 4.2.6B).

Metastases were observed during gross necropsy and identified by morphology and size, and represented as number of mice with metastasis to a specific organ (Fig. 4.2.6C). The number of organs with metastases per mouse is also represented (Fig. 4.2.6D). Mice from the restraint stress group appeared to have more metastatic sites per mouse as well as on a wider range of organs compared to the control group. Mice treated with L-NAME alone or with restraint stress had less metastatic sites on fewer organs.

Fig. 4.2.6 Inhibition of NOS reduces primary tumour growth and affects potential metastatic spread in stressed mice

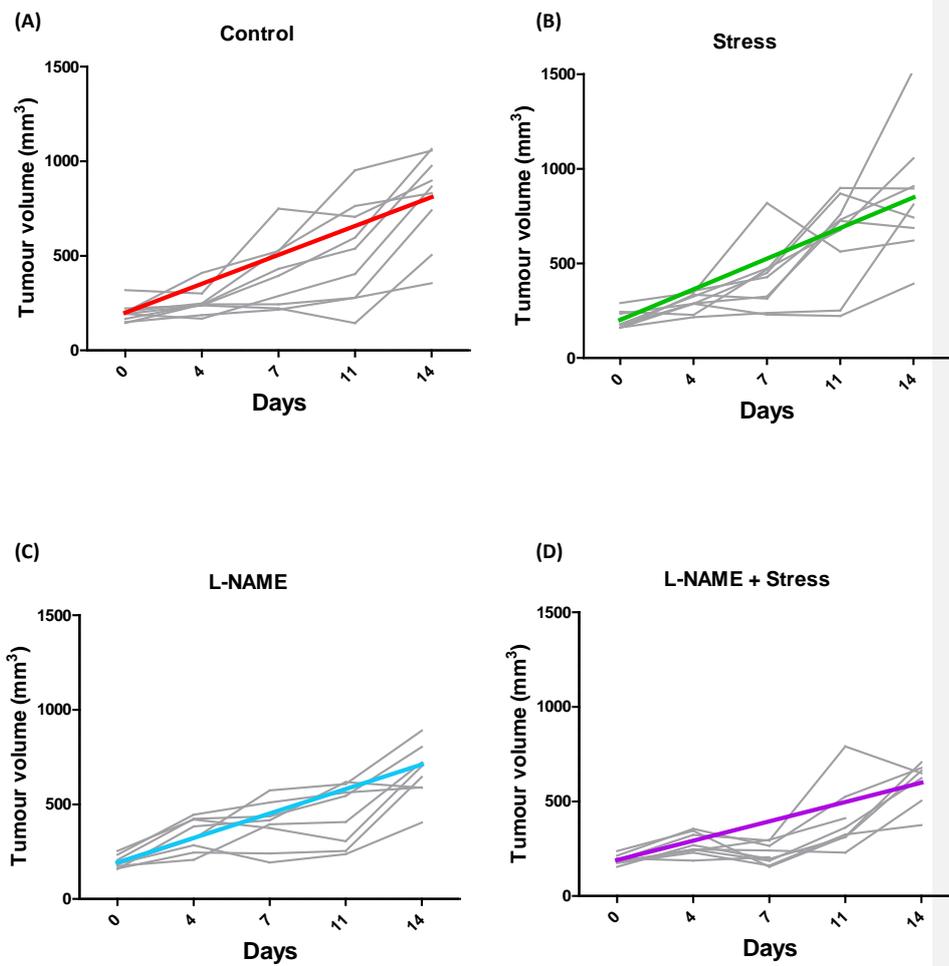


Fig 4.2.6 (A-D) 66CL4 mouse mammary tumour cells were transplanted into the fourth mammary fat pad of female BALB/C mice. Groups were exposed to restraint stress (2hrs) or no stress, in combination with L-NAME treatment (80mg/kg). Data expressed as individual growth rates (grey) and mean growth rate (coloured).

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Fig. 4.2.6 Inhibition of NOS reduces primary tumour growth and affects potential metastatic spread in stressed mice

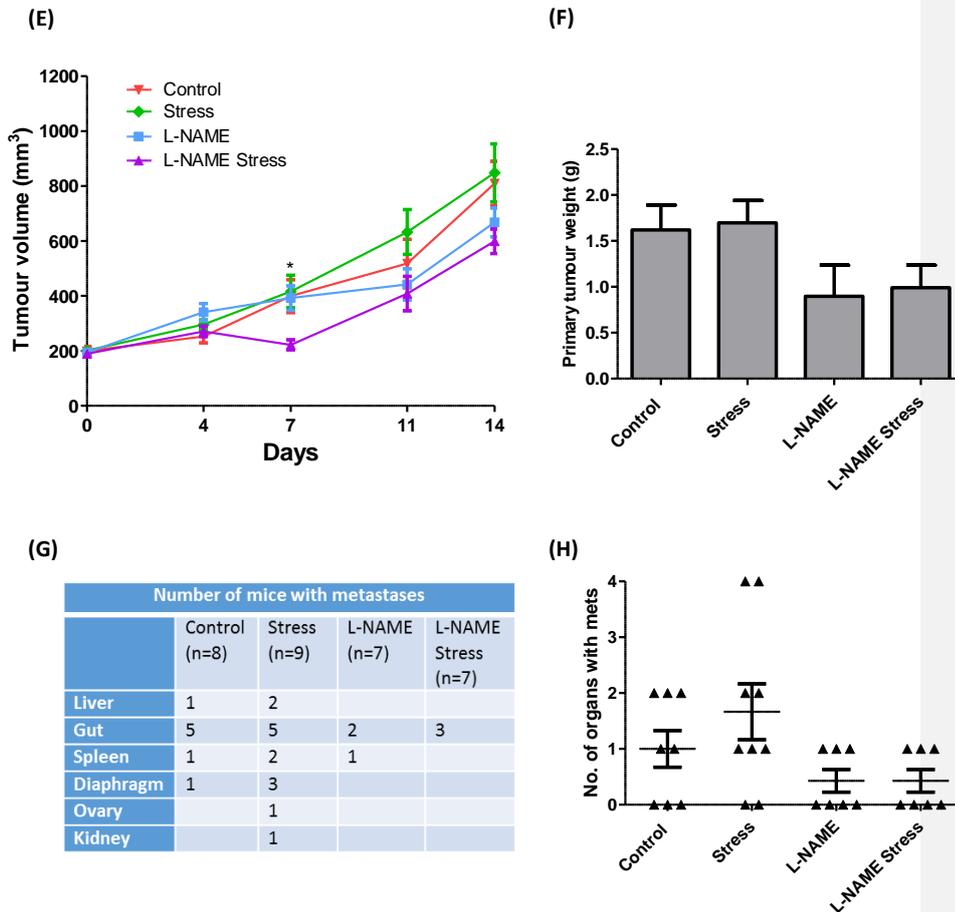


Fig 4.2.6 (A-D) 66CL4 mouse mammary tumour cells were transplanted into the fourth mammary fat pad of female BALB/C mice. Groups were exposed to restraint stress (2hrs) or no stress, in combination with L-NAME treatment (80mg/kg). **(E)** Mean \pm SEM expressed and statistical significance was determined using two-way ANOVA (Bonferroni post-tests). * indicates a significant difference between stress and stress + L-NAME groups. **(F)** Primary tumour weight of excised 66CL4 allografts. **(G)** Metastases were observed by gross necropsy, data presented indicates number of mice with metastases in specific organs. **(H)** Data points represent individual mice plotted according to how many organs had metastases. Statistical significance of frequency data was determined using Chi-Squared. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

4.2.7 Inhibition of NOS had no effect on survival proportions of mammary tumour-bearing mice

The weight of the mice was measured once a week to assess the condition of the mice. There was a significant decrease after 14 days in the stress group compared to the control ($p < 0.01$). There was also a significant decrease in the weight of mice treated with L-NAME alone ($p < 0.001$), and L-NAME in combination with restraint stress after 14 days ($p < 0.001$) (Fig. 4.2.7A).

The survival of mice in this study was not measured as an endpoint, however some mice were sacrificed for welfare reasons before the designated endpoint of the study. Restraint stress used as a model for chronic psychological stress had no effect on the survival proportions of mammary tumour-bearing mice compared to a control group. Similarly, treatment with the NOS inhibitor L-NAME to mammary tumour-bearing mice undergoing restraint stress had no effect on the survival proportions (Fig. 4.2.7B).

Fig. 4.2.7 Inhibition of NOS had no effect on survival proportions of mammary tumour-bearing mice

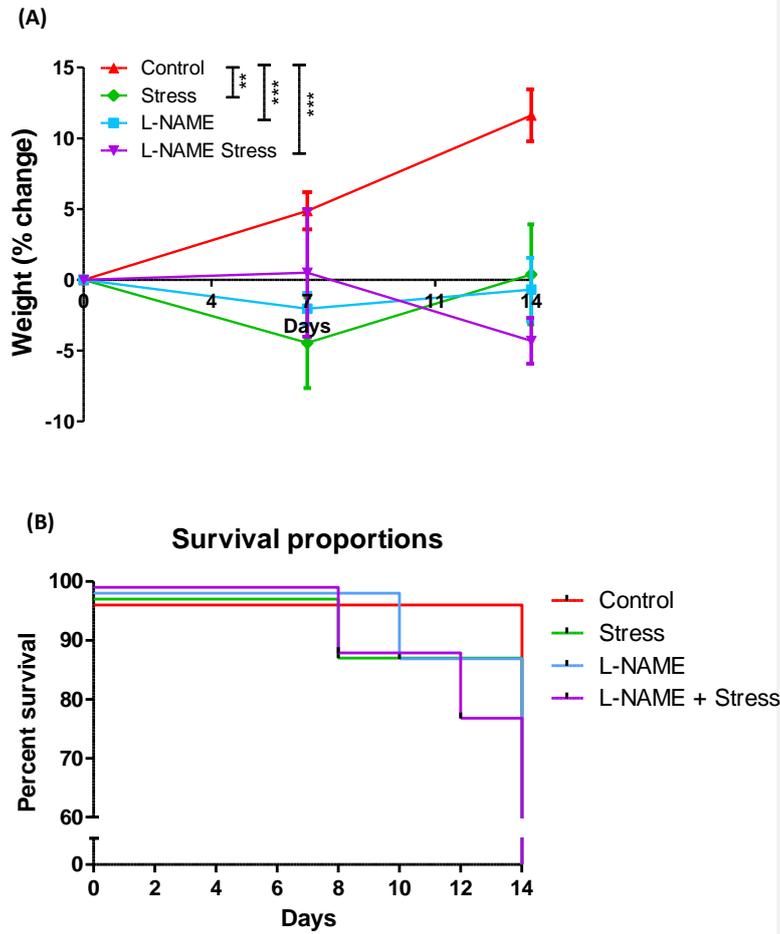


Fig 4.2.7 (A) 66CL4 mouse mammary tumour cells were transplanted into the fourth mammary fat pad of female BALB/C mice. Groups were exposed to restraint stress (2hrs) or no stress, in combination with L-NAME treatment (80mg/kg). Mean % change in weight of the mice \pm SEM is expressed **(B)** Survival proportions are expressed and statistical significance was determined using logrank test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

4.2.8 Inhibition of NOS reduces angiogenesis in induced by stress in mammary tumour bearing-mice

The inhibition of eNOS by L-NAME has the potential to reduce angiogenic signalling through downregulation of NO. CD31 is an adhesion molecule that can facilitate adhesion between endothelial cells and can therefore be used to quantify angiogenesis (318). To evaluate the degree of angiogenesis in the primary tumours CD31 (PECAM1) was immunofluorescently stained and the density of microvessels quantified using high magnification microscopy.

There was no significant difference in microvessel density as determined by CD31+ expression in the primary tumours of the control group compared to the L-NAME treated group. However, there was a significant increase in microvessel density in the primary tumours from the restraint stress group compared to the control ($p < 0.05$), and a significant decrease in the stress + L-NAME group compared to the stress group ($p < 0.05$) (Fig. 4.2.8A). Representative images of CD31 staining are shown (Fig. 4.2.8B)

Fig. 4.2.8 Inhibition of NOS reduces angiogenesis in induced by stress in mammary tumour bearing-mice

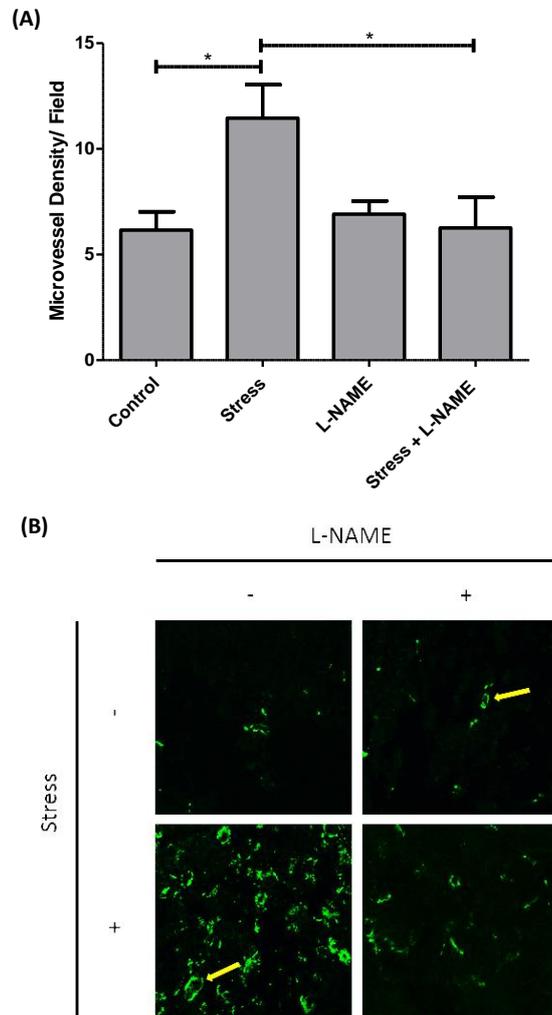


Fig 4.2.8 (A) 66CL4 mouse mammary tumour cells were transplanted into the fourth mammary fat pad of female BALB/C mice. Groups were exposed to restraint stress (2hrs) or no stress, in combination with L-NAME treatment (80mg/kg). Tumours were immunofluorescently stained for CD31. Microvessel density was quantified by counting microvessels per field/image. Mean \pm SEM expressed and statistical significance was determined using one-way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **(B)** Representative panels shown.

4.2.9 High TWIST expression correlates with poor outcome in ER-negative breast cancer subtypes

Analysis of microarray data from large breast cancer patient datasets (EGA and TCGA) can be used to ascertain the impact of expression of a gene on survival proportions. To examine how the expression of TWIST affected Recurrence Free Survival (RFS), breast cancers were first grouped according to their ER status (ER- vs ER+) or molecular subtypes; basal (ER-/HER2-), luminal A (ER+/HER2-/KI67 low), luminal B (ER+/HER2-/KI67 high and ER+/HER+) and HER2+ (ER-/HER2+) before being stratified according to their expression status (high vs low).

In ER-negative breast cancers (n=801) higher expression of *TWIST1* is significantly correlated with a decrease in the probability of RFS ($p=0.0025$) (Fig. 4.2.9A). In ER-positive breast cancers (n=2016) there is no difference in survival probability between higher and low expression ($p=0.17$) (Fig. 4.2.9B). When stratified into molecular subtype high expression of *TWIST1* correlated with poor RFS in basal subtypes (n=618) ($p=0.0087$) and HER2+ subtypes (n=251) ($p=0.028$) (Fig. 4.2.9C-D). There was no significant correlation in survival proportions in luminal A (n=1933) ($p=0.87$) or Luminal B (n=1149) ($p=0.87$) (Fig. 4.2.9E-F).

Fig. 4.2.9 High TWIST expression correlates with poor outcome in ER-negative breast cancer subtypes

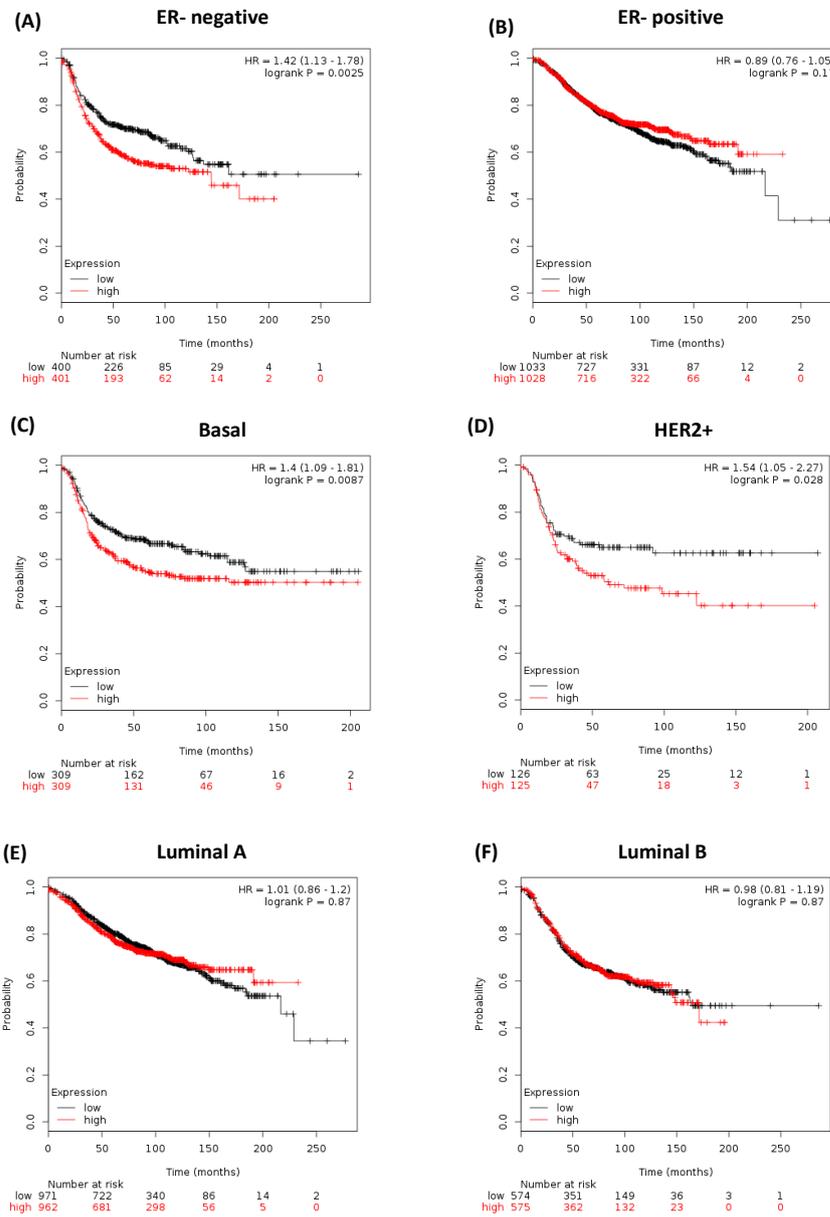


Fig. 4.2.9 The correlation between RFS and *TWIST1* expression in **(A)** ER-negative, **(B)** ER-positive breast cancers, **(C)** basal type, **(D)** HER2+, **(E)** luminal A and **(F)** luminal B was analysed using microarray data form the EGA and TCGA databases.

4.3 Discussion

In this study, glucocorticoids increased the production of ROS/RNS and DNA damage through a NOS-mediated mechanism in mouse mammary tumour cells, as well as upregulated iNOS and markers of metastasis *in vitro*. Furthermore *in vivo* inhibition of NOS was able to negate some of the detrimental effects of psychological stress on tumour growth and metastasis.

4.3.1 Glucocorticoids and oxidative stress

A strong correlation has been shown to exist between oxidative stress, DNA damage and tumourigenesis, however there has been little conclusive evidence to suggest glucocorticoids exert a direct effect on this process. A weak positive association was observed between perceived stress (as assessed by questionnaire) and oxidative stress in a healthy Japanese population (319), whilst a cross-species meta-analysis showed that manipulation of glucocorticoid signalling through administration of endogenous glucocorticoids had a significant impact on redox status, inducing oxidative stress (320). However, the analysis also unsurprisingly revealed that the effect of glucocorticoids varies according to sex, tissue type, duration of treatment and type of glucocorticoid.

Previous work presented in this thesis has shown acute exposure to cortisol has been shown previously to stimulate the production of RNS in human breast cancer cell lines. In this study, it has been shown that cortisol can activate the GR in mouse mammary tumour cells, and through GR activation increase levels of NO_2^- in a similar manner. Pharmacological inhibition of NOS was able to reverse cortisol-mediated NO_2^- production, and furthermore selective inhibition of iNOS proves that cortisol-induced generation of NO_2^- is facilitated through iNOS specifically. In the same cell line, DNA damage and repair, as evidenced by the formation of phosphorylated γ -H2AX foci and RAD51 foci, was also significantly increased in response to cortisol. Inhibition of NOS was able to negate the effect of cortisol on DNA damage indicating that the generation of NO is partly responsible for inducing DNA damage. Taken together these results demonstrate the involvement of cortisol-regulated NO in DNA damage, and strengthens the hypothesis that one of the mechanisms through which exposure to glucocorticoids may influence tumourigenesis is through the upregulation of oxidative stress.

The induction of oxidative stress facilitates transformation through modulation of multiple aspects of cancer cell behaviour. Reactive species are capable of interaction with pathways involved in cell cycle progression, proliferation and survival and can positively regulate tumour cell growth, as well as damaging DNA and producing mutations that sustain progression. In breast cancer markers of oxidative damage are as much as 10 times higher in invasive carcinomas compared to healthy controls, indicating that oxidative stress is a persistent feature

(321). Genomic instability promoted by oxidative stress therefore remains a significant target in breast cancer research.

4.11.2 Psychological stress, NO and metastatic spread

The effect of psychological stress on the growth and metastasis of tumours has proved challenging to study due to the complexity of stress hormone signalling and hormonal fluctuations. Often endogenous administration of glucocorticoids can increase levels above biologically relevant levels and therefore do not reflect realistic conditions (320). Furthermore, models of psychological stress vary, with restraint stress, forced swimming and heat stress all inducing elevated corticosterone levels but also suffering from differing limitations. With regards to the model chosen here, restraint stress it is a well-established model of chronic stress known to induce a hormonal response and maintain levels of glucocorticoids elevated above baseline, however habituation to restraint stress can occur over longer periods of time (61).

In the syngeneic mouse model of mammary tumour used in this study, there was no difference in primary tumour volumes of mice exposed to daily restraint stress compared to the control group. However, this is a typical observation for studies involving psychological stress, carried out over a short time period, and in such an aggressive cancer model (228, 322, 323). The effects of chronic restraint stress on primary tumour volume are instead much more pronounced when combined with chemotherapy, with stress reducing the efficacy of chemotherapies in breast cancer (139), as well as in lung carcinoma (324). Chronic stress alone has however been shown to affect the lymph vasculature surrounding the primary tumour, with restraint stress significantly increasing the lymphatic network and metastasis to the lymph node in a TNBC mouse model (228). Similarly, in this study restraint stress significantly increased the microvasculature of the primary tumour compared to the control, indicating that whilst the tumours grew at the same rate, the primary tumours in stressed mice were more aggressive and had an increased propensity for metastasis. Indeed, a higher number of metastatic sites per mouse was observed in the stress group, as well as a greater potential metastatic spread compared to the control. Stressed mice also weighed significantly less than control mice after 14 days. Weight loss is a common symptom of psychological stress (263), and as such this difference in weight indicates that the restraint stress regime is having an effect on the physiology of the mice.

Inhibition of NOS using L-NAME did not alter the primary tumour volume significantly from the control. This finding is not surprising as in previous studies using NOS inhibitors reductions were seen after longer time courses as well as in combination with conventional chemotherapies (172, 306). There was also no difference in microvasculature between control and L-NAME groups, however there was a reduction in potential metastatic spread in the L-NAME treated

group. The inhibition of NOS in this instance may not have been carried out over a long enough time period, and since it was not administered continuously using osmotic pumps, the single daily dosage may have been too low to have an effect on the growth of the primary tumour.

However, L-NAME treatment was able to exert a significant effect on primary tumour growth when administered prior to restraint stress. There was a significant reduction in primary tumour volume in the L-NAME + stress group compared to the stress alone group after 7 days, as well as a reduction in potential metastatic spread. There was also a significant decrease in microvasculature indicating an inhibition of angiogenic NO signalling. As such the data gathered from this *in vivo* trial suggests that inhibition of NOS may be able to reduce the pro-tumourigenic effect of psychological stress in breast cancer, through reduction of NO-mediated angiogenesis. It also supports the data gathered *in vitro* that proposes that glucocorticoid signalling upregulates NO generation through iNOS, and that an increase in oxidative stress is responsible for the DNA damage that may drive mutational changes linked to tumour aggressiveness.

Furthermore, the *in vitro* data also demonstrates that cortisol can upregulate the expression of VEGF and Twist, two pro-metastatic markers heavily involved in the transformation into an aggressive phenotype. The deregulation of growth factor signalling is a hallmark of tumourigenesis, and is usually observed in invasive tumours (239). The production and signalling of the potent angiogenic factor VEGF is often upregulated in the hypoxic tumour microenvironment and plays a role in the increased NO signalling within tumours. VEGF binding mobilizes intracellular calcium which induces eNOS and the production of NO, increasing the angiogenic potential by creating a feedback mechanism whereby VEGF induces NO, and NO in turn upregulates VEGF (174). Therefore the increased NO signalling stimulated by glucocorticoids may serve to promote angiogenesis through VEGF in a chronic stress model.

The upregulation of Twist by glucocorticoids may be explained by interaction of the GR with Src, whereby activation of the GR releases Src, and Src can act to regulate Twist expression in breast cancer, promoting invasiveness and metastasis (325). Overexpression of Twist is known to correlate with poor survival and can drive metastasis in breast cancer through the dysregulation of multiple pathways (326). In analysis of microarray datasets, high Twist expression denotes poor outcome in ER-negative breast cancers, specifically the ER-negative subtypes basal and HER2+. The GR is also upregulated in ER-negative breast cancers (240), potentially linking tumours that are more susceptible to glucocorticoid signalling, to a shift towards an aggressive metastatic phenotype through increased Twist signalling. The cells used in this model have been confirmed by immunocytochemistry to be ER-negative (Appendix 5 Fig.1.1). The upregulation of Twist by cortisol may therefore exert potentially detrimental effects on this breast cancer subtype, which is responsive to glucocorticoid signalling.

It is also worth considering that in a model of psychological stress, glucocorticoids are not the only hormone secreted. The stress response also invokes catecholamine mediated signalling. Norepinephrine can upregulate VEGF in cancer cells promoting angiogenesis through induction of eNOS (112). Furthermore although the primary focus of the stress induced iNOS/NO response has been glucocorticoid signalling, it has been shown that hydrogen peroxide can act to augment cytokine induction of iNOS expression in mesothelial cells (327). Since exposure to NE produces a rise in the levels of hydrogen peroxide in both human and mouse breast cancer cell lines, this raises the possibility that NE signalling, as well as glucocorticoid, influences iNOS activity and therefore tumour progression.

Stress also has well-characterised effects on the immune system (105), an aspect which has not been explored in this study. Much of the research has focused the effects of stress on the adaptive immune system, such as T and B lymphocytes, and the innate immune system such as NK cells. However, perhaps more pertinent to this research is the role of macrophages. Treatment of macrophages in culture with the stress hormone NE, and subsequent exposure of mouse mammary tumour cells (4T1) to the supernatant from treated macrophages, increased the invasive potential of the tumour cells. Furthermore, induction of chronic stress in a syngeneic mouse model of breast cancer increased the number of tumour-associated macrophages (TAM's) in the primary tumour (328). TAM's are now understood to play an important role in breast cancer progression, and since iNOS expression is upregulated in TAM's (329), tumour cell-macrophage interactions are thought to be in part regulated by the release of NO. In the early stages of tumour development, macrophages produce high concentrations of NO in an effort to initiate tumour cell apoptosis, however should tumour cells escape, macrophages are reprogrammed by the tumour microenvironment to produce low, pro-angiogenic levels of NO/RNS (330). Upregulation of iNOS in TAM's can also suppress T-cell function and proliferation (331). In a mouse model of breast cancer this was observed specifically under hypoxic conditions, indicating that immunosuppression mediated by the expression of iNOS in TAM's is an important process in the progression and eventual spread of cancer (332). The addition of L-NAME in this study may have inhibited NOS in stromal tissue, including iNOS expressed in macrophages. This inhibition could conceivably prevent some of the immunosuppressive pro-tumour effects of TAM's, and contribute to the reduction in tumourigenesis observed in stressed mice treated with L-NAME.

4.11.3 Future perspectives

In this study, NOS inhibition was able to decrease the effect of stress on tumour volume, potential metastatic spread and angiogenesis in an aggressive syngeneic breast cancer model. However the data poses the further question; is the reduction in tumour growth and metastasis

by L-NAME due to inhibition of eNOS in endothelial cells resulting in decreased angiogenesis and metastasis, the inhibition of iNOS in tumour cells resulting in decreased NO signalling/ oxidative damage, or a combination of both.

To address these questions and further work will be carried out to establish the role of NOS isoforms in relation to stress. Specific inhibition of iNOS seems a logical step to take, with previous evidence building regarding its benefits in the context of cancer therapy. However use of 1400W *in vivo* poses some problems, with a much shorter half-life it must be administered through an osmotic pump, a procedure outside the scope of the research presented here. L-NAME was chosen as the inhibitor of choice due to its extensive use in the field, however clinically L-NAME can cause acute elevation of blood pressure and as such co-treatment with an anti-hypertensive should be also be considered in future, as well as routine measurement of blood pressure. Excision of the primary tumours from stress and non-stressed mice and culture *ex-vivo* would also allow further investigations into the extent of NO signalling within the tumour cells. To establish if oxidative stress driven by stress plays a role in tumour progression, an excreted marker of oxidative stress such as 8-OH-dG would also be measured, as would markers of DNA damage such as γ -H2AX at a protein level in the primary tumour.

It is also important to establish if a shift caused by stress in the phenotype of the primary tumour correlates with the phenotype of the metastases. The change in cancer cell populations can adversely affect treatment in recurrent breast cancer, as a therapy that was effective for the primary tumour may no longer work for the metastatic population, and metastatic disease is still the main cause of cancer-related mortality. Recent studies have suggested that tumour cells may disseminate earlier than previously thought and develop in parallel with the primary tumour, while also developing a genetic diversity making them phenotypically distinct (333, 334). Since stress can adversely affect metastatic spread, it is proposed that it may also affect the phenotype of the metastatic populations, further enhancing the damaging effect of stress on the responsiveness to treatment of breast cancer.

**Chapter 5 – The effects of stress hormone signalling on
the efficacy of Faslodex in breast cancer**

5.1 Introduction

5.1.1 Endocrine therapy

Endocrine or hormone therapies are used in the treatment of hormone receptor positive breast cancers whose growth are driven by endogenous hormone signalling, specifically oestrogenic signalling. Oestrogen (E2) acts a potent growth factor in breast tissue by binding to the oestrogen receptor (ER) and facilitating the transcription of oestrogen regulated genes involved in a wide range of signalling pathways (335). This diversity is thought to be mediated in part by differential recruitment of coregulators, coactivators and corepressors to the E2-ER complex which act to direct the transcription of certain target genes (336). In this way, oestrogenic signalling can have wide reaching implications in the context of deregulated growth signalling in breast cancer, affecting many facets of cancer cell biology, including differentiation, proliferation, apoptosis and invasion. To target this signalling, ER-positive breast cancers are treated with endocrine therapies which include two approaches: antagonising the ligand binding at the receptor level using anti-oestrogens, or inhibiting the synthesis of E2. Several classes of hormone therapies have so far been developed to target ER signalling, including selective oestrogen receptor modulators (SERM's) and selective oestrogen receptor downregulators (SERD's), as well as aromatase inhibitors (AI's) which work to block E2 production (337). However, despite the success of endocrine therapies some breast cancers display *de novo* resistance, showing no response to first line endocrine therapies, or go on to acquire resistance after initially responding well. Resistance to endocrine therapies poses a major problem in the treatment of breast cancers, and especially in the recurrent setting where up to half of patients develop resistance

5.1.2 Faslodex and endocrine therapy resistance

Faslodex (Fulvestrant) is currently the only approved SERD available for use in the treatment of breast cancer (see 1.1.3). Faslodex is a pure anti-oestrogenic compound and unlike tamoxifen, which can act as an agonist, Faslodex is an exclusive ER antagonist. The mechanism through which Faslodex works to inhibit ER signalling is twofold; firstly binding to the ER renders it inactive by blocking dimerization and translocation to the nucleus, and secondly targeting the ER for ubiquitination and accelerating degradation by the proteasome resulting in a downregulation of ER expression.

Faslodex is most commonly used in treatment of ER-positive metastatic breast cancers in post-menopausal women, following acquired resistance to anti-oestrogen therapies such as tamoxifen and aromatase inhibitors, and is administered by a monthly intramuscular injection

(338). Resistance to fulvestrant is therefore a significant clinical problem as it is often confers a second line of defence in treatment regimes. The mechanisms through which Fulvestrant resistance is acquired are thought to include mutations in the ER gene *ERS1*, the loss of ER α signalling pathways, the consequential upregulation of compensatory growth-stimulatory pathways and the global changes in gene expression (339, 340).

Although loss of expression of the ER seems a likely explanation for endocrine therapy resistance, this only occurs in a relatively small percentage of tumours - approximately 10% (340). As such, mutations in *ERS1* are thought to account for a proportion of metastatic and pre-treated ER-positive breast cancers going on to develop secondary resistance. Overexpression and amplification of growth factor receptors are also associated with endocrine resistance. The shift from E2-dependant growth to growth in a steroid-deprived environment is thought to be as a result of the upregulation of 'escape pathways' such as growth factors including HER2 and EGFR signalling (341, 342). The ability of growth factor receptors to stimulate growth in the absence of ER-mediated signalling largely relies on crosstalk pathways such as phosphorylation of the ER by EGFR and HER2 (343), and modulation of the ER by kinases such as protein in the MAPK family (344). Phenotypic changes stimulated by the development of Fulvestrant resistance can also manifest in the form of increase invasiveness and aggressiveness (345), and a decreased response to other therapies such as radiotherapy, through disruption of DNA repair pathways (346). Endocrine resistance can also be brought about as a result of cell cycle dysregulation through the over expression or continuous activation of proteins responsible for progression through the cell cycle (347).

5.1.3 Stress and resistance to breast cancer therapies

Initial observations by our collaborator J. Gee et al (personal communication), demonstrated that loss of the ER in acquired Fulvestrant resistant cells correlated with an upregulation of the glucocorticoid receptor (GR), in a similar manner to the compensatory upregulation of EGFR. An inverse relationship has been shown to exist previously between the ER and GR in breast cancer, with decreasing expression of the ER mirrored by increased GR expression (348). Furthermore, in a clinical setting, high expression of the GR has been associated with a shorter relapse-free survival time in ER-negative patients, as well as association between activation of the GR and epithelial-to-mesenchymal transition (EMT) pathways (240). These findings prompted questions regarding the effect of glucocorticoids on endocrine resistant cells with loss of ER expression.

Glucocorticoids have also been shown to interfere with the efficacy of some chemotherapies in breast cancer, promoting resistance to chemotherapeutic agents such as doxorubicin and

paclitaxel (139, 140). Glucocorticoids can exert their effects endogenously, through cortisol produced as part of the stress response, or exogenously in synthetic forms such as dexamethasone which is often administered alongside chemotherapy and endocrine therapies to reduce hypersensitivity reactions. Glucocorticoids have numerous reported effects on the cell cycle, DNA damage and the transactivation/repression of target genes (269). Specifically, glucocorticoids are capable of promoting DNA damage, and facilitating circumnavigation of cell cycle checkpoints which function to halt the cell and allow for repair (125). This interference with the cell cycle by stress hormones negated the effect of paclitaxel in a mouse model of TNBC exposed to restraint stress (139), and prevented paclitaxel-induced apoptosis of tumour cells (138). For this reason, the role of glucocorticoids in the treatment of breast cancer needs further investigation in relation their effects on DNA damage and repair.

5.1.4 DNA damage and repair pathways

Damage to DNA can come in the form of single strand breaks (SSB's), double strand breaks (DSB's) replication errors and chemical adducts. The range of types of damage is mirrored by the range of highly complex and interlinked mechanisms employed to repair DNA in the most efficient and precise manner (349). DNA damaging agents including chemotherapeutic agents, UV, ROS/RNS, IR and mechanical stress and can cause DSB's, where the two complementary stands are broken simultaneously. These are particularly dangerous as the ends can become distorted and inappropriately recombined in other parts of the genome. This inappropriate recombination can lead to genetic instabilities, deregulated gene expression and carcinogenesis (350). DSB's are repaired in one of two ways; by homologous recombination (HR) or non-homologous end joining (NHEJ).

HR uses a sister chromatid or homologous chromosome as a template for DNA resynthesis and re-joining. The ends of the break are resected by several complexes including the MRN complex (MRE11–RAD50–NBS1) and the single strand overhangs are coated in replication protein A (RPA). RPA is then replaced by RAD51 nucleoprotein filaments which are assembled with the help of BRCA1 and BRCA2. The RAD51-single strand DNA nucleofilament then invades a homologous sequence to form a displacement D-loop where synthesis can then be initiated to replace the break site (351).

NHEJ re-joins DNA strands directly by recognising single strand overhangs called microhomologies at the ends of DSB's. The heterodimeric protein Ku70/Ku80 initiates NHEJ by binding to the ends of the DSB and recruiting other factors involved in DNA repair, including DNA-dependant Protein Kinase (DNA-PK). In turn the MRN complex is recruited to the site and signals ATM to hold the ends of the DSB together while ligation of the DSB is carried out by

various ligation complexes. If the ends of the strands cannot be joined perfectly then they must undergo processing by nucleases and/or polymerases before joining (352). The recruitment of ATM also results in the phosphorylation the histone H2AX which generates binding sites for adaptor proteins including CHK2 and p53. Activated CHK2 phosphorylates phosphatase CDC25A, which is degraded thereupon, and can no longer dephosphorylate CDK2-Cyclin, resulting in cell-cycle arrest (Fig. 1.6). If the DSB cannot be repaired during this rapid response, ATM additionally phosphorylates MDM2 and p53. p53 is also phosphorylated by the effector kinase CHK2 (134). These phosphorylation events lead to the stabilization and activation of p53, and subsequent transcription of numerous p53 target genes including the CDK inhibitor p21, which leads to long-term cell-cycle arrest or apoptosis (353).

Deregulation of DNA damage response and repair pathways signals an important change in malignant cells, as the ability to maintain genomic integrity is compromised in favour of proliferation. In breast cancer, DNA repair capacity and tumourigenesis are closely linked, with mutations in *BRCA1*, a gene involved in DNA repair, increasing the risk of breast cancer markedly (354). ER signalling is also involved in the DNA damage response, modulating a range of targets within DNA repair pathways to dampen their response and allow the accumulation of genomic changes which may contribute to tumour initiation (355). Decreased DNA repair capacity has been correlated with breast cancer risk in women (356), and yet conversely some studies have linked increased ER expression with increased capacity for DNA repair (357). The conflicting data regarding the role of ER in DNA damage repair therefore poses the question; do ER-negative acquired endocrine resistant breast cancer cells have altered DNA damage response pathways? This hypothesis is supported by the finding that Faslodex resistant breast cancer cells were able to withstand radiation damage and repair damaged DNA resulting in reduced sensitivity to radiation (346).

5.1.5 Aims

This study aims to:

- Examine the effects of glucocorticoids on oxidative/nitrosative stress and DNA damage in Faslodex resistant cells that have increased expression of the GR.
- Explore the deregulation of DNA damage/repair pathways in Faslodex resistant cells in response to cortisol, which is known to induce DNA damage.

5.2 Results

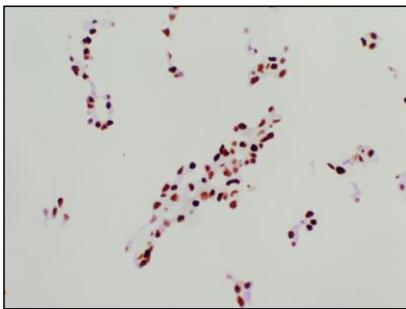
5.2.1 Acquired Faslodex resistance cells (FAS-R) lose expression of the oestrogen receptor

The oestrogen receptor (ER) status of breast cancer cells is closely associated with aggression, invasiveness and overall prognosis, and its role in breast cancer treatment has long been studied. Signalling mediated by the ER directs a number of processes in the cell including proliferation, and therefore by reducing the binding capability of the ER or interfering with its activity the growth of ER-positive breast cancers can be slowed or halted (358). However resistance to Faslodex is associated with degradation and downregulation of the ER and simultaneous upregulation of other growth factor signalling pathways such as EGFR, HER2 and Src (359).

The ER status of FAS-R cells, derived from an ER-positive parental cell line (MCF-7), was determined using immuno-staining for the ER. The ER is present in MCF-7 cells however in Faslodex resistant cells the ER is no longer present (Fig. 5.2.1A-B).

Fig. 5.2.1 Acquired Faslodex resistance cells (FAS-R) lose expression of the oestrogen receptor

(A)



(B)

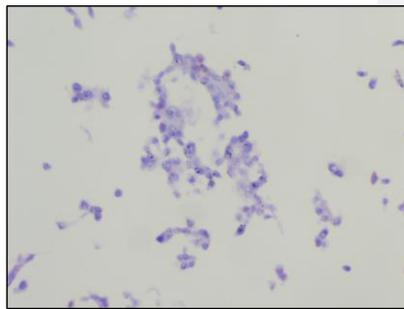


Fig. 5.2.1 MCF-7 **(A)** and FAS-R **(B)** cells were fixed and immunocytochemically stained for the oestrogen receptor (ER) (brown) and the nuclei counterstained with haematoxylin (purple). Representative images shown - magnification x20. *Courtesy of J Gee, Cardiff University.*

5.2.2 ROS/RNS Faslodex resistant (FAS-R) cells and parental MCF-7 cells produce ROS/RNS in response to cortisol.

The stress hormone cortisol has been shown previously to induce the production of damaging ROS/RNS, and therefore increase oxidative stress in TNBC and ER-positive breast cancer cells (310). To examine the effects of cortisol on ROS/RNS production, transformed Fasoldex resistant MCF-7's (FAS-R), which have an altered phenotype compared to parental MCF-7's, were incubated with cortisol alongside the parental cell line in the absence or presence of the glucocorticoid receptor antagonist RU486 for 30mins. Lysates were then collected and ROS/RNS quantified using electrochemical sensors and multistep amperometry.

The effects of cortisol on NO_2^- and H_2O_2 in MCF-7 cells are shown in Fig. 5.2.2A-B and on FAS-R cells in Fig. 5.2.2C-D. A significant increase in levels of NO_2^- was observed in MCF-7 cells in response to cortisol compared to the control ($p < 0.001$). Pre-incubation with RU486 was able to reduce this effect ($p < 0.001$) (Fig. 5.2.2A). A less prominent, although still statistically significant increase was also seen in H_2O_2 levels in response to cortisol in MCF-7 cells ($p < 0.001$), and this was also blocked by GR inhibition ($p < 0.001$) (Fig. 5.2.2B). In FAS-R cells a similar pattern was observed, with cortisol inducing a significant increase in levels of NO_2^- ($p < 0.001$) (Fig. 5.2.2C) and H_2O_2 ($p < 0.001$) (Fig. 5.2.2D), which was abrogated with RU486 ($p < 0.001$).

Fig 5.2.2 Faslodex resistant (FAS-R) cells and parental MCF-7 cells produce ROS/RNS in response to cortisol.

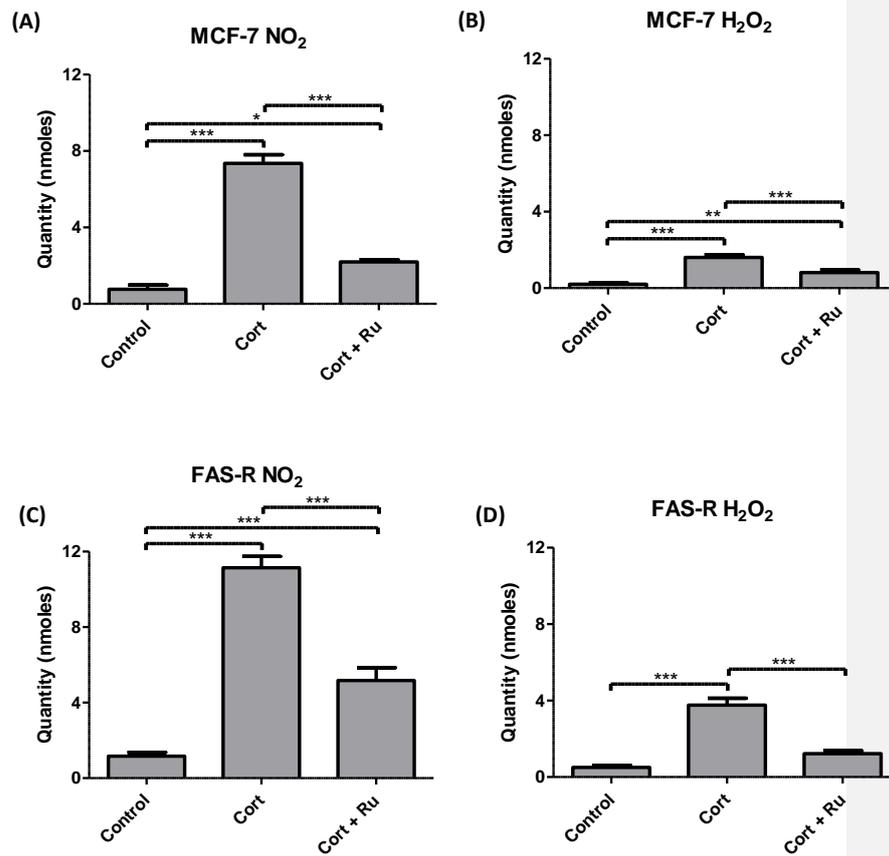


Fig. 5.2.2 MCF-7 (A-B) and FAS-R (C-D) cells were exposed to cortisol (Cort) in the presence and absence of RU486 (Ru) for 30mins. Cells were lysed and levels of NO₂⁻(NO₂⁻) and hydrogen peroxide (H₂O₂) were measured using electrochemical sensors. Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant increase where * = p<0.05, ** = p<0.01, *** = p<0.001.

5.2.3 Faslodex resistant cells (FAS-R) produce higher levels of ROS/RNS in response to cortisol compared to parental MCF-7 cells

The levels of intracellular ROS/RNS in MCF-7 and FAS-R cells in response to acute cortisol treatment with and without RU486 pre-incubation were measured using multistep amperometry as presented above. The data generated was subsequently compared using grouped analysis.

There was a significant increase in the production of NO_2^- in FAS-R cells compared to the MCF-7 parental cells when treated with cortisol ($p < 0.001$). Furthermore levels of NO_2^- in cells treated with RU486 and cortisol remain significantly higher in FAS-R cells ($p < 0.001$) (Fig. 5.2.3A). Levels of H_2O_2 generated after exposure to cortisol are also significantly higher in FAS-R cells compared to MCF-7 ($p < 0.001$) (Fig. 5.2.3B).

Fig. 5.2.3 Faslodex resistant cells (FAS-R) produce higher levels of ROS/RNS in response to cortisol compared to parental MCF-7 cells

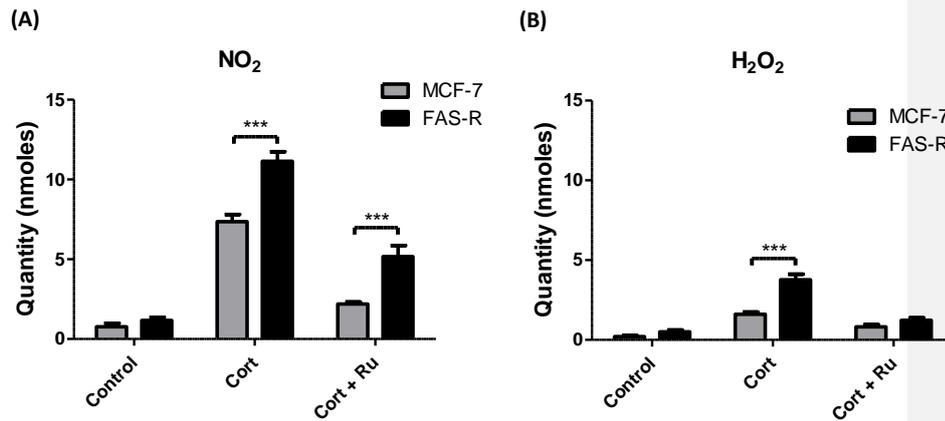


Fig. 5.2.3 MCF-7 and FAS-R cells were exposed to cortisol (Cort) in the presence and absence of RU486 (Ru) for 30mins. Cells were lysed and levels of NO₂-(NO₂) (A) and hydrogen peroxide (H₂O₂) (B) were measured using electrochemical sensors. Mean ± SEM expressed and statistical significance was determine using two way ANOVA (Bonferroni's post-test). * indicates a significant difference where * = p<0.05, ** = p<0.01, ***= p<0.001.

5.2.4 Cortisol induces increased levels of DNA damage in FAS-R cells compared to MCF-7 cells

The histone H2AX is phosphorylated upon activation of ATM which detects double strand breaks in DNA and signals repair processes. Phosphorylated γ -H2AX can therefore be considered a reliable marker of the detection of DNA damage caused by DSB's (191). MCF-7 and FAS-R cells were treated with cortisol in the presence or absence of the GR antagonist RU486 for 2hrs to induce DNA damage. Cells were subsequently fixed and immunofluorescently stained for phosphorylated γ -H2AX foci.

After incubation with cortisol there was a significant increase in the percentage of phospho- γ -H2AX positive cells, indicating an increase in DNA damage in MCF-7 cells ($p < 0.001$). This was blocked by pre-incubation for 30mins with RU486 ($p < 0.01$) (Fig. 5.2.4A). In FAS-R cells there was also a significant increase in DNA damage ($p < 0.001$) which was reversed with RU486 treatment ($p < 0.001$) (Fig. 5.2.4B). When compared to each other there was no significant difference between MCF-7 and FAS-R cells in baseline constitutive DNA damage levels, however there was a significantly higher level of DNA damage in FAS-R cells compared to MCF-7 cells in response to cortisol ($p < 0.05$). There was no difference observed between cell lines in response to cortisol with RU486 pre-incubation. (Fig. 5.2.4C).

Fig. 5.2.4 Cortisol induces increased levels of DNA damage in FAS-R cells

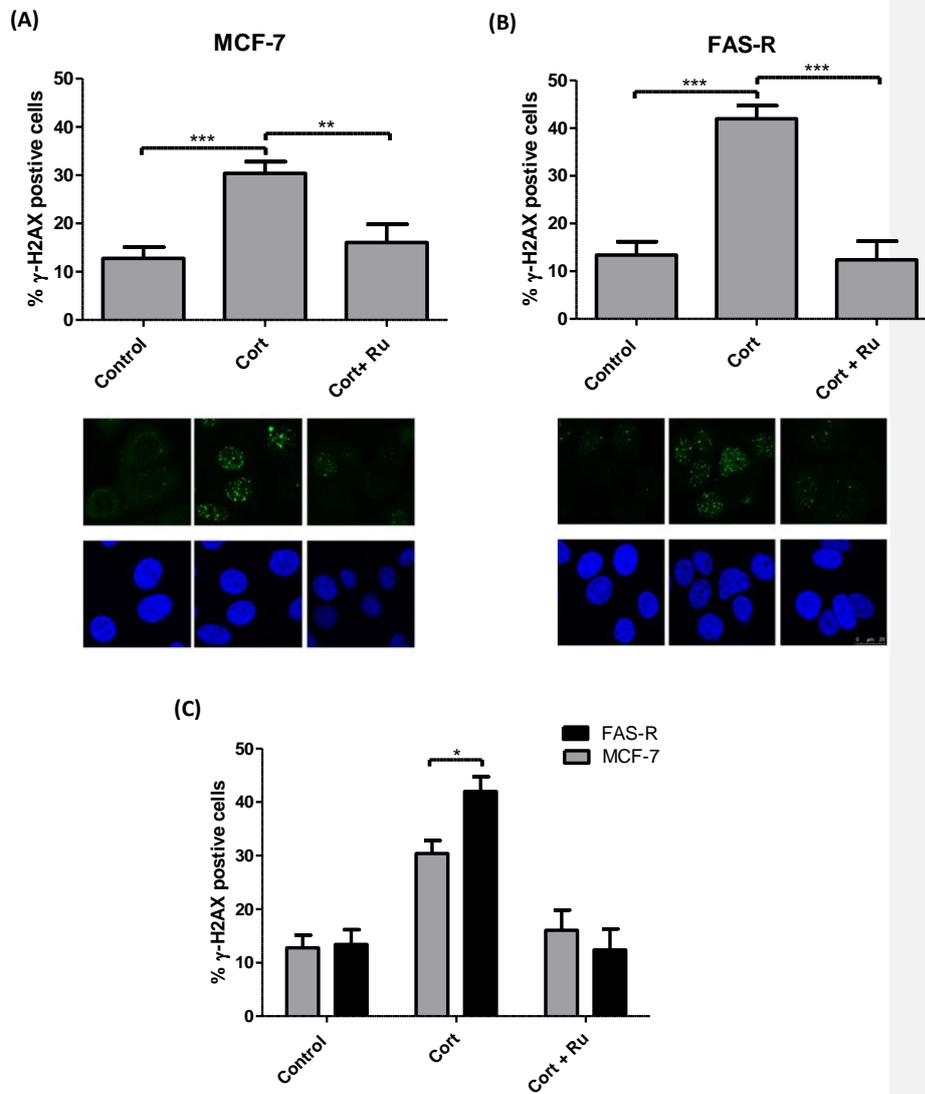


Fig. 5.2.4 MCF-7 (A) and FAS-R (B) cells were exposed to cortisol (Cort) in the presence and absence of RU486 (Ru) for 30mins. Cells were immunofluorescently labelled and scored as positive with >5 foci. Representative images shown. Mean \pm SEM is expressed and significance was determined using a one way (A) or two way (C) ANOVA (*post hoc* Tukey's multiple comparisons). * indicates a significant increase where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.5 Cortisol decreases the capacity for repair in FAS-R cells

To determine if repair pathways were affected in either the parental or Faslodex resistant cell line, MCF-7 and FAS-R cell lines were incubated with cortisol in the presence and absence of RU486 for 30 mins. Cells were then left to repair for 30mins before undergoing to single cell gel electrophoresis using the comet assay. Damage to DNA was assessed by scoring the tail intensity of the comets (0-4), which indicates the amount of single and double strand breaks, and therefore damaged DNA present. The amount of DNA damage can be used to indirectly assess the extent of repair, as cells are treated in parallel and then allowed a period of repair.

The effects of cortisol on DNA repair is displayed in Fig. 5.2.5A, and in FAS-R cells in Fig. 5.2.5B. Cortisol induced DNA damage in MCF-7 cells after 30mins ($p < 0.001$), and this was significantly reduced by the addition of RU486 ($p < 0.001$). There was a significant reduction in DNA damage in cells left to repair for 20mins post-treatment (repair group), compared to the cortisol treatment (no repair) group ($P < 0.05$) (Fig. 5.2.5A).

In FAS-R cells, cortisol caused an increase in DNA damage ($p < 0.001$) which was also significantly reduced by pre-incubation with RU486 ($p < 0.01$) (Fig. 5.2.5B). However, in contrast to the parental MCF-7 line, there was no significant difference between FAS-R cells exposed to cortisol, and those cells treated with cortisol and allowed to repair. Cells in the cortisol-repair group continued to display significantly increased levels of DNA compared to the control ($p < 0.05$).

Fig. 5.2.5 Cortisol decreases the capacity for DNA repair in FAS-R cells

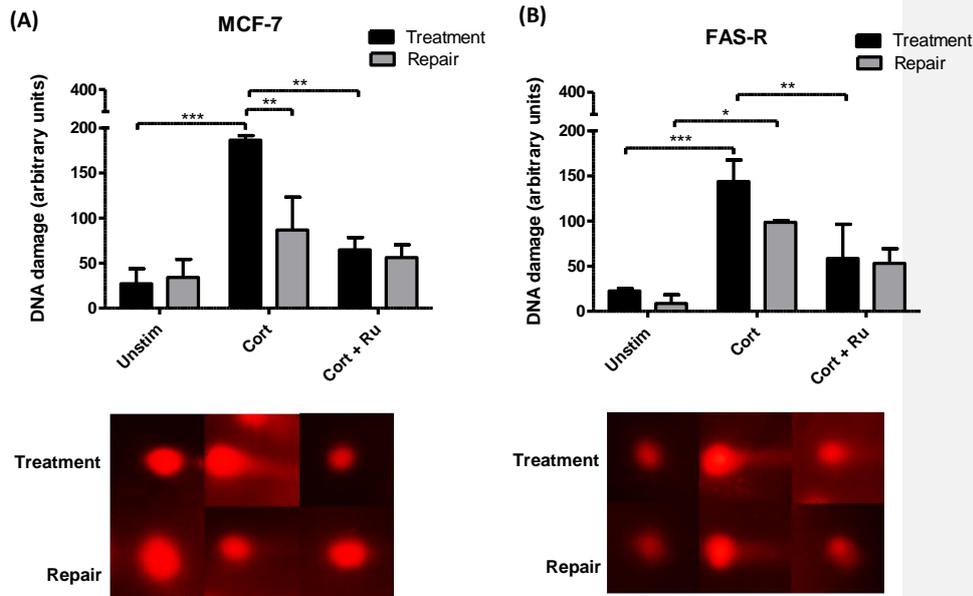


Fig. 5.2.5 MCF-7 (A) and FAS-R (B) cells were exposed to cortisol (Cort) in the presence and absence of RU486 (Ru) for 30mins. Treatment was removed and cells were left to repair for 30mins, then assessed for DNA damage using the Comet assay. Comet tails indicating DNA strand breaks were visually scored according to intensity (0-4). Representative images shown. Mean \pm SEM is expressed and significance was determined using a one way ANOVA (*post hoc* Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.6 Expression of the GR is upregulated in FAS-R cells constitutively and in response to cortisol

An inverse relationship between the ER and the GR in breast cancer has been shown previously (348), with an increase in GR expression correlating with a decrease in ER expression.

To examine the expression of the GR, mRNA from MCF-7 and FAS-R cells in an unstimulated state, and after stimulation with cortisol for 24hrs was extracted and amplified using qRT-PCR. In unstimulated cells, there was a significant difference in the basal expression of the GR, with over a 3-fold increase in FAS-R cells compared to MCF-7 cells ($p < 0.001$) (Fig. 5.2.6A). In FAS-R cells exposed to cortisol the expression of GR increased to approximately 30-fold compared to the parental MCF-7 cell line ($p < 0.05$) (Fig. 5.2.6B).

Fig. 5.2.6 Expression of the GR is upregulated in FAS-R cells constitutively and in response to cortisol

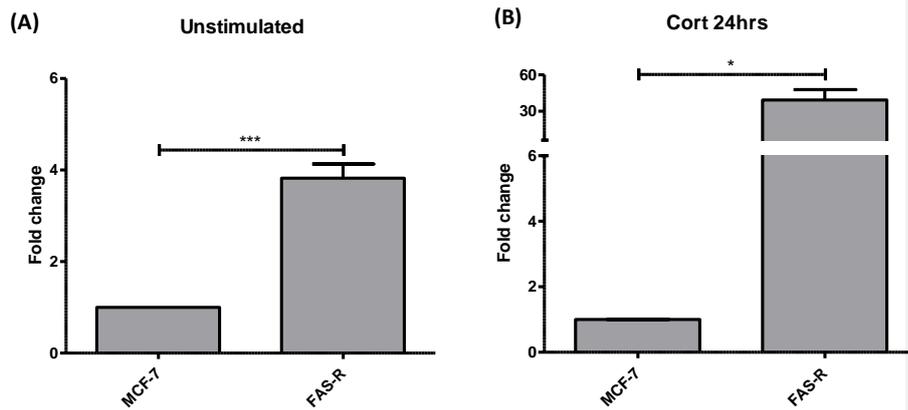


Fig. 5.2.6 MCF-7 and FAS-R cells were left untreated (A) or treated with cortisol for 24hrs (B) and mRNA was extracted. cDNA was synthesised and amplified in the presence of gene specific primers for *NR3C1* and *ACTB* using qPCR. Data are shown as mean \pm SEM. Statistical significance was determined using student's t-test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.7 GR expression in ER-negative breast cancers correlates with poor prognosis

Meta-analysis has linked an increase in GR expression in breast cancer with a short-relapse free survival in those with early stage ER-negative breast cancer, whilst in ER-positive cancers higher levels of GR expression were associated with a better outcome relative to low levels of expression (240). Bioinformatic analysis was used to corroborate this assertion, and examine the survival proportions of ER-positive/ER-negative breast cancers with high/low expression of the GR gene *NR3C1*. Gene expression microarray data from large breast cancer patient datasets (EGA and TCGA), was explored in relation to recurrence free survival, using Kaplan-Meier analysis (360).

In ER-negative breast cancers (n=801) higher expression is significantly correlated with a decrease in the probability of recurrence free survival (RFS) ($p=0.0015$) (Fig. 5.2.6A). In ER-positive breast cancers (n=1443) there is no difference in survival probability between higher and low expression ($p=0.76$) (Fig. 5.2.7B).

Fig. 5.2.7 GR expression in ER-negative breast cancers correlates with poor prognosis

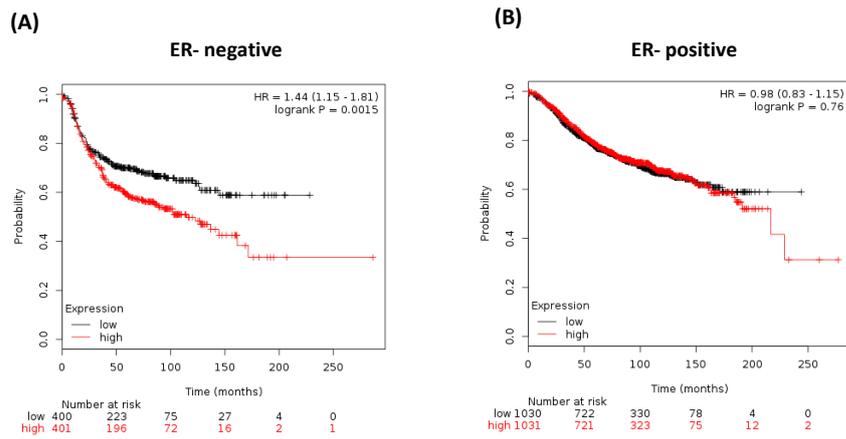


Fig. 5.2.7. Microarray data from the EGA and TCGA databases was examined in relation to the gene *NR3C1* in **(A)** ER-negative and **(B)** ER-positive patients. Patients were stratified into high and low expressors and RFS of the two cohorts is compared using a Kaplan-Meier survival plot.

5.2.8 FAS-R cells have an altered DNA damage response gene expression profile

A DNA damage profile array was performed by J. Gee *et al* at Cardiff University to examine the profile of FAS-R cells in relating to MCF-7 cells. FAS-R cells were first separated into EGFR-negative (1.2) and EGFR-positive (22.2) subtypes before being analysed using PCR array. Expression of 5 genes was analysed; *ATM*, *ATR*, *CHEK1*, *CHEK2*, *CDKN1A*.

Heatmap analysis of differentially expressed genes shows that in both clones there is higher expression (in red) of DNA damage response genes when normalised to parental MCF-7 cells (Fig.5.2.8).

Fig 5.2.8 FAS-R cells have an altered DNA damage response gene expression profile

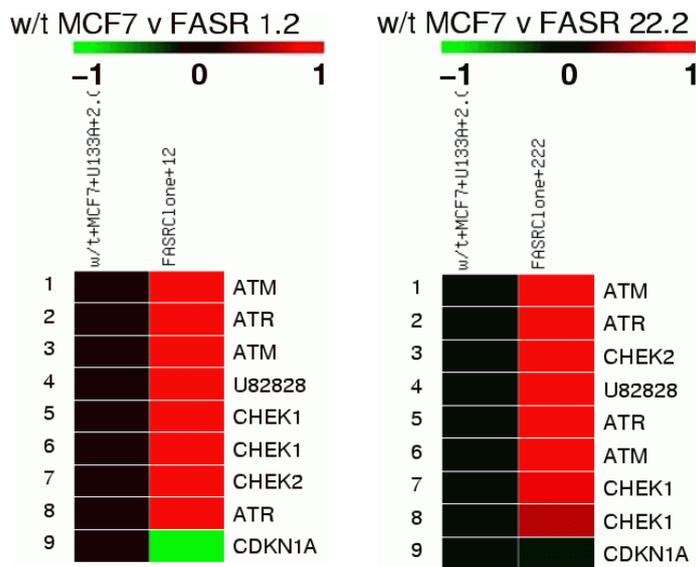


Fig. 5.2.8. FAS-R cells were first separated into EGFR-negative (1.2) and EGFR-positive (22.2) subtypes before being analysed using a DNA damage profile array. Expression of 5 genes was analysed; *ATM*, *ATR*, *CHEK1*, *CHEK2*, *CDKN1A*. Data is presented as a heatmap analysis of FAS-R gene expression compared to MCF-7, scale expresses magnitude of gene expression. Courtesy of J Gee, Cardiff University.

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5.2.9 Cortisol induces upregulation of DNA damage response elements in FAS-R cells

Detecting DNA damage is crucial in order for cells to maintain genetic integrity and continue to pass on genetic information. As such, cells have evolved a highly complex response capable of detecting DNA damage and activate appropriate DNA repair or direct unrepairable cells into apoptosis. The DNA damage response (DDR) uses the signal sensors ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-Related), which are phosphorylated upon activation in response to DSB's. In turn they orchestrate a large network of cellular signalling pathways through phosphorylation cascades to ameliorate genotoxic stress (353).

The expression of ATM and ATR as well as the state of phosphorylation provides useful insight into the activation of DDR pathways. Western blotting was used to determine the protein levels of ATM and ATR in response to acute (30 mins) and prolonged (24 hrs) cortisol exposure in MCF-7 and FAS-R cells (Fig. 5.2.9A). In MCF-7 cells ATM is not phosphorylated in response to cortisol treatment. In contrast, in unstimulated FAS-R cells ATM appears to be phosphorylated and there is no obvious change the level of phosphorylation with the addition of cortisol.

Expression of ATR in MCF-7 cells increases in response to cortisol exposure for 30mins, but not 24hr treatment. In FAS-R cells the expression of ATR is lower than in unstimulated MCF-7 cells, and similar in response to 30mins cortisol treatment. However after 24hrs cortisol exposure expression levels double in FAS-R cells compared to 24hr cortisol treated MCF-7 cells (Fig. 5.2.9B). Phosphorylation of MCF-7 cells increases in response to 30mins and 24hr exposure to cortisol, however in FAS-R cells levels of phosphorylated ATR are high in unstimulated cells and then drop after exposure to cortisol for 30mins and 24hrs (Fig. 5.2.9C).

Fig.5.2.9 Cortisol induces upregulation of DNA damage response elements in FAS-R cells

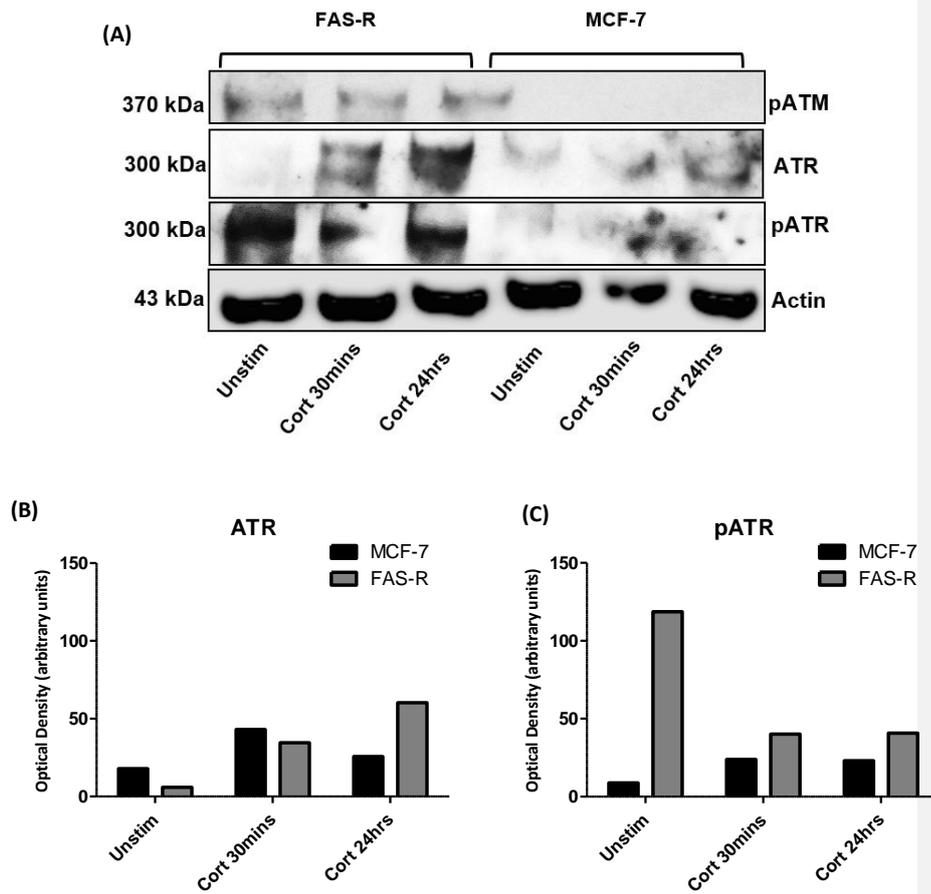


Fig. 5.2.9 MCF-7 and FAS-R cells were treated with cortisol for 30mins or 24hrs. Protein was extracted and expression was visualised using western blotting. Optical density values were normalised against β -actin. Representative images shown, data are shown are representative of n=1.

5.2.10 Cortisol increases the expression of p53 in FAS-R cells compared to MCF-7 cells

Levels of p53 protein are rapidly increased in response to DNA damage through inhibition of p53 degradation pathways and stabilizing post-translational modification by DNA damage sensors such as ATM and ATR. The subsequent p53 accumulation signals a range of downstream effectors involved in the cellular response to DNA damage, including growth arrest, DNA repair and apoptosis (361).

Expression of p53 was examined using qRT-PCR in MCF-7 and FAS-R cells exposed to cortisol. In MCF-7 cells there was a significant decrease in expression of p53 in response to 24hr cortisol exposure compared to unstimulated cells ($p < 0.05$) (Fig. 5.2.10A). In FAS-R cells there was a non-significant increase in cortisol treated cells compared to control (Fig. 5.2.10B). In unstimulated cells there was no significant difference in p53 expression across the two cell lines (Fig. 5.2.10C). However in response to cortisol there was a significant 4-fold increase in p53 in FAS-R cells relative to cortisol treated MCF-7 cells ($p < 0.05$) (Fig. 5.2.10D).

Fig. 5.2.10 Cortisol increases the expression of p53 in FAS-R cells compared to MCF-7 cells

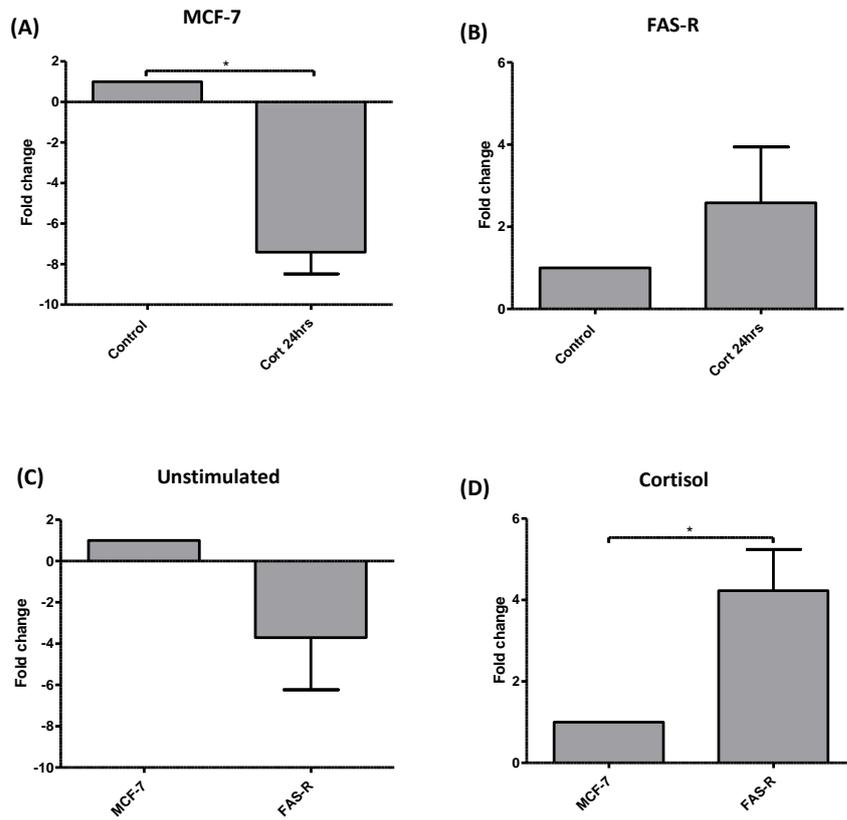


Fig. 5.2.10. MCF-7 (A) and FAS-R cells (B) were left untreated (C) or treated with cortisol for 24hrs (D) and mRNA was extracted. cDNA was synthesised and amplified in the presence of gene specific primers for *TP53* and *ACTB* using qPCR. Data are shown as mean \pm SEM. Statistical significance was determined using student's t-test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.11 Cortisol induces the expression of DNA damage response genes in FAS-R cells

A regulator of cell cycle progression p21, encoded by the gene *CDKN1A* and controlled by p53 transactivation, plays an important role in DNA damage repair by halting progression of the cell cycle and inhibiting DNA replication. The amount of p21 protein therefore increases following DNA damage, and provides useful insight into the capacity for DNA damage response and subsequent repair (362).

The expression of p21 in unstimulated and cortisol treated MCF-7 and FAS-r cells was assessed using qRT-PCR. In MCF-7 cells, p21 expression was higher in cells treated with cortisol compared to unstimulated controls, although this did not reach significance (Fig. 5.2.11A). However in FAS-R cells exposed to cortisol for 24hrs there was a significant 2-fold increase in p21 expression ($p < 0.01$) in compared to unstimulated FAS-R cells (Fig. 5.2.11B). In unstimulated cells there was no significant difference in mRNA levels of p21 between FAS-R cells and MCF-7 (Fig. 5.2.11C). When treated with cortisol for 24hrs there was an average 3-fold increase in p21 mRNA levels in FAS-R cells relative to cortisol treated MCF-7 cells, however this is not significant (Fig. 5.2.11D).

These results were further corroborated by determination of the expression of p21 at a protein level using western blotting. In MCF-7 cells, p21 was only present in cells treated for 24hrs with cortisol. p21 was constitutively expressed in unstimulated FAS-R cells, with a slight decrease after 30mins cortisol treatment and then an increase after 24hrs (Fig. 5.2.11E).

Fig. 5.2.11 Cortisol induces the expression of DNA damage response gene p21 in FAS-R cells

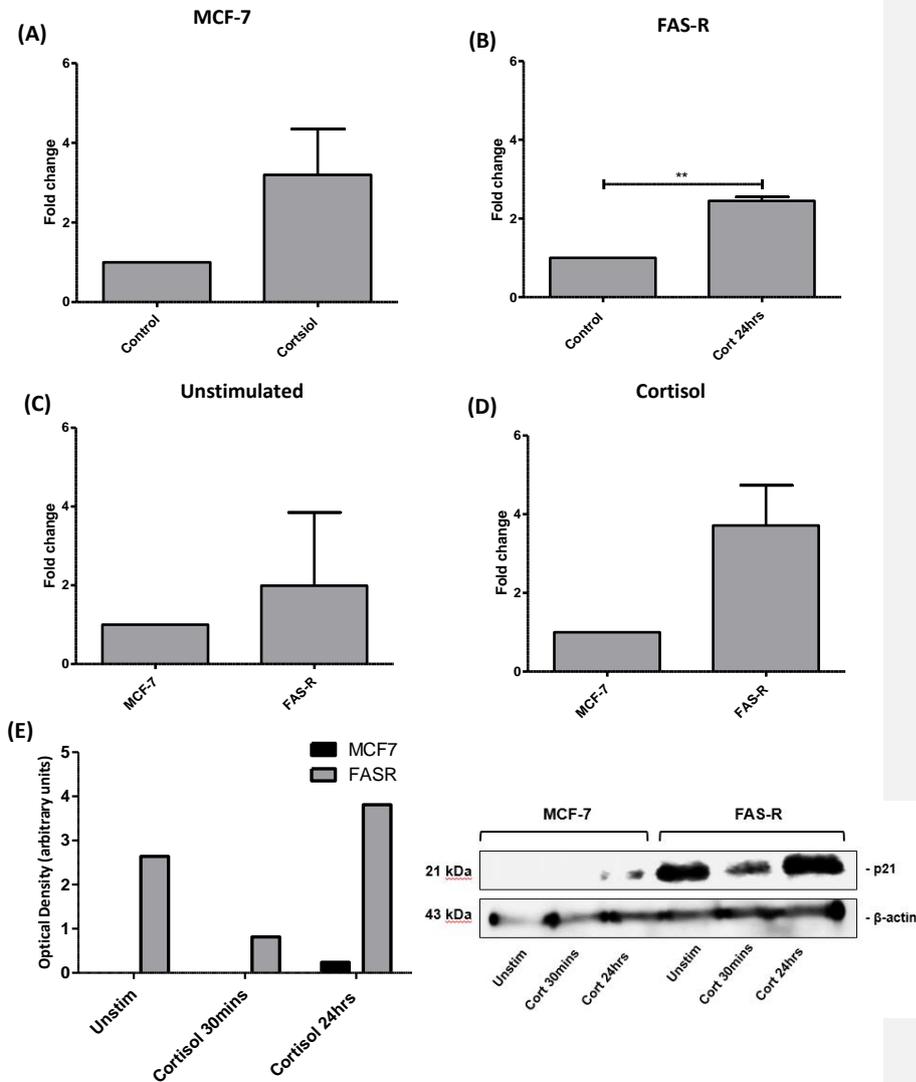


Fig. 5.2.11 MCF-7 **(A)** and FAS-R cells **(B)** were left untreated **(C)** or treated with cortisol for 24hrs **(D)** and mRNA was extracted. cDNA was synthesised and amplified in the presence of gene specific primers for *CDKN1A* and *ACTB* using qPCR. Data are shown as mean \pm SEM. Statistical significance was determined using student's t-test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. **(E)** Protein expression from cells exposed to cortisol for 30mins or 24 hours was visualised using western blotting. Optical density values were normalised against β -actin. Representative images shown, $n=1$.

5.2.12 Cortisol induces expression of Rad51 within the HR pathway in FAS-R cells

The protein encoded by the gene *RAD51* assists in the repair of double strand breaks via homologous recombination (HR). The detection of DSB's by damage sensors recruits BRCA1 amongst others, which promotes HR through the resection of DSB ends. The ssDNA overhang is bound by RAD51 forming a nucleoprotein filament which is then capable of invading a homologous region of dsDNA and repairing the break (363). RAD51 is known to play a role in cancer progression with overexpression correlating to poor clinical outcome in TNBC (364).

The expression of RAD51 in response to cortisol, which is known to induce DSB's, was explored using qPCR (Fig. 5.2.12A-D). In MCF-7 cells there was a significant downregulation of RAD51 mRNA levels in response to exposure to cortisol for 24hrs ($p < 0.01$) compared to unstimulated controls (Fig. 5.2.12A). Conversely in FAS-R cells, incubation with cortisol increased the expression of RAD51 2-fold ($p < 0.01$) (Fig. 5.2.12B). When compared to the parental cell line MCF-7 in an unstimulated state, the expression of RAD51 in FAS-R cells was lower ($p < 0.05$) (Fig. 5.2.12C). However in response to cortisol there was significantly higher levels of expression in FAS-R cells, compared to cortisol treated MCF-7 cells ($p < 0.01$) (Fig. 5.2.12D).

Fig. 5.2.12 RAD51 expression is increased in response to cortisol in FAS-R cells

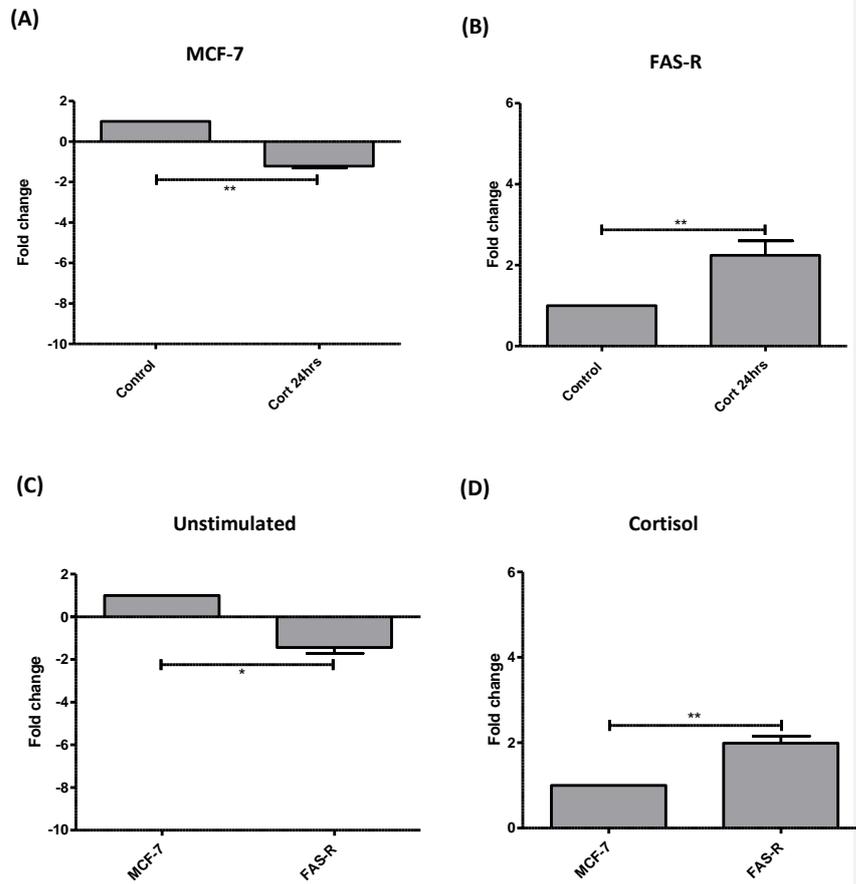


Fig. 5.2.12 MCF-7 (A) and FAS-R cells (B) were left untreated (C) or treated with cortisol for 24hrs (D) and mRNA was extracted. cDNA was synthesised and amplified in the presence of gene specific primers for *RAD51* and *ACTB* using qPCR. Data are shown as mean \pm SEM. Statistical significance was determined using student's t-test. * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.13 PARP inhibition blocks cortisol induced DSB detection in FAS-R cells

Single strand breaks in DNA are detected by PARP enzymes, specifically PARP-1, which functions to modify and activate other target proteins involved in repair pathways such as base excision repair and nucleotide excision repair. PARP-1 can also signal the repair of DSB's by activating ATM and subsequently NHEJ or HR. Inhibitors of PARP have been proven highly effective in the treatment of HR-deficient breast cancers such as BRCA1 and BRCA2 mutated cancers. Since activation of the HR pathway is defective these cancers rely on PARP-dependant activation of BER and NHEJ to repair damaged DNA (365).

The ability to repair DNA damage through several interconnected pathways is crucial in order for cells to survive and maintain genetic integrity. In order to explore the ability of MCF-7 and FAS-R cells to detect cortisol induced DNA damage, a PARP inhibitor (PJ34) was added 30mins prior to cortisol treatment for 30mins. Cells were immunofluorescently stained for phosphorylated γ -H2AX as a marker of DNA damage detection. In MCF-7 cells cortisol significantly increases phospho- γ -H2AX foci formation ($p < 0.001$) and this is significantly reduced by pre-incubation with RU486 ($p < 0.01$). Incubation with the PARP inhibitor PJ34 alone for 24hrs had no effect on levels of detected DNA damage, and incubation with PJ34 prior to cortisol did not significantly alter the detection of cortisol induced DNA damage compared to cortisol alone (Fig. 5.2.13A). In FAS-R cells cortisol induces DNA damage signalling ($p < 0.001$) which is negated by pre-incubation with RU486 ($p < 0.001$). Incubation with the PARP inhibition PJ34 for 24hrs does not significantly affect DNA damage detection, however PJ34 treatment prior to cortisol significantly decreased the formation of phospho- γ -H2AX foci compared to cortisol alone. ($p < 0.001$) (Fig. 5.2.13B).

Fig. 5.2.13 PARP inhibition blocks cortisol induced DSB detection in FAS-R cells

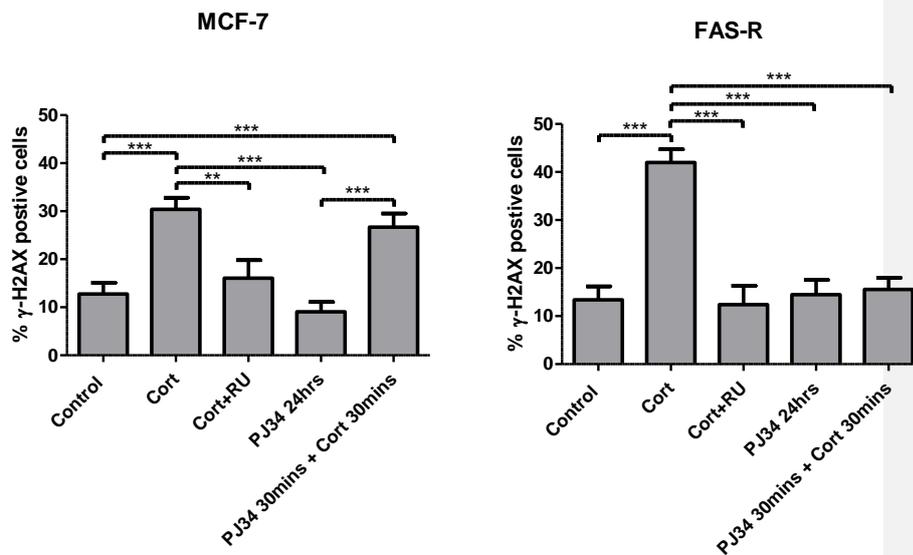


Fig. 5.2.13 MCF-7 (A) and FAS-R (B) cells were exposed to cortisol (Cort) in the presence and absence of RU486 (Ru) and PJ34 for 30mins. Cells were immunofluorescently labelled and scored as positive with >5 foci. Representative images shown. Mean \pm SEM is expressed and significance was determined using a one way ANOVA (*post hoc* Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.3 Discussion

Previous work presented here and in published studies has implicated cortisol as a cause of DNA damage in breast cancer, mediated through the GR and its role in oxidative stress. This data shows that cortisol has enhanced effects in a Faslodex resistant breast cancer model, promoting increased generation of ROS/RNS and DNA damage compared to the parental cell line. Furthermore, cortisol adversely affects DNA repair in FAS-R cells, and promotes dysregulation of the DNA damage response (DDR).

5.3.1 The change in phenotype

The parental cell line MCF-7 used in this study is a well characterised ER-positive line which also expresses some of the highest levels of the GR amongst breast cancer cell lines (139). As a result of continuous exposure to Faslodex, which acts to degrade the receptor, FAS-R cells no longer express the ER, however this has been shown to be reversible upon withdrawal of Faslodex (341). This study demonstrates that there is an increased basal expression of the GR in FAS-R cells, and upon stimulation of the GR this increases to a 30-fold increase compared to the parental cell line. The mechanism through which the acquisition of antioestrogen resistance mediates an upregulation the GR is not yet known, however similar relationships between the ER and EGFR as well as HER2 have been observed in resistant cell lines and tumours (342, 366).

Crosstalk between the ER and GR has been highlighted as a factor determining the way breast cancers respond to oestrogen, with ER/GR status playing a role in breast cancer outcome (240), and co-treatment modulating different effects on gene expression compared to single hormone treatment (367). Receptor mediated gene expression is regulated by specific binding elements which are in turn tightly controlled by chromatin structures. The chromatin landscape is therefore a large determinant in deciding which binding elements are available for transcription factor binding. Recently it has been shown that ER and GR can affect the binding of one another at specific sites that overlap through an assisted load mechanism and chromatin remodelling (368). This crosstalk allows GR signalling to influence ER activity, with studies showing that glucocorticoid treatment inhibits growth in oestrogen stimulated ER-positive MCF-7 cells (369). However the shift in phenotype from an ER-positive to an ER-/GR+ as found in this study, may be acting as a compensatory pathway, affording the cell a new route stimulated by alternative steroid hormone signalling and crosstalk between growth pathways.

There are many potential implications arising from this phenotypic change observed in FAS-R cells, including an increase in aggressiveness and invasive capability, as ER-negative breast cancers are intrinsically more aggressive than ER-positive cancer with fewer effective treatments and a tendency to relapse earlier (370). Importantly, it has also been shown that increased endogenous glucocorticoid exposure leads to greater ER-negative mammary tumours growth *in*

vivo (230). Therefore the role of glucocorticoids in endocrine resistance breast cancer becomes particularly relevant when it is considered that resistance occurs predominantly in breast cancer relapses, which are highly stressful events for patients and may increase circulating levels of cortisol for a prolonged period. Moreover glucocorticoids play a role in resistance to conventional chemotherapies, which bears further relevance to patients with acquired Faslodex resistance as they transition to an ER-/GR+ phenotype, and are treated with chemotherapy as a last line of defence. As such, the expression of the GR may prove a useful tool in the evaluation of the efficacy of treatment, and may emerge as a novel therapeutic target for ER-/GR+ subtypes or predictive biomarker for relapse. Previous work has demonstrated inhibition of the GR can reduce tumour volume in a TNBC model, when combined with paclitaxel (371). The loss of ER in Faslodex resistance could therefore indicate potential benefits from treatment strategies involving GR antagonism.

5.3.2 Oxidative stress, DNA damage and repair

The data presented here demonstrates that acquired Faslodex resistance promotes a change in phenotype resulting in an increase in oxidative species production, increased DNA damage and decreased DNA repair capacity in response to cortisol.

In MCF-7 cells cortisol induced a significant increase in NO_2^- , as measured by electroanalytical sensing, which was negated in the presence of RU486, indicating this effect is GR mediated. A similar pattern was observed in FAS-R cells however there was a significant difference between the two cell lines, with FAS-R cells producing more NO_2^- in response to cortisol than MCF-7 cells treated in the same way. Furthermore, increased levels of NO_2^- were still generated in FAS-R cells even with receptor blockade by RU486. There was also higher levels of the damaging ROS H_2O_2 produced in FAS-R cells treated with cortisol compared to MCF-7 cells which produced non-significant levels. These data demonstrate that glucocorticoids increase levels of oxidative stress in Faslodex resistant cells compared to their parental cell line. This may be as a result of increased expression of the GR in FAS-R cells, which is 3 fold higher in an unstimulated state and therefore could account for increased response to treatment. There is currently a lack of research exploring the role of oxidative stress in endocrine resistance, however one pre-clinical study regarding antioestrogen resistance has shown an association between increased oxidative stress and tamoxifen resistance *in vivo*. Tamoxifen resistant tumours were found to express higher levels of antioxidants and increased AP-1 activity, a transcription factor involved in mitogenic signalling pathways (372).

This enhanced production of ROS/RNS in FAS-R cells may also be responsible for the increase in DNA damage as observed using quantification of phosphorylated $\gamma\text{-H2AX}$ foci. In FAS-R cells there is a significant increase compared to MCF-7's after treatment with cortisol, which again

can be reversed with inhibition of the GR. The efficacy of DNA repair was also examined in response to cortisol using the comet assay. In MCF-7 cells cortisol produced an increase in DNA damage and did not significantly affect the repair. However, in FAS-R cells exposure to cortisol produces an increase in DNA damage which is not significantly reduced during repair. This indicates that whilst repair may be occurring, the rate of repair is affected, and as such cells may proliferate with damaged DNA. This aberrant DDR can contribute to genomic instability, which is closely linked to tumour progression, and may represent a potential mechanism for the growth of endocrine resistant tumours.

In order to further probe the differences in DNA repair mechanisms, FAS-R cells were profiled for DNA damage response genes. Overall there is a marked increase in the basal expression of genes involved in DNA damage detection NHEJ pathways. This finding prompted the hypothesis that resistance to Faslodex may be linked to, or induce, a deregulation of the DNA damage detection and repair pathways conferring genetic instability and promoting pro-tumourigenic alterations.

To confirm results from the profile the expression of DDR genes was examined at a protein level, as was their activity by phosphorylation. In FAS-R cells in an unstimulated state the DNA damage signal sensor ATM is phosphorylated, and this does not drastically change in response to acute or long term cortisol exposure. Similarly while ATR expression increases in FAS-R cells in response to cortisol, phosphorylated ATR is present at a basal level in FAS-R cells and then decreases in response to cortisol. These results are unexpected considering the effect cortisol has on the phosphorylation of γ -H2AX downstream and the DNA damage observed by the comet assay. One explanation may be the persistent phosphorylation of ATM and ATR is a result of deregulation of the DNA damage sensing machinery. However these results are representative on an n=1 and thus cannot be used to confirm deregulation of the DDR.

Commented [RF9]: Comment 64 – text clarified to point out that the results are n=1.

The expression of downstream targets of the DNA damage sensors were also evaluated, with p53 expression downregulated in MCF-7 cells treated with cortisol, but significantly upregulated in FAS-R cells treated with cortisol in comparison. This further demonstrates the difference in the response to DNA damage between cell lines, as classically a p53 mediated response is achieved through post-translational modification, with no induction of p53 mRNA (373). The role of p53 as a tumour suppressor is to promote cell cycle arrest or apoptosis when signalled by stress. Loss of expression or mutation of p53 is closely linked with tumour initiation acceleration of tumour growth, as well as increased metastasis in breast cancer (374, 375). The p53 signalling pathways is controlled by the proto-oncogene MDM2 which binds and degrades p53 continuously in the absence of upstream triggers, as such an upregulation of p53 could indicate a dysfunction in the MDM2 feedback loop allowing high levels of p53 to accumulate.

Overexpression of p53 has also been found to correlate with poor outcomes in younger patients with TNBC, although in this case it is proposed that the accumulation of p53 is as a result of a mutation (376). It is not yet known whether FAS-R cells possess wild type or mutated p53, however mutated p53 would correlate with the shift in FAS-R cells to a more aggressive ER-negative phenotype.

Another part of the DNA damage response downstream of p53 is p21, which acts in a number of ways to halt the cell cycle and inhibit replication of damaged DNA. In FAS-R cells expression of p21 protein is higher in unstimulated cells compared to MCF-7, however mRNA levels are not significantly different. Treatment with cortisol significantly increases levels of p21 mRNA and protein in FAS-R cells, and produces a modest rise in MCF-7 cells. The disparity in results between protein and mRNA levels of p21 could be attributed to post-transcriptional regulation of p21, affecting its stability and localization (377). Furthermore proteins may also remain stable and have longer half-lives compared to mRNA, which can result in poor correlation between mRNA and protein levels (378). In breast cancer, high levels of expression of p21 at both an mRNA and protein level, have been found to correlate with adverse pathological parameters and poor prognosis (379). Conversely, loss of p21 expression defined histopathologically, indicated worse survival rates in a cohort of breast cancer patients (380). The exact mechanism of p21's involvement in tumorigenesis is uncertain, however it has been suggested that the localization of p21 is important, with nuclear p21 promoting senescence, and the accumulation of cytoplasmic p21 associated with oncogenic activity (377). In this model of endocrine resistance, FAS-R cells proliferate a steady rate in a parallel with the parental cell line (341), therefore it would appear that the constitutive upregulation of p21 in unstimulated cells does not induce senescence. This could potentially implicate p21 in oncogenic signalling in endocrine resistance, and suggests that a glucocorticoid-induced upregulation in p21 could exacerbate this.

It is difficult to elucidate the full extent of the types of DNA damage caused by cortisol, and as such it is also hard to separate the DNA repair pathways that may be activated and affected, due to the pleiotropic effects of cortisol. To determine if HR processes were involved, RAD51 expression was quantified, and again FAS-R cells showed a distinctly different pattern than their parental counterparts. In response to cortisol, RAD51 expression was upregulated in FAS-R cells compared to unstimulated FAS-R cells, as well as compared to cortisol treated MCF-7 cells, indicating that HR was also being stimulated. The activation of HR machinery suggests that single strand breaks are also being generated as well as double strand breaks, and that FAS-R cells are responding to the damage despite the evidence from the comet assay that repair rates seem to be affected in FAS-R cells. Interestingly there has been shown to be an increase in rates of HR in

breast cancer cells (381) and RAD51 is known to play a role in cancer progression with overexpression correlating to poor clinical outcome in TNBC (364).

Since the apparent deregulation of repair in acquired Faslodex resistant cells cannot be wholly attributed to either the inactivation of HR or NHEJ pathways, the response to inhibition of PARP was investigated. PARP inhibition was used to determine if the capacity for repair was altered due to aberrations in the sensing of damage by BRCA1/2. PARP inhibitors are used to treat breast cancers that carry BRCA1/2 defections, or have a 'BRCAness' profile i.e. they may or may not carry mutation but behave in a similar manner. PARP inhibitors target mechanisms employed to repair DNA damage and can be administered in combination with cytotoxic therapy or radiation to block DNA repair, or as a single agent. In MCF-7 cells which are not BRCA1/2 mutated and which do not display 'BRCAness', inhibition of PARP for 24hrs has no effect on DNA damage, indicating that in these cells even though PARP cannot effectively signal SSB's to be repaired, if DSB's form they can still be repaired. Inhibition of PARP prior to cortisol treatment also does not affect DNA damage sensing machinery, as the same levels of phosphorylated γ -H2AX are induced when compared to cortisol treatment alone. In FAS-R cells PARP inhibition alone does not induce phosphorylated γ -H2AX indicating that repair of constitutive levels of DNA damage is not affected. However PARP inhibition prior to cortisol-induced DNA damage significantly reduces the percentage of phosphorylated γ -H2AX positive cells compared to cortisol treatment. This suggests PARP inhibition may block the detection of cortisol induced DNA damage, specifically DSB's. Since the activity of ATM which acts to signal DSB's appears to be deregulated in FAS-R cells this may provide an insight into the differing response to DNA damage, as ATM and PARP1 are closely linked. In the absence of PARP-1 the activity of ATM in response to ionizing radiation has been shown to be compromised, with a decrease in H2AX phosphorylation (382). It is therefore proposed that inhibition of PARP further compromises the already deregulated function of ATM in an acquired Faslodex resistance cell line.

5.3.3 Future perspectives

Whilst the work presented here gives an insight into the change in phenotype induced by endocrine resistance, specifically the deregulation in DNA damage response and repair, as of yet a clear pathway or mechanism cannot be proposed.

The obvious difference in the machinery of the DDR in FAS-R cells compared to MCF-7 highlights a potential target for therapy in the face of endocrine resistance. Previous work has shown inhibition of HR can sensitize cancer cells to radiotherapy (383), while transcriptionally targeted therapies such as that of the *RAD51* gene can prove much less cytotoxic to normal tissue due to its upregulation in comparison. Furthermore p21 has also been touted as a potential target and sensitizer due to its propensity for overexpression in cancer and anti-apoptotic effects (384).

Upstream p53 is another obvious target due to its frequent mutation in aggressive tumours, with p53 inhibitors currently undergoing clinical trial and drugs capable of reverting wild type p53 to mutant in order to restore apoptotic signalling the focus of extensive research (385). As such targeting these proteins involved in the DRR may prove especially attractive and expand the repertoire of treatment for endocrine therapy resistant breast cancers.

Future work on this topic hopes to explore the role of ER the effect of loss of ER on DNA damage signalling, as well a DNA damage and repair mediated by glucocorticoids. In order to do so, and to also allow for crosstalk between the ER and GR to be examined, FAS-R and MCF-7 cells will be cultured in the presence of oestrogen and both receptors inhibited pharmacologically. This data hopes to provide information on whether cortisol has the same effect in the presence of oestrogen, or whether it's signalling is interfered with. Furthermore since glucocorticoids are known to play a role in cell cycle progression and DNA repair pathways are so critically linked to regulation of the cell cycle, the growth and cell cycle distribution of FAS-R and MCF-7 cells will be examined in response to cortisol. Lastly whilst indirect measurements have indicated dysfunction in DNA repair in FAS-R cells, it is planned that direct analysis of repair by NHEJ and HR will be undertaken using reporter construct assays.

**Chapter 6 – Dexamethasone, an exogenous
glucocorticoid, negatively impairs chemotherapy
induced cytotoxicity in breast cancer**

6.1 Introduction

6.1.1 Breast cancer therapies and resistance

Chemotherapy refers to the use of cytotoxic drugs in the treatment of cancer. Anthracyclines and taxanes are some of the chemotherapies available to breast cancer patients, and are routinely prescribed to treat aggressive or advanced breast cancers.

The taxane, Paclitaxel (Pac) is a microtubule-stabilising agent, which acts to bind to the β -subunit of tubulin preventing microtubule assembly, and therefore mitosis. Cells then enter cell cycle arrest, and subsequently cell death occurs by apoptosis (386). Paclitaxel is therefore an effective chemotherapeutic which can act on rapidly dividing cells, and is primarily used for treatment of metastatic breast cancers (387). However, resistance can occur over time following exposure to paclitaxel, and an increase in the treatment of early breast cancers with taxanes has seen an increase in resistance in recurrent disease (388, 389). Several mechanisms have been proposed to explain the development of resistance to paclitaxel, including overexpression of multi-drug resistance (MDR) genes such as *MDR-1*, which encodes for a membrane transporter that can transport chemotherapeutics such as paclitaxel out of cells (390). Altered expression of β -tubulin isoforms can foster resistance to paclitaxel, possibly by decreasing binding, and alteration in pro-apoptotic genes have also been observed in the development of paclitaxel resistance (391). Co-treatment with glucocorticoids, such as dexamethasone, has been shown to act in a protective capacity, inducing resistance and reducing the efficacy of paclitaxel in solid tumours such as breast and ovarian (392).

Doxorubicin (Dox) is a non-selective anthracycline widely used in the treatment of breast, bladder and ovarian cancer as well as types of leukaemia and lymphoma, often in combination with other therapies (393). The cytotoxic effects of Dox are mediated through multiple mechanisms; the intercalation of DNA which forms doxorubicin-DNA adducts that induce cell death, and the disruption of topoisomerase-II activity, thereby preventing DNA resealing and subsequently preventing replication in rapidly dividing cells (394). Furthermore, the oxidation of Dox to a semi-quinone by NAD(P)H-oxoreductases also generates free radicals increasing oxidative stress and promoting protein, lipid and DNA damage (39, 393). Although highly effective, and considered a mainstay of chemotherapy regimens, Dox treatment also triggers many side effects including acute cardiotoxicity (395), vomiting, hair loss and inflammation (39). As such it is often prescribed alongside the potent synthetic glucocorticoid dexamethasone (Dex) to treat hypersensitivity. Dex, when used as a co-medication, can reduce inflammation, dampen immune responses and to relieve nausea and boost appetite, as well as protecting normal tissues from toxicities induced by chemotherapeutics (396).

Resistance to Dox poses a significant problem in the treatment of aggressive breast cancers, and since there is currently no marker to predict the efficacy of Dox, it is often prescribed without a full understanding of the therapeutic gain (397). Resistance to Dox has been associated with a number of factors related to its mechanisms of action, including MDR-associated proteins, alterations in DNA repair and topoisomerase activity and increased detoxification capacity (39, 398, 399). The ability of a cell to increase protection from oxidative stress is particularly relevant in the context of Dox resistance as oxidative stress plays a role in its mechanism of action. An increase in intracellular reduced glutathione (GSH) which has anti-oxidant capacities has been observed in cells with reduced Dox sensitivity (400). Furthermore, studies have positively correlated expression of genes associated with increase antioxidant capacity with chemoresistance - with particular focus on nuclear factor erythroid 2-related factor (Nrf2), a transcription factor that controls cellular redox homeostasis and detoxification by regulating several antioxidant response related genes (401).

6.1.2 NRF2, resistance and oxidative stress

Under normal homeostatic conditions NRF2 levels are suppressed by a Kelch-like ECH-associated protein 1 (KEAP-1)-dependant mechanism, whereby KEAP-1 promotes the ubiquitination and proteasome degradation of NRF2. However in response to oxidising conditions, the binding of NRF2 to KEAP-1 can be disrupting allowing NRF2 to accumulate in the nucleus and heterodimerize with small Maf proteins which allows transactivation of antioxidant response element controlled genes. These include genes can encode for glutathione synthases, detoxification enzymes, antioxidant and drug transport proteins (402, 403).

Expression of Nrf2 is upregulated in several cell lines resistant to chemotherapy drugs including cisplatin and etoposide, as well as Dox resistance breast cancer lines, and its inhibition is capable of restoring sensitivity (401, 404). Tamoxifen resistance was also reversed by the blockade of NRF2 in an MCF-7 derived cell line which possessed an enhanced antioxidant expression profile (405). Furthermore, the inhibition of downstream targets of NRF2 such as heme oxygenase-1 (HO-1) have been shown to be able to decrease cell viability in leukaemia cells (406). In NRF2 knockout mice studies showed an increased susceptibility to tumour formation in response to exposure to carcinogens (407), supporting the current consensus that the NRF2 pathway functions a cell survival pathway protecting against oxidative stress that can be deregulated in tumourigenic transformation.

6.1.3 Glucocorticoids and oxidative stress

Glucocorticoids have been shown promote cell survival signalling pathways in mammary epithelial cells (408) and reduce the cytotoxic potential of common chemotherapeutic agents in

breast and ovarian cancer cell lines (140) (409) . Multiple mechanisms have been proposed to explain the pro-survival effects of glucocorticoids including increased cell adhesion and inhibition of apoptosis via the induction of anti-apoptotic proteins (409) (410). Dex has also been shown to modulate immunological effects in patients receiving chemotherapy, increasing the activation of regulatory T cells which are capable of suppressing anti-tumour responses (411). Dex has been shown to mediate conflicting effects on Dox specifically, acting as a chemosensitiser *in vivo* enhancing the inhibition of tumour growth by Dox (412), and promoting chemoresistance in cancer cell lines and models (413).

Glucocorticoids are also known to play a role in oxidative stress in cancer. Through genomic and non-genomic pathways the glucocorticoid receptor (GR) is involved in the transactivation and transrepression of multiple anti-inflammatory responses that suppress oxidative stress (269). Activation of the GR can inhibit NRF2 activity through repression of transcription of the NRF2 gene, and subsequently suppress the antioxidant response including the expression of antioxidant response controlled genes (414). Conversely glucocorticoids have also been shown to increase levels of damaging free radicals in the context of breast cancer in a rapid non-genomic manner (310). In this way glucocorticoids may serve to increase oxidative stress which can promote decreased sensitivity to Dox through upregulation of the antioxidant response.

6.1.4 Aims

This investigation aims to:

- Determine the effects of exogenous glucocorticoids on the efficacy of breast cancer therapeutics.
- Explore a mechanism through which dexamethasone acts to interfere with doxorubicin-induced cytotoxicity.

6.2 Results

6.2.1 Dexamethasone reduces the efficacy of chemotherapies but not endocrine therapies

To explore the effect of Dex on breast cancer chemotherapies and endocrine therapies, MCF-7 cells were exposed to the LD₅₀ of paclitaxel, doxorubicin, tamoxifen and fulvestrant (Faslodex) in combination with doses of Dex for 24hrs. Paclitaxel alone has a significant cytotoxic effect on MCF-7 cells ($p < 0.001$), and this is not negated by the addition of Dex at 0.1 μ M or 1 μ M. Co-treatment with 10 μ M Dex significantly increased cell viability ($p < 0.01$) compared to paclitaxel alone, matching viability levels observed in untreated control cells. There was also a significant difference between paclitaxel combined with 0.1 μ M or 1 μ M Dex, and paclitaxel with 10 μ M Dex ($p < 0.05$) (not shown) (Fig. 6.2.1A).

Similarly, when incubated with the LD₅₀ of doxorubicin (1 μ M) for 24hrs, cell viability was significantly decreased ($p < 0.001$) and this was not reversed by incubation concomitantly with 0.1 μ M dexamethasone. However, higher concentration of Dex (1 μ M and 10 μ M) significantly abrogated the cytotoxic effect of Dox ($p < 0.01$) (Fig. 6.2.1B).

Treatment with the endocrine therapies tamoxifen and fulvestrant also resulted in a decrease in cell viability, although a 50% decrease in cell viability was not achieved after 24hrs. Incubation with Dex at 0.1 μ M, 1 μ M or 10 μ M alongside the endocrine therapies did not significantly affect cell viability compared to tamoxifen or fulvestrant alone (Fig. 6.2.1C-D).

Fig 6.2.1 Dexamethasone reduces the efficacy of chemotherapies but not endocrine therapies

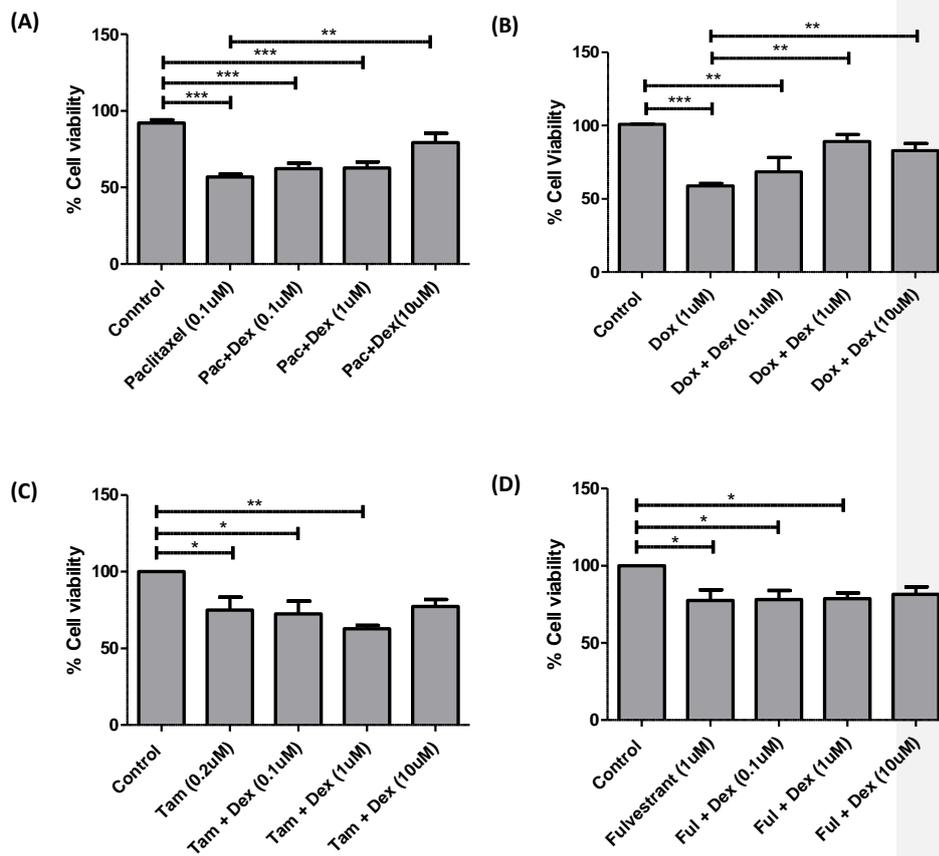


Figure 6.2.1 MCF-7 cells were exposed to **(A)** 0.1 μM paclitaxel, **(B)** 1 μM doxorubicin, **(C)** 0.2 μM tamoxifen and **(D)** 1 μM fulvestrant alone, and in combination with dexamethasone (0.1 μM, 1 μM, 10 μM). Cell viability was assessed using MTT assays and the results are expressed as a percentage of cell viability compared to the control. Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

6.2.2 Dexamethasone reduces the cytotoxicity of Doxorubicin through a glucocorticoid receptor mediated mechanism in ER+ and TNBC cell lines

Once the optimal concentration of Dex required to reduce the efficacy of Dox had been determined, further cell viability assays were carried out to explore if a similar effect could be observed in an aggressive triple negative breast cancer cells, and to confirm this effect was mediated through the GR. MDA-MB-231 cells were incubated with 10 μ M doxorubicin, a higher concentration than MCF-7 cells as they are more inherently resistant to the cytotoxic effects of Dox (Appendix 6 Fig. 1.1). Both cell lines were further treated with Dex (1 μ M) alone, and in combination with Dox for 24hrs, as well as with a 30min pre-treatment with the glucocorticoid receptor antagonist RU486 (1 μ M)

In MDA-MB-231 cells, 10 μ M Dox significantly reduced the percentage of cell viability compared to untreated cells ($p < 0.001$). Dex alone has no effect on cell viability compared to untreated cells. In combination Dex significantly reduced the efficacy of Dox, with cell viability levels significantly higher than Dox alone ($p < 0.01$). Incubation with RU486 prior to Dex and Dox treatment blocked the protective effect of Dex on Dox induced cytotoxicity, reducing the cell viability to a similar level as seen with Dox alone (Fig. 6.2.2A).

The same pattern is observed in MCF-7 cells exposed to 1 μ M doxorubicin, where Dex alone has no effect, however in combination Dex is able to significantly reverse the effect of Dox on cell viability ($p < 0.001$). Pre-treatment with RU486 significantly reduces viability when compared to Dox and Dex ($p < 0.001$), indicating that the observed cytoprotective effect of Dex is mediated through glucocorticoid receptor binding and signalling (Fig. 6.2.2B).

Fig 6.2.2 Dexamethasone reduces the cytotoxicity of Doxorubicin through a glucocorticoid receptor mediated mechanism

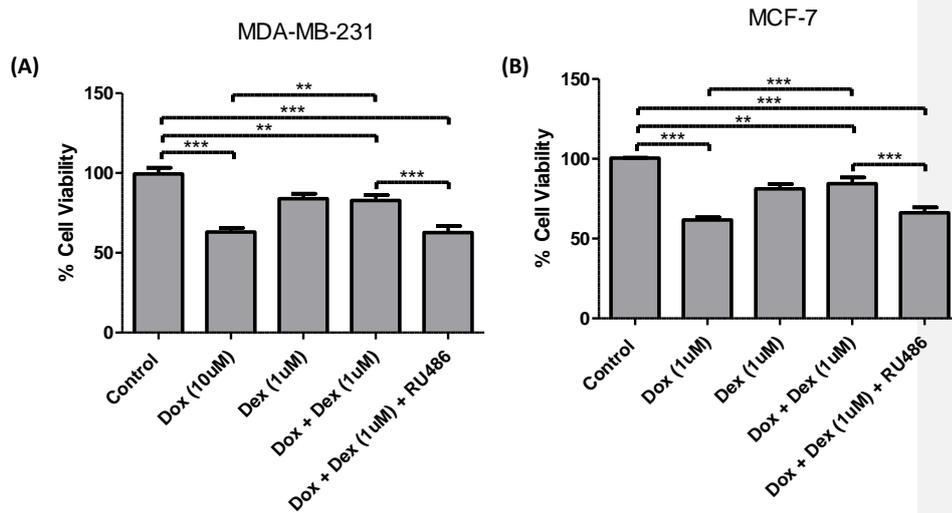


Fig. 6.2.2 MDA-MB-231 **(A)** and MCF-7 **(B)** cells were exposed to doxorubicin or dexamethasone alone or in combination, as well as with RU486 pre-treatment. Cell viability was assessed using MTT assays and the results are expressed as a percentage of cell viability compared to the control. Mean \pm SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Fig. 6.2.3 Dexamethasone in combination with Doxorubicin increases levels of extracellular nitrite

Dox is known to promote the production of reactive species in the cell, increasing the levels of oxidative stress (39). Similarly, previous work has shown that glucocorticoids can induce the production of reactive nitrogen species (RNS) in breast cancer cells, through an iNOS mediated pathway (310). To understand how the combination of Dox and Dex may alter levels of nitrosative stress, extracellular NO_2^- released into the growth medium was measured.

The cell lines MCF-7 and MDA-MB-231 were exposed to Dox and Dex alone and in combination for 24hrs, and the Griess assay was used to measure NO_2^- levels in the growth medium. In MCF-7 cells, incubation with Dox and Dex individually produced an increase in extracellular NO_2^- , although this was not significantly different compared to unstimulated cells. However, when Dex and Dox were added in combination, a significant increase in NO_2^- was observed ($p < 0.05$) (Fig. 6.2.3A). In MDA-MB-231 cells treated with the same concentrations of Dox and Dex ($1\mu\text{M}$), a similar pattern is observed, with Dox and Dex alone increasing levels of NO_2^- but not significantly. Whilst in combination there is a significant increase compared to the baseline levels ($p < 0.05$) (Fig. 6.2.3B).

Fig. 6.2.3 Dexamethasone in combination with Doxorubicin increases levels of extracellular nitrite

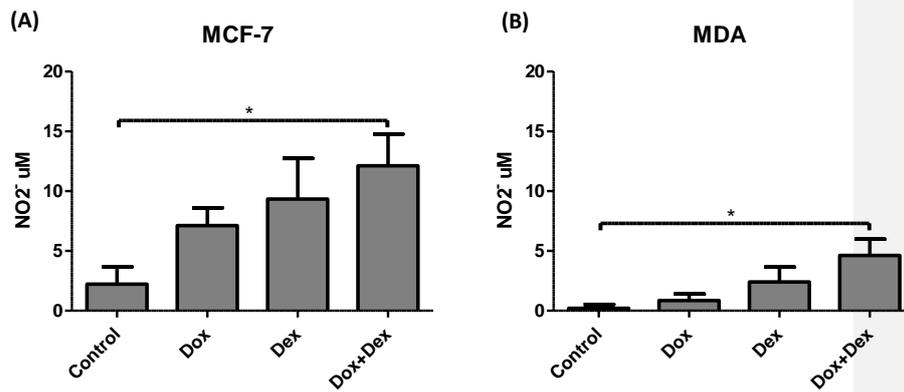


Figure 6.2.3 MCF-7 (A) and MDA-MB-231 (B) cells were exposed to doxorubicin or dexamethasone alone or in combination. Extracellular nitrite (NO₂⁻) levels were quantified using the Griess assay. Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = p<0.05, ** = p<0.01, *** = p<0.001.

6.2.4 Dexamethasone in combination with Doxorubicin increases levels of intracellular ROS/RNS

In order to examine the generation of further reactive species, electrochemical quantification of intracellular ROS/RNS was employed using multi-step amperometry. MCF-7 cells were exposed to Dox and Dex for 24hrs and subsequently lysed. The lysates were analysed for levels of NO_2^- and H_2O_2 using electrochemical sensors.

Exposure of MCF-7 cells to Dox alone did not have a significant effect on levels of NO_2^- compared to unstimulated cell. However, Dex alone, and in combination with Dox, resulted in a significant increase in NO_2^- ($p < 0.001$). Dox alone did not have a significant effect on NO_2^- levels (Fig. 6.2.4A). Conversely the generation of H_2O_2 was significantly increased in response to Dox treatment ($p < 0.01$), but not Dex (fig. 6.2.4B). Combined treatment with Dox and Dex also significantly increased levels of H_2O_2 compared to unstimulated cells ($p < 0.001$).

Fig 6.2.4 Dexamethasone in combination with Doxorubicin increases levels of intracellular ROS/RNS

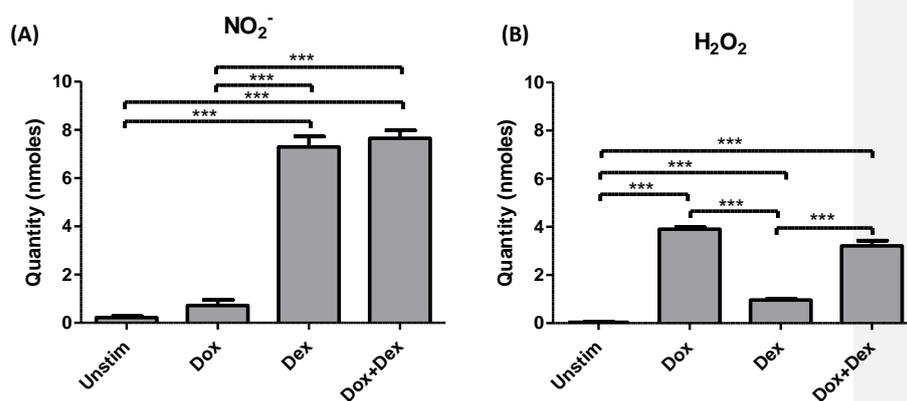


Figure 6.2.4. MCF-7 cells were exposed to doxorubicin or dexamethasone alone or in combination. Levels of nitrite (NO₂) (A) and hydrogen peroxide (H₂O₂) (B) were measured using electrochemical sensors. Mean ± SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = p<0.05, ** = p<0.01, *** = p<0.001.

6.2.5 NRF2 expression is downregulated in response to Doxorubicin and Dexamethasone

NRF2 is transcription factor capable of regulating the transcription of genes involved in cellular detoxification and antioxidant responses (402). Generation of reactive species triggers the release of NRF2 from KEAP-1, which in the absence of oxidative stress promotes the ubiquitination and degradation of NRF2. The expression of NRF2 in MCF-7 cells was measured in response to Dox and Dex, which when treated concomitantly induced the production of oxidative/nitrosative species.

The relative fold change in mRNA levels was assessed using qPCR, and a significant down regulation of NRF2 mRNA was observed in response to Dex ($p < 0.05$), but not Dox when administered alone. Combined treatment also downregulated NRF2 compared to control, however not significantly so (Fig. 6.2.5).

6.2.5 NRF2 expression is downregulated in response to Doxorubicin and Dexamethasone

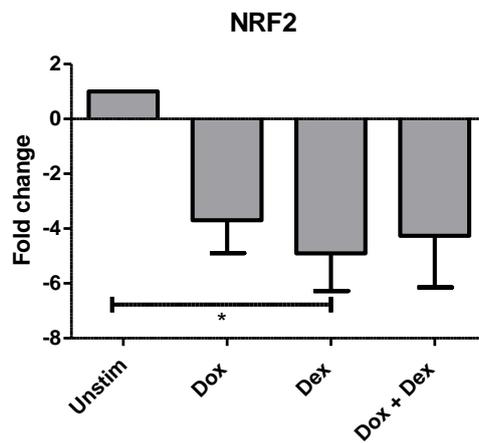


Figure 6.2.5 MCF-7 cells were exposed to doxorubicin (Dox) or dexamethasone (Dex) alone or in combination. RNA was extracted and cDNA synthesised and amplified in the presence of gene specific primers for *NFE2L2* and *ACTB* using qPCR. Ct values were normalised against β -actin and fold change calculated using the delta-Ct method. Mean \pm SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

6.2.6 Dexamethasone in combination with Doxorubicin increases the expression of antioxidant response element controlled genes

Part of the cellular antioxidant response is mediated by NRF-2's activation of the antioxidant response element (ARE), which controls transcription of target genes involved in cytoprotection (402). The downstream targets of NRF2 include NAD(P)H quinone dehydrogenase 1 (NQO1), a quinone reductase enzyme that can prevent redox cycling and the generation of radicals (415), and HO-1, a potent antioxidant that catalyses the degradation of heme and increases reduced glutathione levels (416). The response to oxidative stress and induction of antioxidant pathways, was explored in MCF-7 cells treated with Dox and Dex by analysis of the expression of NQO1 and HO-1 using qRT-PCR.

The relative fold change in expression of mRNA for NQO1 did not significantly differ in response to Dox or Dex compared to unstimulated cells. Combined treatment with both Dex and Dox had a significant effect on expression with over a 2-fold increase in mRNA levels ($p < 0.05$) (Fig. 6.2.6A). Dox and Dex alone also do not have an effect on the expression of HO-1, however together they significantly increased expression (over 2-fold) compared to the control ($p < 0.01$) and to Dex alone ($p < 0.05$) (Fig. 6.2.6B).

Fig. 6.2.6 Dexamethasone in combination with Doxorubicin increases the expression of antioxidant response element controlled genes

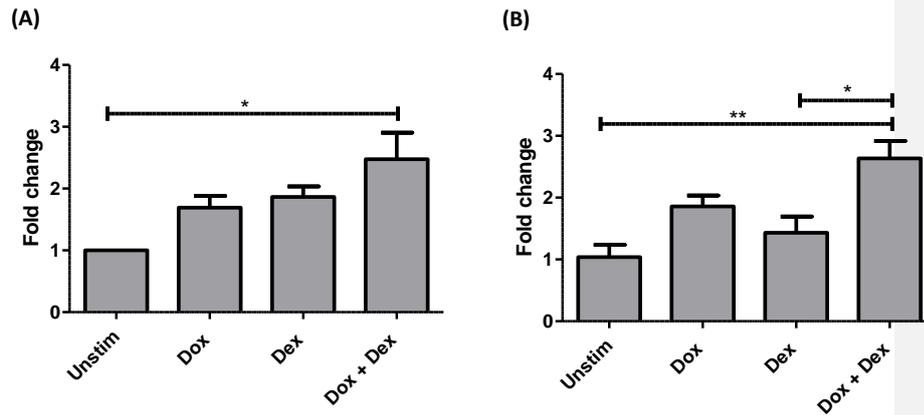


Figure 6.2.6 MCF-7 cells were exposed to doxorubicin (Dox) or dexamethasone (Dex) alone or in combination. RNA was extracted and cDNA synthesised and amplified in the presence of gene specific primers for *NQO1* (A), *HMOX1* (B) and *ACTB* using qPCR. Ct values were normalised against β -actin and fold change calculated using the delta-Ct method. Mean \pm SEM expressed and statistical significance was determine using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

6.2.7 Increased expression of ARE-controlled genes is correlated with poor RFS in ER+/HER2 – breast cancers

Bioinformatic analysis of mRNA microarray data from breast cancer patients can be utilised to provide information on how the expression specific genes correlates to overall survival (OS) and recurrence survival (RFS) (360). Analysis of microarray data from large patient datasets (EGA and TCGA) was used to examine the survival proportions of ER positive/ HER2 negative breast cancers, which were split into high and low expressers of NRF2, NQO1 and HO-1 (n=494). High expression of NRF2 (*NFE2L2*) correlates with poor RFS ($p=0.0084$) (Fig. 6.2.7A), as does the downstream targets of NRF2, NQO1 ($p=0.023$) (Fig. 6.2.7B) and HO-1 ($p=0.037$) (Fig. 6.2.7C).

Further analysis of Oncomine microarray data from the Curtis Breast dataset shows a significantly increased expression of NQO1 in invasive ductal and lobular breast carcinomas (n=90) compared to normal breast (n=144) ($p=0.027$) (Fig. 6.2.7D). Similarly in analysis of the TCGA data set and increased expression of HO-1 is observed in invasive breast carcinoma (n=76) compared to normal (n=61) ($p=1.67 \times 10^{-5}$) (Fig. 6.2.7E).

Fig. 6.2.7 Increased expression of ARE-controlled genes is correlated with poor RFS in ER+/HER2 – breast cancers

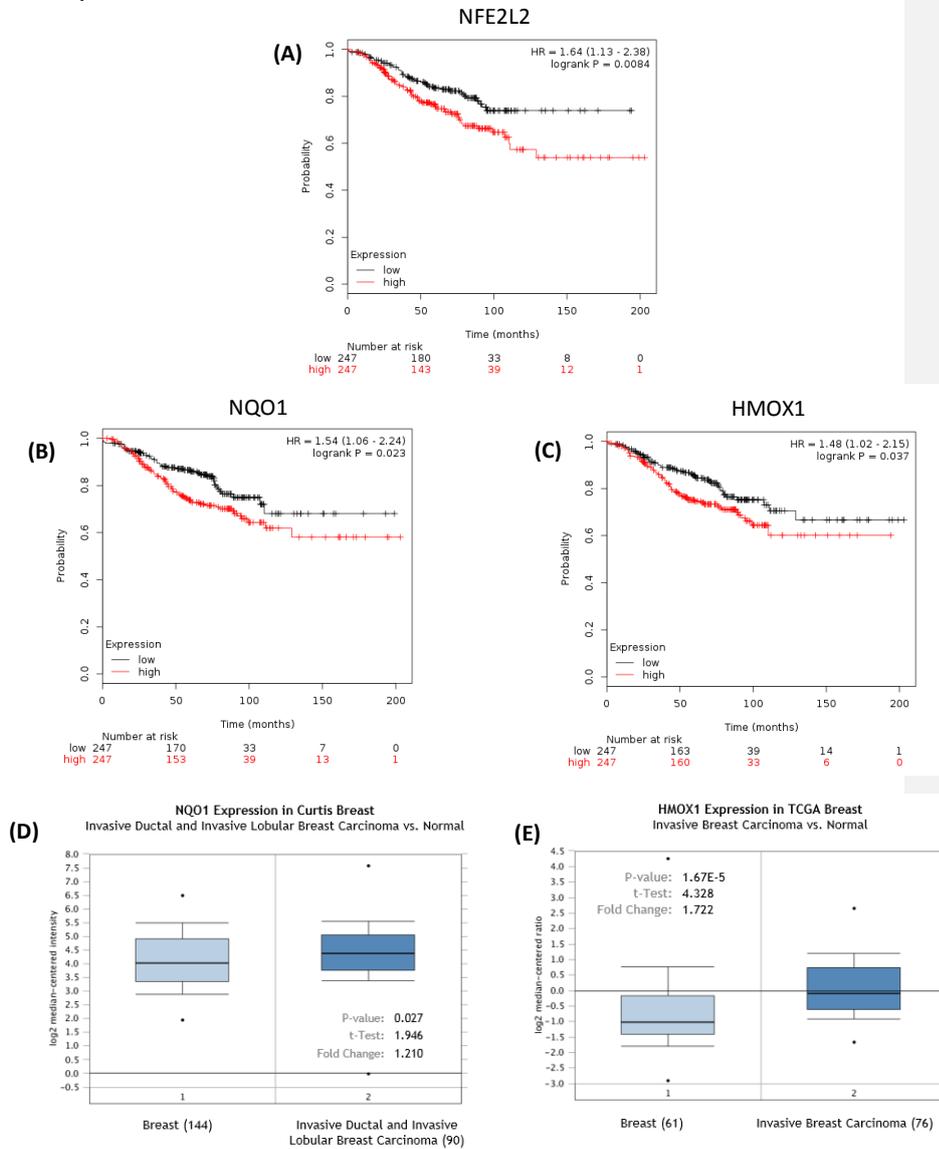


Fig. 6.2.7 Microarray data from the EGA and TCGA databases was examined in relation to the genes (A) *NFE2L2*, (B) *NQO1* and (C) *HMOX1* in ER-/HER2+ patients. Patients were stratified into high and low expressers and RFS of the two cohorts is compared using a Kaplan-Meier survival plot. (D) Oncomine analysis of (D) *NQO1* and (E) *HMOX1* expression in the Curtis and TCGA breast databases respectively was carried out.

6.2.8 Dexamethasone does not alter the effect of Doxorubicin on the cell cycle

Glucocorticoids have been shown to exert an effect on the cell cycle, including inducing progression delay through blockade of the G1 phase (417). In some cases this inhibition of progression through the cell cycle may play a role in glucocorticoid-mediated resistance to cytotoxic agents, reducing the efficacy of drugs that target rapidly dividing cells. Doxorubicin meanwhile, has been shown to arrest cells at both the G0/G1 checkpoint and at G2/M checkpoint (418).

Cell cycle analysis was performed on MCF-7 cells treated with Dox and Dex alone and in combination for 24hrs (Fig. 6.2.8). The proportion of cells in each phase of the cell cycle was determined using the intensity of DAPI staining of the nuclei. In logarithmically replicating untreated cells there was a normal distribution of cells in G0/G1, S phase and G2/M phase. Treatment with the cytotoxic agent Dox resulted the proportion of cells in shifted into a pro-apoptotic distribution, with the largest percentage of cells in sub-G1 phase. Dex had no significant effect on the distribution of cells compared to untreated cells, and Dex in combination with Dox did not alter the distribution compared to Dox alone.

Fig. 6.2.8 Dexamethasone does not alter the effect of Doxorubicin on the cell cycle

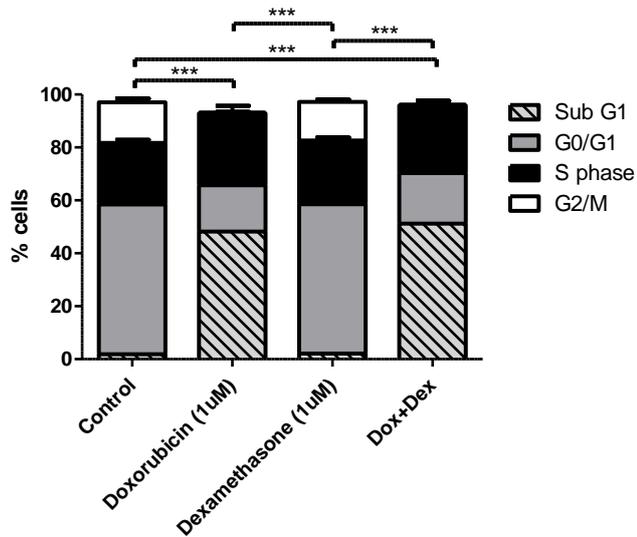


Figure 6.2.8 MCF-7 cells were exposed to doxorubicin (Dox) or dexamethasone (Dex) alone or in combination. Cell cycle distribution was determined using fluorescent intensity of DAPI staining. Mean \pm SEM expressed and statistical significance was determined using two way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

6.2.9 Potential pathway through which Dexamethasone may reduce the efficacy of Doxorubicin

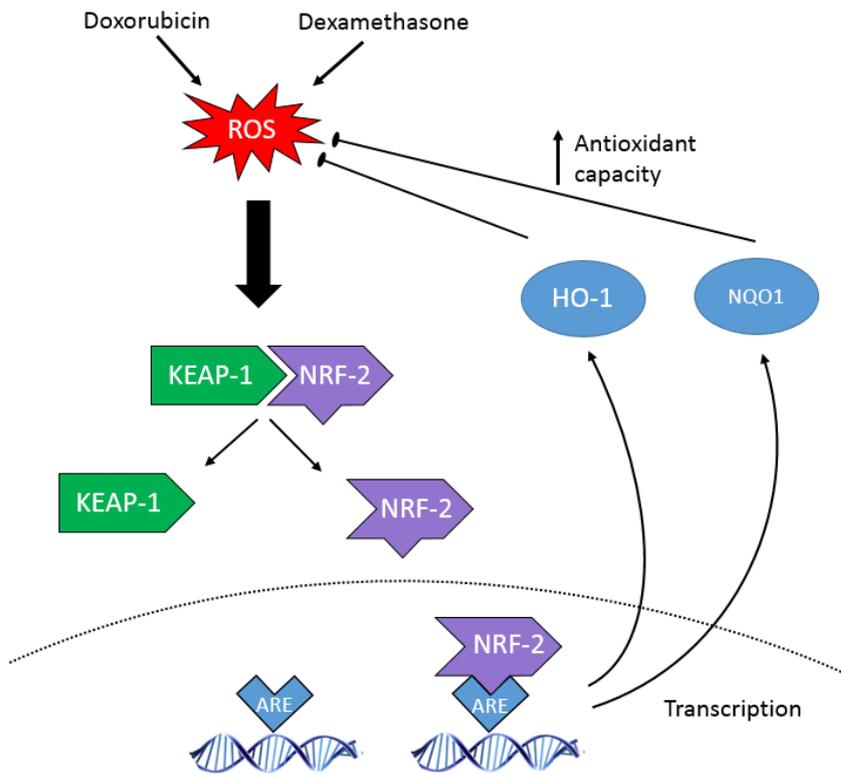


Fig. 6.2.9 Dexamethasone may reduce the efficacy of doxorubicin through the upregulation of ROS/RNS, which in turn promotes an increased anti-oxidant response resulting in a chemoprotective effect.

6.3 Discussion

This data shows that glucocorticoid treatment can significantly reduce the efficacy of Dox on breast cancer cell lines, and that this is mediated through the glucocorticoid receptor. Furthermore, Dox and Dex in combination increase levels of oxidative stress, and increase the expression of genes involved in the antioxidant response. These findings point to the use of glucocorticoids as co-medication for breast cancer as a potentially harmful desensitizer for chemotherapeutic treatments.

6.3.1 Glucocorticoids and chemosensitivity

The use of Dex in conjunction with chemotherapy is clinically relevant, as many treatment regimens for breast cancer include the use of synthetic glucocorticoids to manage the side effects of chemotherapy. Dex at concentrations of 1 μ M and 10 μ M has a significant cytoprotective effect on the viability of MCF-7 cells treated with doxorubicin, comparable to that of untreated cells. Furthermore Dex has a similar effect on the efficacy of paclitaxel, returning percentage of viable cells to a baseline level, although only at the higher concentration of 10 μ M. This finding is in keeping with previous work demonstrating that pre-incubation or incubation alongside glucocorticoids can have a significant effect on the efficacy of chemotherapeutics, desensitizing tumours which may result in faster growth. Several mechanisms have been proposed to explain this effect, including glucocorticoid-mediated regulation of anti-apoptotic genes (140), interference with cell cycle checkpoints (138), and increases in extracellular adhesion (409). In this study, the data indicates that Dex does not interfere with cell cycle progression or the ability of Dox to arrest cell cycle progression and hold cells in a sub-G1, early apoptotic state. However the work presented here does add a further dimension, indicating that the burden of oxidative stress is increased by the addition of dexamethasone, subsequently activating a stronger antioxidant response which may facilitate increased cell survival in Dox treated cells.

Increasing concentrations of Dex had no effect on the efficacy of the endocrine therapies, tamoxifen or fulvestrant. This is in keeping with current literature that shows co-treatment with glucocorticoids does not reduce the efficacy of endocrine therapies in breast cancer, and can in some cases enhance the response to endocrine therapy (419). Endocrine therapies work to block hormone receptors that stimulate proliferation and although there is evidence to suggest cross talk between GR and ER signalling, in this study Dex did not appear to promote a stimulatory effect either. However, since these cells were not cultured in the presence of oestrogen the true efficacy of anti-oestrogenic compounds could not be fully evaluated, and therefore the effect of Dex could also not be assessed.

With regard to resistance to therapies in breast cancer, there is data that demonstrates the NRF2 signalling pathway is persistently activated in tamoxifen resistant MCF-7 cells, and the enhanced antioxidant response is partially responsible for the acquisition of resistance (405). In these resistant cells the activation of the GR and its ability to downregulate NRF2 may actually restore sensitivity, although further work is needed to explore this potential mechanism.

6.3.2 Oxidative stress and the antioxidant response

In response to Dox and Dex alone there was an increase in the production of extracellular NO_2^- , however in combination they produced a statistically significant increase in both cell lines. Using multistep amperometry which provides greater sensitivity and specificity, intracellular levels of ROS/RNS could be measured to further establish the effect of Dox and Dex on the oxidative state of breast cancer cells. There was no significant difference in levels of NO_2^- between Dex treated and combination treated cells. Similarly, there was no significant difference in levels of H_2O_2 between Dox treated and combination treated cells, however taken together the overall levels of ROS/RNS in the concomitantly treated cells is greater than Dox or Dex alone. Increased ROS/RNS levels can promote DNA damage which can in turn promote tumourigenesis in breast cancer, as demonstrated previously (420). Here the increased burden of oxidative stress in cells that are already damaged by Dox treatment may mean further genomic instability and promote a shift to a pro-survival anti-oxidant phenotype.

This conclusion is supported by the finding that only combined treatment had a significant effect on the expression of the antioxidant response element- controlled genes NQO1 and HO-1. The ability to maintain redox homeostasis is crucial for cell survival, and these processes are often deregulated in cancer cells, with the balance tipped in favour of survival and proliferation. As such this data draws together the understanding that NRF2 and the antioxidant response plays a role in Dox resistance, and that glucocorticoid mediated generation of ROS/RNS can also stimulate an antioxidant response, linking these pathways in a way that has not been done so previously. This therefore highlights the role of a NRF2-driven response specifically in Dex induced cytoprotection of chemotherapy treated breast cancer cells. In these results, the downregulation of NRF2 but simultaneous upregulation of its downstream target genes in response to short term Dox and Dex indicates a possible feedback mechanism whereby NRF2 is activation results in silencing of its transcription. To fully assess the extent of NRF2 activation its dissociation from KEAP1 could be quantified using immunoprecipitation and immunoblotting. Interestingly, in MCF-7 cells continuously treated with Dox to induce resistance, NRF2 levels are seen to increase (404, 421). This suggests a potential shift in phenotype, with aberrant activation of NRF2 conferring growth advantage in the face of doxorubicin-induced cytotoxicity.

Bioinformatic analysis of patient sample databases also provided further insight into the role of the antioxidant response in breast cancer. In ER+/HER2- breast cancer patients treated systemically with chemotherapy a higher expression of NRF2 correlated with a decrease survival probability. One explanation for this may be that an increased expression of NRF2 increases capacity for oxidative stress and signals a greater antioxidant response – which may promote cell survival and confer resistance to chemotherapy, thus decreasing survival prospects. Analysis of the same patient datasets revealed that higher expression of NQO1 and HO-1 were also associated with poor recurrence free survival. An increase in expression of NQO1 and HO-1 was elevated in invasive carcinomas compared to breast, supporting the assertion that tumourigenic cells have an increased capacity for oxidative stress (239).

6.3.3 Future perspectives

This data suggests that current treatment strategies involving glucocorticoid co-treatment may need reevaluating to consider the benefits versus the potential risk of reducing the efficacy of chemotherapies. The benefits of glucocorticoids include protection of normal tissues and an increased quality of life for the duration of treatment, however given the protection glucocorticoids may confer to tumour cells, the efficacy of cytotoxic treatment may be compromised. Therefore the replacement of glucocorticoids with non-steroidal anti-emetics, which do not promote resistance is currently being treated as a potential avenue (141).

It is hoped that the capacity of glucocorticoids to interfere with chemotherapy will be further probed using an *in vivo* model of aggressive breast cancer. Co-treatment with Dox and Dex mimicking current treatment regimens and achievable plasma concentrations would be used to evaluate the efficacy of cytotoxic therapy when combined with exogenous glucocorticoids. This model could also be used to explore the role of endogenous glucocorticoids in the form of the endocrine stress response, which are already known to affect the efficacy of other cytotoxic agents such as paclitaxel (139). The model will further allow for exploration of the gene profiles in solid breast tumours which will give much greater detail regarding the changes in the genomic landscape which are known to occur in response to Dox treatment (422). More robust antioxidant activity assays would also be carried out in order to support the findings here, as well as investigation into protein activity and the generation of a Dox resistant line.

Chapter 7 – General discussion and conclusion

7.1 Aims of the thesis

This study set out to explore and identify mechanisms through which psychological stress can play a role in the tumourigenesis of breast cancer. By building upon the existing literature in the field which highlights the biological effects of stress hormones, the research presented here attempts to elucidate a signalling mechanism linking the action of stress hormones, to the generation of oxidative/nitrosative species and DNA damage. The data generated in this thesis also endeavours to consolidate and expand upon understanding of the roles stress hormones may play in breast cancer progression and response to treatment.

Exploring the link between stress and breast cancer is important, as both clinicians and patients often believe that psychosocial factors may impact the cancer progression. Contradictory evidence from studies has shown that psychological interventions may not improve breast cancer patient outcomes (423), but previous life stressors can affect risk and incidence (424). The majority of the literature regarding the effects of stress hormones in cell and animal models also points to the conclusion that chronic endocrine responses have a part to play in the formation and progression of cancer (71). The role of stress is therefore undoubtedly complex, and there is a disparity between mechanistic findings and the translation into meaningful results for patients. This gap in knowledge is the focus of the research presented here, which aims to use information gathered regarding the effects of stress hormones *in vitro*, and translate it into an *in vivo* mouse model. Furthermore, the research aims to study the effects of stress hormones in combination with current treatments, as well as models of drug resistance.

7.2 Conclusions

Findings from this study have resulted in the following conclusions:

- i) The stress hormones, glucocorticoids and catecholamines induce DNA damage through the generation of ROS/RNS in breast cancer cells. Specifically, cortisol induces production of RNS through a non-genomic interaction with iNOS.
- ii) Inhibition of NOS in an *in vivo* syngeneic model of breast cancer reduces the detrimental effects of psychological stress on tumour growth and may affect potential metastatic spread.
- iii) Glucocorticoids promote an increase in oxidative stress and DNA damage, as well as decrease efficacy of DNA repair in endocrine resistant cells.
- iv) Exogenous glucocorticoids in combination with chemotherapeutics can potentially reduce the cytotoxic capabilities of chemotherapy agents, through the promotion of oxidative stress and upregulation of the antioxidant response.

This study can be considered successful in answering the research questions posed, regarding the effects of stress on oxidative stress, tumourigenesis and breast cancer therapeutics. It has been demonstrated that the stress hormones catecholamines and glucocorticoids can generate production of intracellular ROS/RNS, as detected by a novel application of electrochemical sensing. The actions of both NE and cortisol were mediated through receptor signalling, and the pattern of the generation of ROS/RNS was the same across breast cancer cell lines. Furthermore, a mechanism through which glucocorticoids can increase RNS has been established, drawing together a novel signalling pathway activated by GR, and resulting in an increase in the generation of NO by iNOS. This represents an important finding, as oxidative and nitrosative stress are crucial mediators of a number of processes related to cancer initiation and progression (261, 425).

The effects of stress on tumour progression were examined using an *in vivo* model of metastatic breast cancer. Psychological stress was induced using an established method of repetitive restraint, and tumour growth, metastasis and aggressiveness were measured. Stress alone had no effect on primary tumour growth or survival, however there was an increase in the angiogenic marker CD31, as well as a wider spread of metastasis in stressed mice. The use of a NOS inhibitor significantly reduced primary tumour growth in stressed mice, as well as reducing angiogenesis and metastasis, but did not reduce growth or spread in non-stressed mice. These results indicate that NO signalling may contribute to stress-induced changes in the tumour, and could be a driving factor in stress-mediated spread of metastases.

Drug resistance poses a major threat in the treatment of breast cancer, and previous studies have linked resistance to a deregulation of the DNA damage response pathways (346). The effect of stress hormone signalling on DNA damage and repair was further explored in an acquired endocrine resistance model. In a SERD resistant breast cancer cell line, expression of the GR was found to be constitutively expressed at a higher levels compared to the parental cell line, and produced a more pronounced response to cortisol stimulation. Exposure to cortisol produced an increase in oxidative stress and DNA damage, as well as impairing the rates of DNA repair. Proteins involved in the detection and repair of DNA were also deregulated compared to the parental cell line in response to cortisol. Taken together, this data suggests the development of resistance promotes a change in phenotype (ER-/GR+) that is more responsive to the actions of glucocorticoids on DNA damage and repair.

For triple negative cancers, or cancers in which endocrine therapies have failed, drug resistance severely limits the number of treatment options from an already small pool. In this study, the effects of glucocorticoids were examined in relation to chemotherapy efficacy. In line with previous studies the synthetic glucocorticoid dexamethasone, which is regularly prescribed alongside conventional chemotherapies, reduced the efficacy of doxorubicin and paclitaxel in breast cancer cells. Combined treatment with doxorubicin and dexamethasone promoted an increase in oxidative/nitrosative stress, and a subsequent increase in elements of the antioxidant response. These results suggest that chemoresistance, induced by exogenous glucocorticoids, may be a result of increased antioxidant capacity, driven by an increase in ROS/RNS. Furthermore, this data also implies a possible role for glucocorticoids generated through the stress response in the development of doxorubicin resistance.

Overall the data presented provides valuable insight into a range of cellular mechanisms influenced by stress hormone signalling. This data, and the body of data that precedes it, has begun to characterise the effects of psychological stress through stress hormone exposure, and makes a meaningful contribution to the epidemiological and empirical data in the literature. However, the mechanisms studied here represent a fraction of the full effects of stress hormones, which stand at the centre of a complex network of physiological processes. To understand the real life impact of psychological stress may have on cancer progression, and to translate these findings in to practical strategies to improve the prospects of cancer patients, further extensive work needs to be undertaken in sophisticated breast cancer models, and in the clinic.

7.3 Experimental limitations

7.3.1 Limitations of cell culture models

Cell lines are widely used to model cancer and have many advantages, such as the ease with which they can be handled, the large quantities that can be produced and the many publications characterising their phenotypes. However, the use of cell lines in breast cancer research has come under scrutiny. Cell lines are prone to genotypic and phenotypic drift due to continuous culture, which can result in selection for rapidly growing subpopulations. This was highlighted in a study identifying biological variation between MCF-7 cells from different laboratories (426). The problem of cross-contamination of cells lines resulting in ‘false’ cell lines has also been raised in several studies, with HeLa cells the most prolific cross- contaminants in a study of 252 banked cell lines (427). To overcome this we employ several standards of operations in our laboratories, ensuring one cell line is handled at a time, and when cell lines are purchased they are split early with one handler at a time.

The relevance of cell lines to the study of human breast cancer has further been called into question, as studies have sought to explore the differences in gene expression between cell lines and primary tumours. A panel of 51 breast cancer cell lines were compared to primary breast tumours, and while overall the cell lines tended to mirror the genetic aberrations seen in primary tumours, there were important differences observed between lines and primary tumours of specific subtypes. This indicates that single cell lines considered luminal A for example, may not be representative of the subtype of primary breast tumours (428). One explanation for this may be the fact that many of the most commonly used breast cancer cell lines, including MCF-7 and MDA-MB-231, are not derived from primary breast tumours but from pleural effusions of tumour metastases. Therefore these lines often represent the most malignant variants and may skew results towards overrepresentation of late stage disease (429).

In relation to the work presented here, the use of monolayer cell culture methods to study oxidative/nitrosative stress presents specific limitations. Oxidative balance is strongly influenced by hypoxia within the tumour microenvironment, with reoxygenation of hypoxic tumours causing increased oxygen radical production. Hypoxia and oxidative stress also act cooperatively to induce pro-angiogenic signalling through VEGF (430). The way in which NO functions within a tumour is further affected by hypoxia, as the metabolism of NO is much slower under low oxygen conditions, producing increases in local concentrations of NO (177). Cells cultured under normoxic conditions in a 2D monolayer are not exposed to the same fluctuations in oxygenation as seen in solid tumours, and as such the response to the induction of oxidative/nitrosative stress may differ. A way in which the effects of hypoxia on ROS/RNS could be studied is through the use of 3D culture systems. Breast cancer cells grown in spheroids – either in suspension or

with the aid of a matrix – are able to more accurately mimic the complexity of the tumour microenvironment. Spheroids can contain populations of cells in varying stages, including proliferative on the outer layers, and quiescent, apoptotic and hypoxic in the centre (431). This cellular heterogeneity more closely resembles the processes *in vivo*, and could prove advantageous in establishing how oxidative/nitrosative stress induced by stress hormones may affect tumourigenesis.

To further combat some of the problems faced when using cell lines, primary cells can be derived from tumours and established in culture. This allows for more a more confident classification of tumour subtype, as often detailed pathology is also available. However, the growth of explant cultures can be limited to short periods of time, as the fibroblast cells quickly outgrow the malignant epithelial cells. Therefore the separation of defined cell populations is necessary, with several methods such as enzymatic stromal degradation and sedimentation developed, to permit the expansion of tumour epithelial cells (429, 432). Successful protocols have also been established to culture slices of breast tissue in order to maintain the tissue architecture and more closely examine the cell-cell interactions without disruption (433). The use of human primary tissue culture was outside the scope of the research presented here, however further work in this laboratory is ongoing, with the aim of developing an *ex vivo* mouse mammary tumour model and patient-derived xenografts to explore the role of stress and oxidative stress.

7.3.2 Limitations of DNA damage and repair assays

In this study, DNA damage was assessed using immunofluorescent quantification of the DNA damage markers, phosphorylated γ -H2AX and RAD51. Analysis of the formation of phospho- γ -H2AX foci is considered a highly sensitive measure of the induction of DNA damage repair cascades, providing a useful and low-cost tool to assess DNA damage (434). However, some may argue that the assay is an indirect, rather than direct assessment of DSB's, as phospho- γ -H2AX foci may form at lesions other than DSB's (191). For example, the formation of foci can be observed in cells progressing through the certain phases of the cell cycle. To negate this problem, markers of specific stages of the cell cycle - such as Centromere protein F (CENPF) which identifies cells in G2 - can be used to exclude cells from the analysis (435). Another potential use for the γ -H2AX assay is the measure of DNA repair. This is achieved by inducing DNA damage and then observing the rates of disappearance of the foci. However this method can be considered less reliable than using the assay purely to quantify damage, as it has been shown that residual foci may exist for longer than the original DSB (436).

Whilst the γ -H2AX assay is useful in identifying DSB's, analysis of RAD51 foci formation can provide more information regarding the specific type of DNA repair induced. RAD51 foci formation signals the induction of homologous recombination, as well as the presence of

functional BRCA2 responses (193). However, the use of RAD51 as a direct measure of DNA repair should also be approached with a degree of caution, as RAD51 foci formation has been observed in the absence of DNA damage in RAD51 overexpressing cancer cells (437).

The identification of foci as a measure of DNA damage also faces some technical limitations. In this study, digital microscope images were taken and cells were manually scored as either positive or negative depending on the number of foci. Although the images were scored blind and negative controls were included, there is still the potential for some subjective bias to be introduced. This could be overcome by employing the use of automated image analysis software developed specifically for the identification of foci, however these software also require high quality images and can be sensitive to staining variation between batches of biological repeats (438). Furthermore, statistical tests in our laboratory and the work of others have shown no differences in data produced by independent viewer analysis versus automated software (439).

The alkaline comet assay was used in a similar vein to the immunofluorescence assays, both as a direct measure of DNA damage and an indirect measure of repair. The comet assay utilises the principals of electrophoresis to visualise breakages in DNA of single cells, making it a highly sensitive method (440, 441). In this case the alkaline version of the comet assay was used, which allows for identification of not only DSB's, but SSB's, cross-links and alkali-labile sites. As such, the levels of DNA damage identified represent total damage within the cell, providing a useful starting point for further exploration the induction of DNA damage and the activation of repair mechanisms.

The comet assay can also serve as an indirect measure of DNA repair, and whilst widely used, is prone to the same limitations as any indirect method. Primarily this is the assumption that a decrease in DNA damage is equal to an increase in repair. However this may not always be the case, as the rates of repair for different types of DNA damage are variable. A pertinent example is the repair of damage induced by ROS, which can take as little as 3 minutes or up to 4hrs (195, 442). Interpretation of the comet assay is also confounded by the subjective scoring of 'comet' tail length. The tails are scored from 0-4 depending on tail length, a well-established practice in published literature (440). Software has been developed to automate the scoring process, however - as with the immunofluorescence assays - this requires high quality images and high degree of replicability between experiments in order to maintain the same settings.

Whilst each of the DNA damage assays discussed above has their own limitations, taken together the use of these complementary techniques provide compelling evidence for the induction of DNA damage in response to stress hormones. Furthermore, the assays employed to substantiate the findings are currently the only assays commonly used to measure DNA damage. With regards

to DNA repair, data gathered from the comet assay indicates stress hormones adversely affect the rates of repair. In order to further establish the mechanisms through which they do so, protein assays were used to examine the activity of proteins involved in the DNA damage response. Western blotting techniques have been a mainstay of molecular biology, and in this study were utilised effectively to demonstrate the expression, as well as the phosphorylation of proteins in DNA damage response cascades. However, the quantification of expression and phosphorylation is limited by the sensitivity and specificity of antibodies, with high molecular weight protein such as ATM and ATR difficult to detect.

Whilst western blotting allows the evaluation of physically expressed protein levels and protein modification, the expression of genes is often studied using qPCR, which allows for much greater sensitivity. Relative mRNA expression correlates to changes in transcription and translation of specific genes between samples. However, qPCR cannot be used as a direct measure of protein expression, as it does not take into account any post-translational modifications affecting expression/activity. Moreover increases in gene expression do not always translate to increases in protein expression, with the half-life of mRNA often varying widely compared to protein, and the translation of proteins controlled by post-transcriptional events (443).

In order to quantify expression of certain genes, mRNA is first extracted, and converted to single strand cDNA. This template is then amplified in the presence of gene-specific primers. Double strand DNA is recognised by fluorescent dyes, and fluorescent intensity can be used as a measure of amplified product, indicating the presence of the target mRNA. The reliability of qPCR is dependent on several factors, the first of which is the quality of the template RNA. RNA is prone to rapid degradation and once it has degraded cannot be reverse transcribed, leading to differences which could be mistaken for experimental results. In this study, the quality of the RNA was assessed using spectrophotometric analysis of absorbance to determine ratios for purity and concentration. However, this is not the current gold standard, and it is recommended that RNA quality be measured using a bioanalyser to inspect the 28S and 18S ribosomal RNA bands (205). Reliability can also be compromised by the variable efficiency of reactions. This was minimised, and the reproducibility of reactions was ensured, by consistently using the same assay kits throughout the RNA extraction, reverse transcription and amplification steps. During PCR, specific detection of templates can be achieved using custom designed fluorescent probes (the TaqMan system), however this method is associated with significantly higher costs, and so in this case non-specific detection was employed. Since this type of detection recognises all dsDNA, a signal can be generated from indiscriminate binding, resulting in false positives. To prevent this, melt curves were run with each cycle to ensure there was no nonspecific signal caused by primer-dimer formation, and no template controls/ RNA only controls were also included.

7.3.3 Limitations of electroanalytical techniques in cancer biology

Classically, analytical techniques such as the Griess assay are used to measure the release of NO indirectly through the detection of NO_2^- in solutions. This assay does not have a detection limit suitable for all biological applications, however, has an important supporting role when used in combination with other techniques (203, 312). In this study the Griess assay was used to measure extracellular release of NO_2^- into cell culture media, and these results were corroborated by electrochemical sensing of ROS/RNS in the cell culture media, which show an increase in RNS in response to cortisol after 30mins (Appendix 4 Fig. 1.2).

Fluorescent probes have also been used as more specific measure of ROS/RNS. Many of these probes exploit oxidation reactions to produce a fluorescent product, however they can also be oxidised by a number of other cellular processes, and as such are not a direct measure of activity (444). Probes are also not highly secretive and can therefore potentially overestimate physiological levels or trends. Currently there are a small number of highly sensitive fluorescent probe based assays available (445, 446), and whilst probes can be useful to explore spatial resolution, they cannot be used to track multiple ROS/RNS simultaneously. As such, for this study the use of fluorescent probes was not appropriate in order to understand the changing pattern of ROS/RNS in response to stress hormones.

More recently, advances in electroanalytical techniques have provided the ability to take selective and simultaneous measurements of various bioactive signalling molecules in biological systems. The basis of electroanalytical measurements relies on the principal that molecules that are easily oxidized can be measured using voltage-dependent detection. Previously very low concentrations of ROS/RNS were virtually undetectable, however with the advent of electrochemical sensors, concentration of ROS/RNS in the femtomolar range can be measured reliably and specifically by applying defined voltages (447, 448). The primary advantage of using electrochemical sensors is the ability to measure dynamic species, such as NO and ONOO^- , as well as cumulative species such as NO_2^- , using an inexpensive and reproducible method. The fabrication of such an electrode that can simultaneously measure H_2O_2 , ONOO^- , NO and NO_2^- in cell lysates has been described previously (199).

In this study, the principals and techniques developed for use in the study of neurotransmitter release in the GI and CNS, were applied to the study of ROS/RNS in cancer biology (447). As such, the limitations of the applications of electrochemical sensing remain the same. The main issue that arises when using electrochemical sensors is the selectivity of the measurements, as molecules with a similar structure - such the neurotransmitters serotonin and dopamine for example - are oxidized at similar potentials (447). With regards to ROS/RNS, H_2O_2 can be

measured very selectively, as its peak oxidation requires a potential to be applied that does not oxidise other species of interest in this study to the same extent. Measurement of NO_2^- is considered the least selective, as the potential that is required also oxidises other species, resulting in an additive signal. However, it is possible to calculate the proportion of the signal made up by each species, as they have been defined through calibration. The selectivity of the measurements of NO_2^- was further corroborated by pharmacological inhibition, which served to demonstrate that specific inhibition of NOS only mediated effects on levels of NO_2^- , and had no effect on ROS (H_2O_2) (Appendix 4 Fig. 1.3A-B). Electrodes can also be prone to fouling, a process whereby an accumulation of proteins is deposited on the surface of the electrode. This leads to the decay of current across replicative measurements. However the electrodes used in this study do not exhibit a decline in the response with an increasing number of measurements using a single sensor. This can be seen to be the case as responses were generated for up to 90mins, which would not be observable or would decline if fouling was present, although it is not certain if fouling would occur over longer measurements.

7.3.4 Limitations of studying resistance

The use of anti-oestrogen resistant cell lines has contributed significantly to the understanding of resistance in breast cancer patients, with several laboratories over the years generating models of each stage of the evolution of resistance. In this study we collaborated with Dr. Gee at the University of Cardiff to explore the effects of stress on endocrine resistant breast cancer, using a model of long term acquired Faslodex resistance, previously generated through continuous exposure (2 years) (341). Since ER-positive patients are routinely treated with 5 years of adjuvant endocrine therapy, this method for developing resistance mimics the *in vivo* processes closely, as the cells are forced to adapt to the loss of ER-driven growth signalling over a prolonged period (449). However, as with the use of any cell line model of breast cancer, there are limiting factors relating to the relevance of the breast cancer subtype chosen. The parental cell line MCF-7 is the most commonly used cell line in anti-oestrogen resistance studies, and whilst it has provided an in depth understanding on the mechanisms relating to ER-stimulated growth and ER loss, it cannot be considered representative of all ER-positive tumours (450).

The ability of glucocorticoids to induce chemoresistance was also studied in MCF-7 cells, as well as a TNBC line. The LD_{50} of each chemotherapy drug was calculated using dose response curves, and the concentration of doxorubicin used fell within the reported range of plasma concentrations levels (399). However, further pharmacokinetics studies are needed to fully establish the clinical relevance of the concentrations of dexamethasone used. Furthermore, a pulsed drug treatment approach mimicking cycles of chemotherapy and steroid treatment may have more clinical relevance than short term or continuous exposure. The generation of a

doxorubicin resistant cell line may have also provided more information regarding the drivers of resistance, as well the effect dexamethasone on the evolution of resistance.

To examine dexamethasone-induced resistance to chemo and endocrine therapies, the MTT assay was used. The MTT assay measures the ability of cells to reduce a dye, and as such is a measure of functioning mitochondria and metabolic activity. This provides reliable, replicable information on cell populations, however it is not a direct measure of cytotoxicity (211). To corroborate the findings presented here, a direct measure of apoptosis such as the LDH assay - which measures the release of lactate dehydrogenase after the induction of apoptosis or necrosis - would be of value. Colony forming assays which measure the proliferative capacity could also be implemented as an indicator of resistance (451).

7.3.5 Limitations of *in vivo* models

Translation of *in vitro* findings into *in vivo* models is a critical step in the discovery of new mechanisms and therapies in breast cancer. The range of different mouse models now available provides rapid physiologically relevant information regarding the initiation, progression and metastasis of tumours, as well as providing a platform to explore and manipulate genetic components.

Human or mouse tumour cells can be implanted subcutaneously or into the mammary fat pad (ectopic or orthotopic) to study primary tumour growth, or injected into the tail vein to study metastatic disease, and these methods have been used extensively for many years to study cancer research (213). Human-derived breast cancer cell lines can be implanted in immunocompromised mice as a xenograft, a commonly used method to study breast cancer genetics. However, they are limited by reduced heterogeneity, and cannot be used to study the effect of the immune system on tumour development. Syngeneic models using strain-matched mouse cells represent a more global model of breast cancer, and are considered superior in the study of tumour-host microenvironment interactions (452). This is particularly important when developing treatments that may interact with the host's immune system (214). Furthermore, syngeneic models can be metastatic, with several models in existence which have been selected for organ-specific metastasis, allowing exploration and identification of factors that lead to specific metastatic routes (213). The high success rate of transplantation and rapid growth of syngeneic cell line-derived models also results in lower housing costs and higher experimental throughput. However, with these benefits comes the compromise that only advanced stages of the disease can be successfully modelled.

To address the early events that lead to the initiation of breast tumours, several classes of genetically engineered mice (GEM) have been developed. In these models, spontaneous tumour

formation arises in an otherwise normal mammary microenvironment, conventionally as a result of transgenic induction of an oncogene. Yet whilst GEM have contributed extensively to breast cancer research, the use of GEM to replicate human cancers has been called into question, as uncertainty surrounds whether the specific oncogenes targeted reflect those observed in human tissue. Furthermore, multiple reports have established that overall, GEM tumours do not share the same histological features as human tumours (453).

By far the most expensive method, but perhaps offering the highest risk to reward ratio, is the use of patient-derived xenograft (PDX), which uses clinical isolates implanted into immunocompromised mice. Tumours grafts from patients implanted into mice often retain histological features and clinical markers, therefore offering the most relevant model in which to study specific subtypes of breast cancer (454). These models have also been utilised successfully to determine the responsiveness of patients to treatments, as well as to examine the processes leading to acquired resistance (455). However, transplantation efficiency remains a significant problem, as does the limited availability of samples.

In the work presented here, the use of GEM or PDX models was beyond the scope of the project. Moreover, as psychological stress is known to mediate effects on the immune system, an immunocompetent syngeneic model was chosen to best represent breast cancer tumourigenesis in this instance. However, as with all animal models, there are some limitations of the experimental *in vivo* work. In relation to the induction of psychological stress, the effects of the restraint stress were not quantified using measures of plasma corticosterone levels. This was a deliberate decision in order to reduce the further stress caused by a daily blood draw. Instead the weight of each mouse was monitored, as it is well established that stress promotes weight loss in breast cancer mouse models (263), an effect that was also observed in this study. It is possible that some of effects of the restraint stress may have been masked, as the no stress control groups were exposed to daily IP injections, which could be considered a stressor. Although experiments in our laboratory have demonstrated that IP and tail vein injections induced cortisol levels approximately 10-fold lower than restraint stress (unpublished data). Since corticosterone levels can peak and then plateau, exposure of the control group to a stressor may have partially confounded the data.

It is also worth considering that in previous studies where induction of stress has been shown to elicit an effect on breast cancer growth, less aggressive models have been used. As such, the highly aggressive nature of the cell line used in this study may also mask some of the more subtle effects of stress. Habituation to chronic stress could also play a role, as it was observed that after day 4-6, mice were more inclined to enter restraint stress apparatus willingly. The effects of chronic exposure to restraint stress on circulating levels of stress hormones are have been well

documented, however there remains a degree of controversy. In rats studies have shown conflicting results, with some finding that there is a normalisation of serum corticosterone levels after chronic stress (456), whilst others show that elevation of corticosterone levels is maintained by chronic stress (457).

There is also evidence to suggest that psychological stress may affect tumour initiation, as well as progression (458), a concept which was not explored in this work. In order to do so, a transgenic model of spontaneously arising mammary tumours would be necessary. The MMTV-PyMT mouse model forms early onset tumours which metastasise to the lung, and is widely used in cancer initiation studies. However, this model would not be appropriate in a study of stress, as the promoter (mouse mammary tumour virus (MMTV)) also contains a glucocorticoid response element which could interfere with true glucocorticoid receptor signalling (459).

7.4 Future perspectives

The work presented in this thesis describes a novel mechanism through which stress hormones can affect tumourigenesis, however it also raises several more questions. The effect of stress hormones varies according to length of exposure and stage of cancer, amongst many other factors. As such, a one size fits all recommendation for stress intervention in breast cancer may not prove to be beneficial for a number of patients. Instead the added pressure of trying to remain stress-free may be counterintuitive, and end up contributing to further psychological distress.

Instead, current shifts in the dynamics of breast cancer research are leaning heavily towards the development of personalised medicine. The use of primary cell culture may represent an avenue for the development of patient-specific treatment, eliminating the need for animal models as well as established cell lines, both of which have their distinct pitfalls. Aspirate from needle biopsies have been shown to contain enough viable biological material to be expanded (460), and recently several protocols have been developed to assess individual drug resistance from cultured primary samples. Tumour specimens, in the form of aspirate or solid biopsy, can be cultured in the presence of chemotherapy agents and assessed for proliferation to provide information about drug resistance (461, 462). In this way therapy regimes with tumour-specific drugs can be tailored to the patient to improve outcome. This approach also has the potential to be able to correlate stressors identified by patients, with stress hormone receptor profiles and polymorphisms in individual tumours, with the aim of promoting personalised stress interventions.

The translation of findings regarding psychological stress and cancer into meaningful results for patients has resulted in several studies on effect of stress hormone receptor inhibition and cancer progression. Retrospective analysis of survival rates of patients with breast cancer, who were prescribed beta-blockers before a cancer diagnosis, revealed significantly lower rates of breast-cancer-specific mortality in women who used propranolol (463). These results were corroborated in another study which determined that beta-blocker use significantly reduced distant metastasis and cancer recurrence in breast cancer patients (464). However, a more recent meta-analysis has shown that beta-blockers had no effect on rates of overall death, cancer-specific death or recurrence in breast cancer (465), although this analysis did not take into account breast cancer subtype or specific beta-blocker. The conflicting opinions on either side of the debate therefore underline the need for prospective assessment of the effect of beta-blockers. Currently clinical trials are ongoing, combining chemotherapy with beta-blockers in breast, ovarian, and lung cancers (466). A clinical trial is also underway to evaluate the efficacy of a novel GR antagonist in combination with paclitaxel in solid tumours (467).

As well as directly targeting the activity of stress hormones, changes in the genomic landscapes of cancer brought about through chronic stress exposure also represent potentially targetable processes. Recent advances in functional genomics have allowed a greater understanding of the evolutionary dynamics of cancer progression and metastasis, and this information is now being developed with a view to target treatments at mutational drivers (468, 469). Phylogenetic modelling of cancer using evolution tree models can identify trunk mutations, which are present in tumour cells and metastatic cells originating from early progenitors. Branching mutations can then be identified, which give rise to subclonal populations of genetically distinct cells present within regions of the tumour or at distant metastatic sites. An understanding of these different cell lineages can provide information about the timeline of mutagenic events, as well as the pattern and spread of cells. It also allows ancestral mutations to be targeted in tumours with a high level of heterogeneity, which tend to be more likely to resist treatment (470).

Personalised genomics also has the potential to predict resistance to treatment, through the analysis of circulating tumour DNA (ctDNA) in blood plasma (471). Comprehensive sequencing of ctDNA allows for the detection of somatic mutations that can appear concurrently with the acquisition of resistance. Novel approaches using personalised PCR probes have shown potential in predicting disease relapse, as well as the detection of micrometastases that are not yet clinically presentable (471). In a similar vein, detection of micro RNA's (miRNA's) present in the plasma has also proven a viable approach for the prediction of disease progression. A panel of 18 miRNA's upregulated in breast cancers has recently been described, which may provide a useful diagnostic tool in the specific detection of early breast cancers (472). Aberrant miRNA's have also been implicated in endocrine resistance through targeting of resistance-associated genes and downregulation of the ER (471). Moreover, some studies have shown miRNA's are also involved in the regulation of the DNA damage response (473, 474), raising the possibility that miRNA profiling could identify cancers that are not only resistant to treatment, but have altered DNA damage responses as a result. With this information there is the potential to target specific elements of DNA repair machinery, and design personalised treatment interventions for cancers that have not responded or relapsed after conventional therapies.

7.5 Novel contribution

Despite the need for ongoing study, the data presented demonstrates a compelling argument for the involvement of stress hormone-mediated signalling in tumourigenic processes. The work carried out in this thesis can therefore be considered to present the following novel contributions:

- i) Elucidation of a mechanism for glucocorticoid induced DNA damage through and iNOS-mediated pathway.
- ii) Demonstration of the effects of stress hormone signalling and inhibition of NOS on metastatic potential in breast cancer
- iii) Evidence for the detrimental effect of glucocorticoids in endocrine resistance breast cancer cells, and a glucocorticoid-mediated reduction in the efficacy of chemotherapeutics.

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Appendices

Appendix 1 – Buffers and solutions

Antigen retrieval buffer (1x): Tris 10mM, EDTA 1mM, TWEEN20 0.05% dissolved in distilled water, pH9.

Blocking buffers: 5-10% BSA in TBST or 5-10% skimmed milk powder (Marvel).

Comet lysis buffer (1): 10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA, 1% Triton, 1% DMSO

Comet electrophoresis buffer (1x): 50mM NaOH, 1mM EDTA, 1% DMSO

Paraformaldehyde fixative 3% (1x): 3% paraformaldehyde (Sigma Aldrich, UK) 2% sucrose dissolved in PBS, pH7.2.

RIPA buffer: 150mM NaCl, 1% NP40/Igepal, 0.5% NaDoC, 0.1% SDS, 50mM protease inhibitor (Sigma Aldrich, UK).

Electrophoresis running buffer (1x): 0.025M Tris, 0.192M glycine and 0.1% SDS dissolved in distilled water.

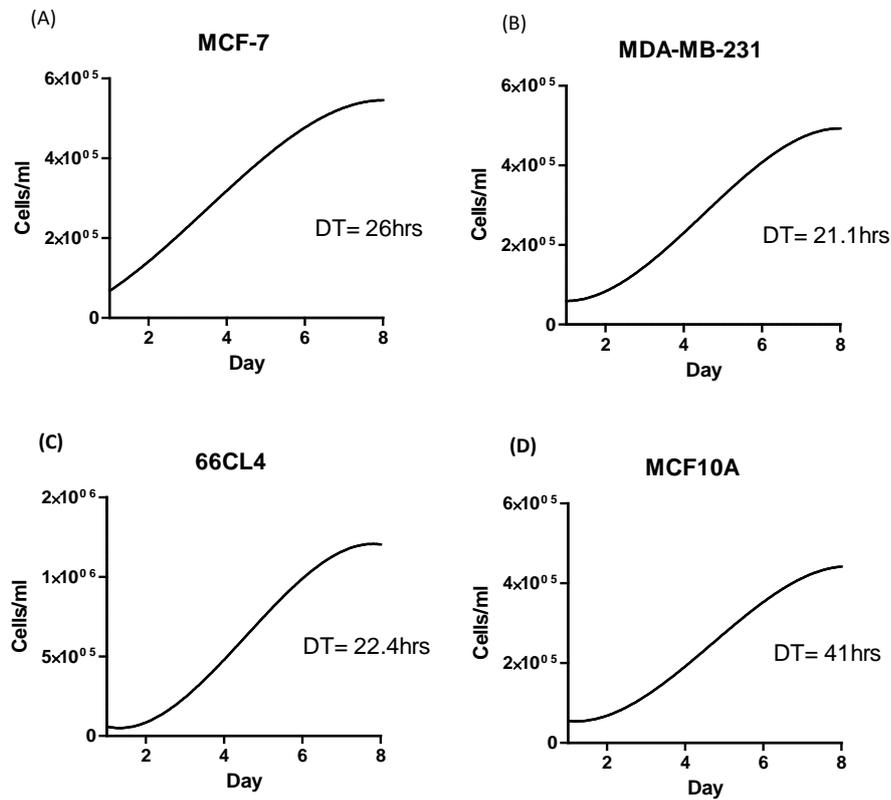
Stripping buffer (1x): 100mM β -mercaptoethanol, 2% SDS dissolved in 62.5mM Tris HCl pH 6.7.

TBST (1x): 0.1% tween 20: 20mM Tris (pH 7.5), 150mM NaCl, 0.1% Tween.

Transfer buffer (1x): 0.025M Tris, 0.192M glycine, 20% methanol made up in distilled water.

Appendix 2 – Cell line growth curves

Fig 1.1 Growth curves



Appendix 2 Fig. 1.1 Growth curves for (A) MCF-7 (B) MDA-MB-231 (C) 66CL4 and (D) MCF10A cell lines. Cells were plated at a density of 5×10^4 cells per well of a 6 well plate and counted at 2 day intervals for 8 days. Experiments repeated in triplicate and data expressed as non-linear regression. Doubling time (DT) was calculated using the formula:

$$\text{Doubling Time} = \frac{\text{duration} \times \log(2)}{\log(\text{Final count}) - \log(\text{Initial count})}$$

Commented [RF10]: Comment 23

Fig. 1.1 Calibration curves

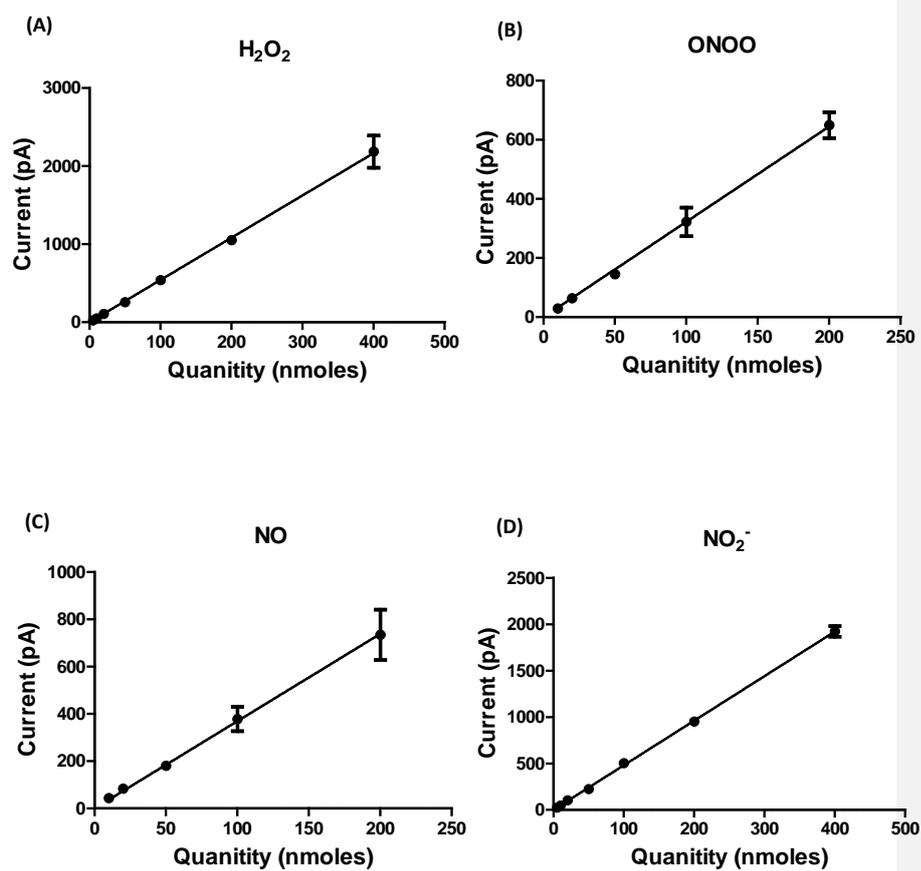
Appendix 3 Fig. 1.1 Calibration curves for (A) H_2O_2 (B) ONOO^- (C) NO and (D) NO_2^- .

Fig. 1.2 worked example of conversion from current to nmoles

(A) Conversion to M =
$$\frac{(\text{Current mA})}{\left(\begin{array}{c} \text{species specific} \\ \text{value} \\ \text{derived from} \\ \text{calibration curves} \end{array} \right)} \times (1 \times 10^{-6})$$

Conversion to moles = $M \times (\text{vol in L})$

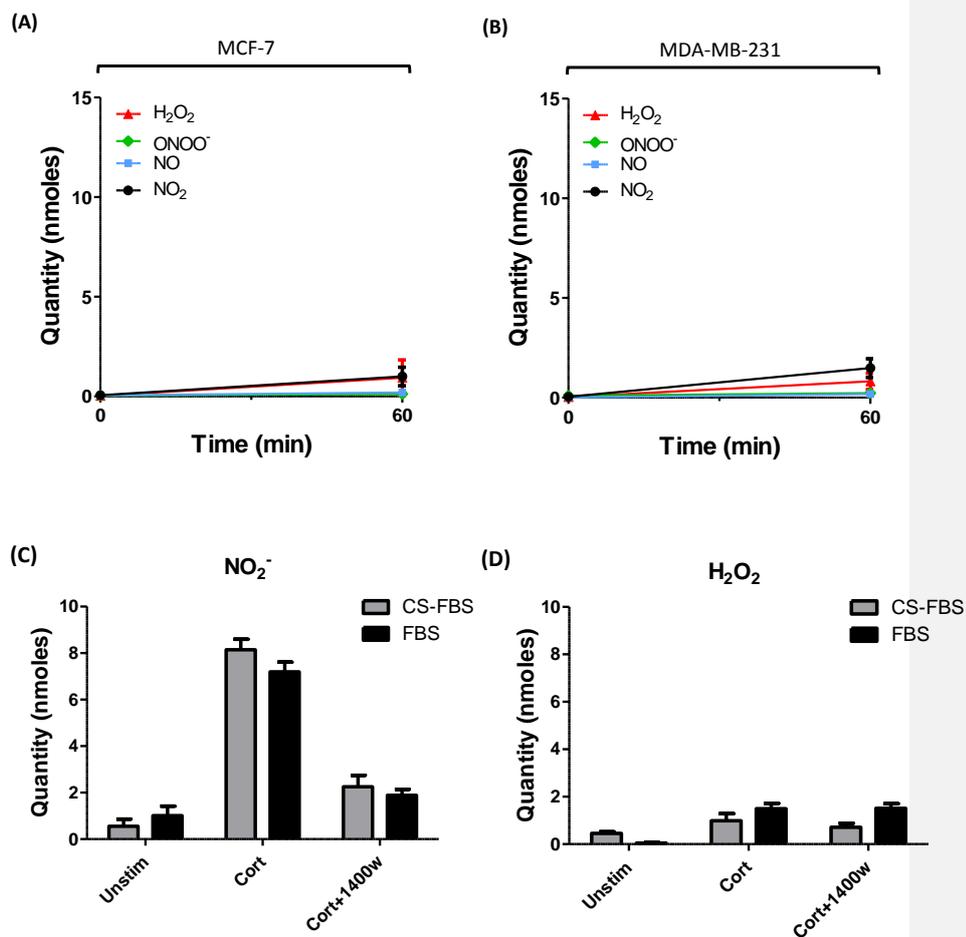
Conversion to nmoles = $\text{moles} \times (1 \times 10^9)$

(B)

Species	Calibration coefficient
H ₂ O ₂	50.9
ONOO ⁻	28.77
NO	36.95
NO ₂ ⁻	48.39

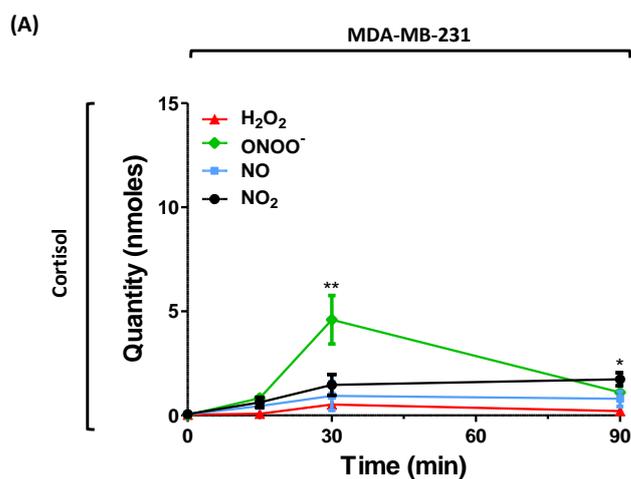
Appendix 3 Fig. 1.2 (A) Conversion of current data to nmoles using species specific values derived calibration curves. **(B)** Calibration coefficient of each species.

Fig 1.1 Electrochemical sensing controls



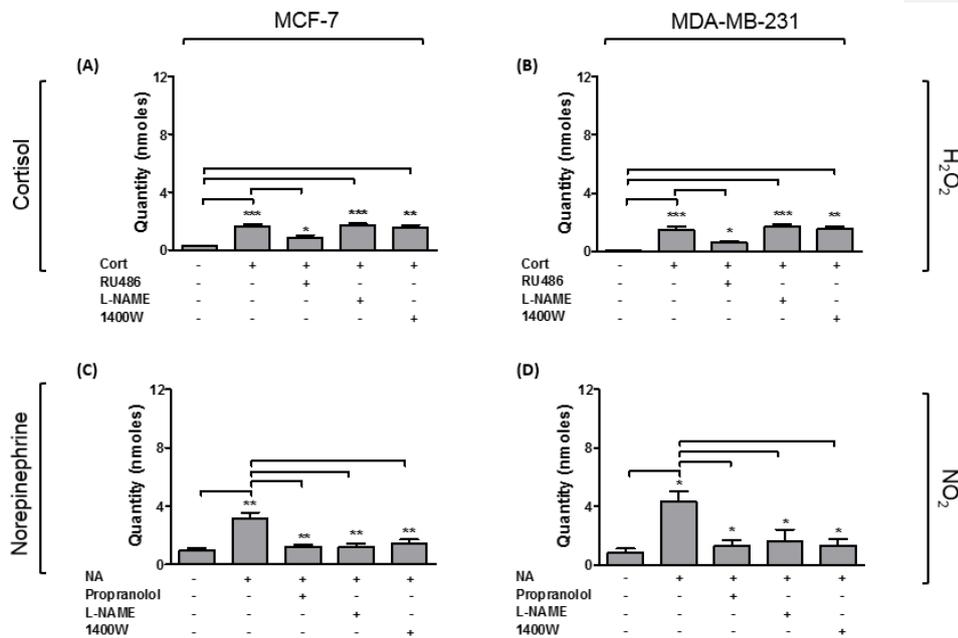
Appendix 4 Fig. 1.1 (A-B) MCF-7 and MDA-MB231 cells were left untreated and lysed at the beginning and end of an experiment. Levels of H₂O₂, ONOO⁻, NO and NO₂⁻ were measured using electrochemical sensors. **(C-D)** MCF-7 cells were cultured in media with 10% FBS or 10% charcoal-stripped (CS) FBS, and exposed to cortisol for 30mins in the presence and absence of 1400W. Levels of H₂O₂ and NO₂⁻ were measured using electrochemical sensing. Mean ± SEM expressed and statistical significance was determine using two-way ANOVA (Bonferroni post-tests). * indicates a significant difference. * = p<0.05, ** = p<0.01, ***= p<0.001.

Fig. 1.2 Electrochemical sensing in cell culture media



Appendix 4 Fig. 1.2 (A) MDA-MB231 cells were exposed to cortisol for 15, 30 and 90 mins, and the cell culture media was removed. Levels of H₂O₂, ONOO⁻, NO and NO₂⁻ were measured using electrochemical sensors. Mean ± SEM expressed and statistical significance was determined using two-way ANOVA (Bonferroni post-tests). * indicates a significant difference. * = p<0.05, ** = p<0.01, *** = p<0.001.

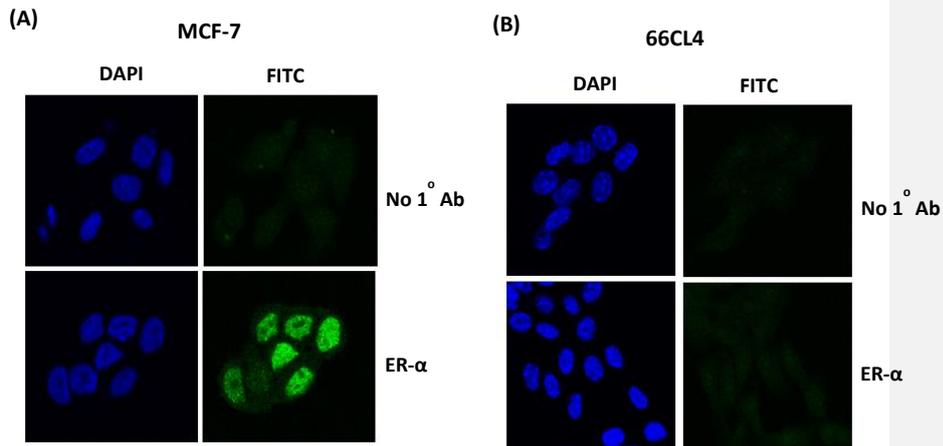
Fig. 1.3 Stress hormones induce production of ROS/RNS



Appendix 4 Fig. 1.3 MCF-7 (A+C) and MDA-MB-231 (B+D) cells were incubated with Cort (1 μ M) and RU486 (1 μ M). Cells were also incubated with NE (1 μ M) and Propranolol (1 μ M), as well as non-specific and specific NOS blockers (L-NAME (100 μ M) and 1400W (10 μ M)) for 30 mins prior to 30 mins stress hormone exposure. Levels of H₂O₂ (A-B) and NO₂⁻ (C-D) were measured using electrochemical sensors. Mean \pm SEM expressed and statistical significance was determined using one way ANOVA (post hoc Tukey's multiple comparisons). * indicates a significant difference cells. * = p<0.05, ** = p<0.01, *** = p<0.001.

Appendix 5 – ER status of 66CL4 cells

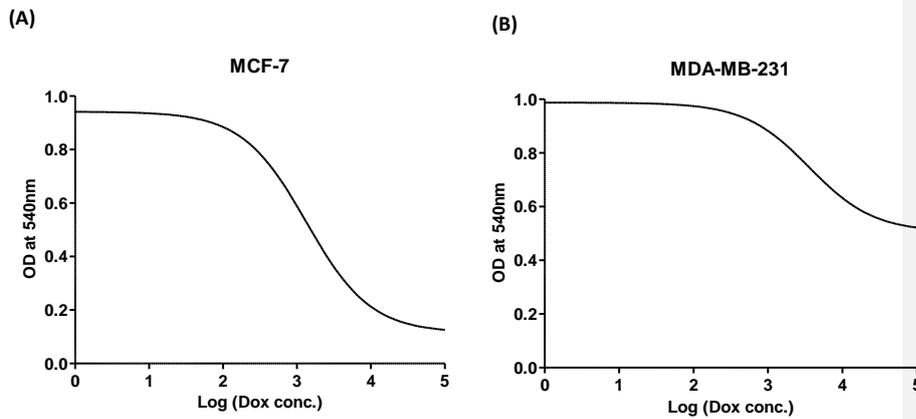
Fig. 1.1 ER status of MCF-7 and 66CL4 cells



Appendix 5 Fig. 1.1 MCF-7 (A) and 66CL4 (B) cells were immunofluorescently labelled for the oestrogen receptor (ER- α) (green) and counterstained with DAPI (blue). Representative images are shown.

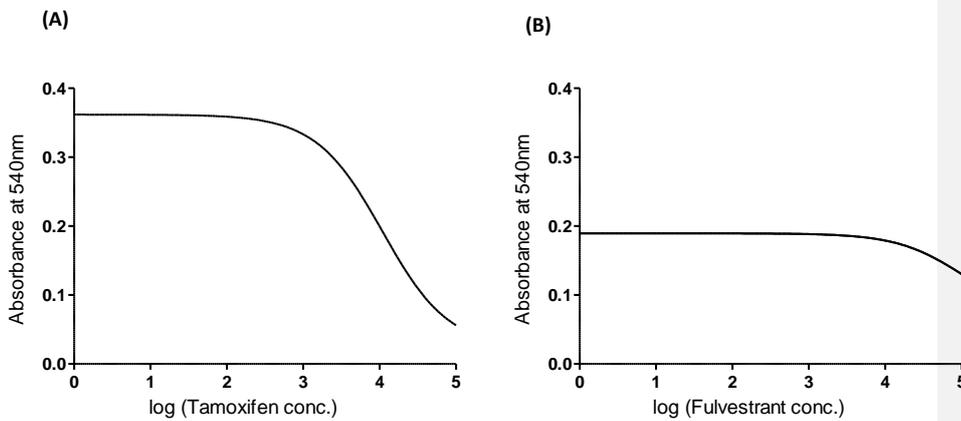
Appendix 6 –Dose response curves

Fig. 1.1 Doxorubicin dose response



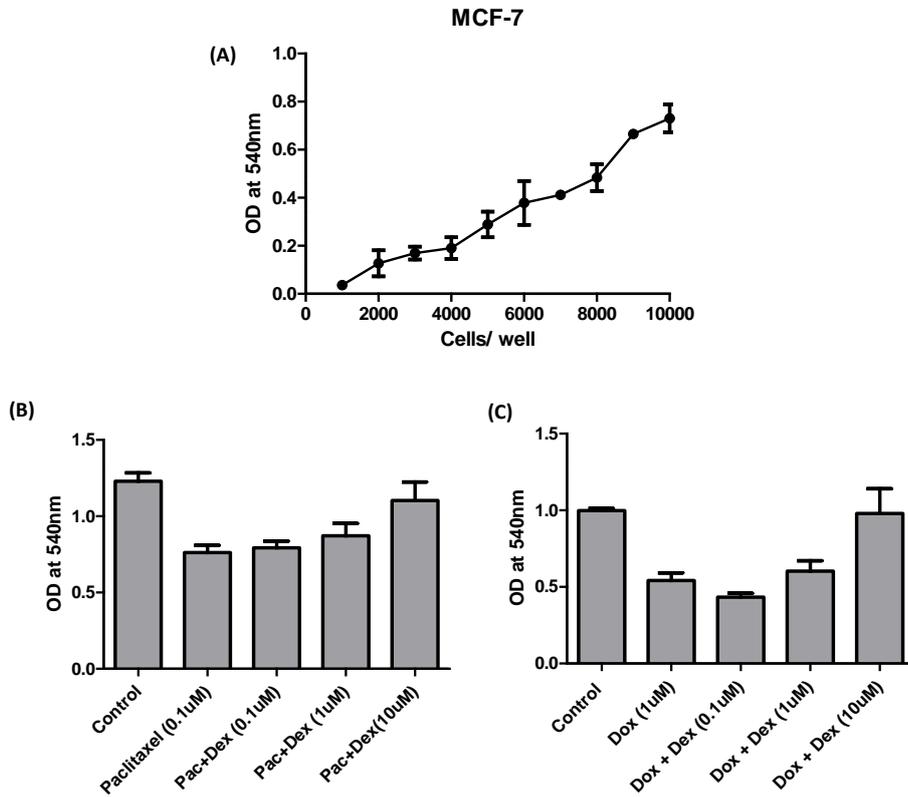
Appendix 6 Fig. 1.1 MCF-7 (A) and MDA-MB-231 (B) cells were exposed to a range of concentrations of doxorubicin (0,0.1,1,10,100 μ M). Cell viability was assessed using MTT assays and the results are expressed as optical density (OD) at 540nm. A logarithmic dose response curve was plotted.

Fig 1.2 Endocrine therapy dose response curves



Appendix 6 Fig. 1.2 MCF-7 cells were exposed to a range of concentrations of (A) Tamoxifen and (B) Fulvestrant (0,0.1,1,10,100 μ M). Cell viability was assessed using MTT assays and the results are expressed as optical density (OD) at 540nm. A logarithmic dose response curve was plotted.

Fig 1.1 OD data for MTT assay



Appendix 7 Fig. 1.1 (A) MCF-7 cells were seeded at increasing densities (1000-10000) in well of a 96 well plate. **(B-C)** MCF-7 cells were exposed to chemotherapeutic agents alone or in combination with dexamethasone. Cell viability was assessed using MTT assays and the results are expressed as optical density (OD) at 540nm.

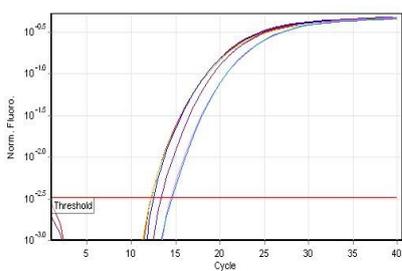
Fig. 1.2 Raw Ct values for qRT-PCR

Quantitation Report

Experiment Information

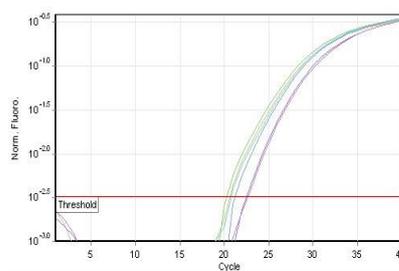
Run Name	66CL4 GR
Run Start	11/14/2016 2:43:47 PM
Run Finish	11/14/2016 3:30:42 PM
Operator	
Notes	
Run On Software Version	Rotor-Gene 2.0.2.4
Run Signature	The Run Signature is valid.
Gain Green	5.

Quantitation data for Cycling A.Green (Actin)



No.	Colour	Name	Ct
1	Red	Unstim	12.25
2	Yellow	Unstim	12.24
3	Blue	Cort 30 mins	12.50
4	Purple	Cort 30 mins	13.38
5	Pink	Cort 24hrs	14.45
6	Light Blue	Cort 24hrs	14.59
7	Green	NTC	

Quantitation data for Cycling A.Green (GR)



No.	Colour	Name	Ct
13	Gold	Unstim	20.61
14	Light Green	Unstim	20.28
15	Teal	Cort 30 mins	20.75
16	Blue	Cort 30 mins	21.24
17	Purple	Cort 24hrs	22.51
18	Light Purple	Cort 24hrs	22.66
19	Dark Purple	NTC	

RESEARCH ARTICLE

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Glucocorticoids induce production of reactive oxygen species/reactive nitrogen species and DNA damage through an iNOS mediated pathway in breast cancer

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Abstract

Background: Psychological stress increases the circulating levels of the stress hormones cortisol and norepinephrine (NE). Chronic exposure to elevated stress hormones has been linked to a reduced response to chemotherapy through induction of DNA damage. We hypothesize that stress hormone signalling may induce DNA damage through the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and interference in DNA repair processes, promoting tumourigenesis.

Methods: Breast cancer cell lines were incubated with physiological levels of cortisol and NE in the presence and absence of receptor antagonists and inducible nitric oxide synthase (iNOS) inhibitors and DNA damage measured using phosphorylated γ -H2AX. The rate of DNA repair was measured using comet assays and electrochemical sensors were used to detect ROS/RNS in the cell lysates from cells exposed to stress hormones. A syngeneic mouse model was used to assess the presence of iNOS in mammary tumours in stressed versus control animals and expression of iNOS was examined using western blotting and qRT-PCR.

Results: Acute exposure to cortisol and NE significantly increased levels of ROS/RNS and DNA damage and this effect was diminished in the presence of receptor antagonists. Cortisol induced DNA damage and the production of RNS was further attenuated in the presence of an iNOS inhibitor. An increase in the expression of iNOS in response to psychological stress was observed *in vivo* and in cortisol-treated cells. Inhibition of glucocorticoid receptor-associated Src kinase also produced a decrease in cortisol-induced RNS.

Conclusion: These results demonstrate that glucocorticoids may interact with iNOS in a non-genomic manner to produce damaging levels of RNS, thus allowing an insight into the potential mechanisms by which psychological stress may impact breast cancer.

Keywords: Breast cancer, Stress, Glucocorticoid, Norepinephrine, iNOS

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