Psychological Stress and Breast Cancer

Investigating the Influence of Psychological Stress and Ageing on the Epigenetic Modifications of Breast Cancer

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Abstract

Almost every cell in the body is responsive to stress through release of hormones termed stress hormones and the response to stress can differ depending on the type and duration of the stressor. Acute stress can facilitate a "fight or flight response" and aid survival, chronic long-term stress on the other-hand with the persistent release of stress hormones has been shown to be detrimental to health. We are now beginning to understand how this stress hormone response impacts important processes such as DNA repair and cell proliferation processes in breast cancer. However, it is not known what epigenetic changes stress hormones induce in breast cancer. Epigenetic mechanisms include modification of DNA and histones within chromatin that may be involved in governing the transcriptional processes in cancer cells in response to changes by endogenous stress hormones. ***The contribution of endogenous acute or long term exposure of glucocorticoid stress hormones, and exogenous glucocorticoids to methylation patterns in breast cancer tissues with different aetiologies is highly innovative and remains to be evaluated***. *In vitro* and *in vivo* models were developed to investigate the epigenetic modifications and their contribution to breast cancer progression and aetiology. As ageing is a risk factor for breast cancer and is highly affected by epigenetic modifications, an ageing in vivo model was developed to examine the influence of stress and ageing on epigenetic regulation in breast cancer. Treating a panel of triple negative breast cancer cell lines with the primary glucocorticoid hormone cortisol resulted in epigenetic alteration characterised by downregulation of DNMT1, loss of methylation on promoter regions of tumour suppressor genes including *ESR1*, and loss of methylation on LINE-1 repetitive element used as a surrogate marker for global methylation. This was verified *in vivo* in MDA-MB-231 xenografts; the model verified the loss of methylation on ESR1 promoter, and subsequent increase in ESR1 expression in primary tumours in mice subjected to restraint stress. With regards to the influence of ageing, a syngeneic breast cancer mouse model was developed; older mice exhibited a decrease in DNMT1 in breast cancer tumours with age increase from 3-18 months. Our study highlights that DNA methylation landscape in breast cancer can be altered in response to stress and glucocorticoid treatment. The study also suggests that stress and ageing can influence epigenetic modifications in breast cancer.

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Contents

1. Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Breast Cancer Epidemiology and Aetiology

Accounting for about 15% of all newly diagnosed cancers, breast cancer is one of the most common cancers in the United Kingdom (UK)[1, 2]. One in seven women will be affected by breast cancer (BC) during their lifetime [2]. With 11,399 deaths resulting from this disease annually in the UK (according to 2017 statistics from Cancer Research UK), breast cancer is the fourth most common cause of cancer mortality among females in the UK[1, 3]. These numbers reflect the urgent need for preventive and treatment measures; and while technology in health care has advanced in the early detection of the disease, several molecular mechanisms that underlie the initiation and progression of this disease remain unknown. Approximately 10% of BC cases are estimated to be hereditary; resulting from inherited germ-line mutations in susceptibility genes. Those mutations commonly occur on tumour suppressors involved in cell cycle, apoptosis, and DNA repair [4]. The rest 90% of BC cases are thought to be due to acquired genetic somatic changes and/or epigenetic modifications[4-6]. Genetic alterations disrupt cell growth, division, and survival through gene deletions, amplification, mutations, and gene rearrangements in key genes [4]. Epigenetic deregulation, mostly aberrant promoter methylation, contributes to altered expression and regulation of genes [7]. Epigenetic alterations, however, including DNA methylation, play critical roles in the progression and development of breast cancer [4].Breast cancer is a heterogeneous complex disease comprised of several types and subtypes based on molecular characteristics, clinical behaviours and outcomes [8].

1.1.2 Molecular Classification

Genetic and epigenetic changes are both commonly correlated to specific biological and clinic-pathological tumour characterisation, which can contribute to the development of personalised therapies that target associated molecular pathways [9]. However, traditional biological characteristics are still used for breast cancer diagnosis today. These include tumour size, lymph nodes metastasis, histological grade, age, and hormonal and the expression of biological receptors, such as oestrogen (ER), progesterone receptors (PR), and epidermal growth factor (HER2). However, this traditional characterization system is currently insufficient to determine the underlying biological changes affecting breast cancer development, progression, and response to therapy. Current research is focusing on distinguishing more defined biological characterisation to help improve patient risk measurements ensuring a higher chance of benefit and the less toxicity from treatment modality[8]. Global Gene Expression Profiling (GEP) research has provided gene expression pattern based evidence for categorising BC into discrete biological subtypes [10]. Today, depending on the location of the tumour, BC can be sub-divided into Invasive Lobular Carcinoma (ILC), Invasive Ductal Carcinoma (IDC), and Ductal Carcinoma *in situ* (DCIS). Ductal Carcinoma *in situ* is a non-invasive BC; where cells from this type of BC are usually confined in the milk ducts, hence termed *in situ*, and do not spread to nearby breast tissues. Invasive cancer cells usually spread from the initial site (either the milk ducts or the lobules) into the nearby breast tissue, and/or to the lymph nodes and/or different parts of the body. Therefore, patients with DCIS have a better prognosis than patients with IDC.

IDC is molecularly divided into five main intrinsic subtypes based on the genes and receptors that the cancer cells express. These classifications are based on the expression of oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and a proliferation marker known as Ki-67 (Table 1). These classifications help determine treatment and predict prognosis[11]:

- **Triple-negative/basal-like** breast cancer (TNBC) is hormone-receptor negative (ER and PR negative) and HER2 negative. It is considered the most aggressive type of breast cancer. This type is more common in younger patients and patients with *BRCA1* mutations.
- **Luminal A** breast cancer is hormone-receptor positive (ER and/or PR positive), HER2 negative, and has low levels of the protein Ki-67. This type grows slowly compared to other types, and luminal A cancers are also low-grade. Since this type expresses the ER, the cells are responsive to hormonal treatment (anti-oestrogen treatment)
- **Luminal B** breast cancers are also hormone-receptor positive (ER and/or PR positive), and either HER2 positive or HER2 negative with high levels of Ki-67. Cancers of this type tend to grow slightly faster than luminal A cancers. Although these cell types can respond to hormonal treatment, patients with this subtype have a worse prognosis
- **HER2-enriched** breast cancer is hormone-receptor negative (ER and PR negative) and HER2 positive. Although these cancers are often successfully treated with targeted therapies aimed at the HER2 protein, they tend to grow

faster than luminal cancers and patients with this subtype can have a poorer prognosis.

- **Normal-like** breast cancer is similar to luminal A type of breast cancer: it is hormone-receptor positive (ER and/or PR positive), HER2 negative, with low levels of Ki-67. Patients with Normal-like breast cancer tend to have a good prognosis. However, the prognosis is worse than luminal A cancers' prognosis.

Table 1 Molecular Classification of Breast Cancer Subtypes

BC Type	Hormonal status	HER2 status	Ki-67	% of Patients	Cell lines used for this thesis
Triple Negative / Basal like (TNBC)	ER- and PR-	–	varies	15-20%	MDA-MB-231 MDA-MB-157 BT-549 Hs-578T
Luminal A	ER+ and/or PR+	_	low levels	40-44%	MCF-7 T47D
Luminal B	ER+ and/or PR+	+ or -	high levels	20-24%	
HER2-enriched	ER- and PR-	+	varies	20-24%	
Normal-like	ER+ and/or PR+	_	low levels	2-4%	

Numerous studies are also being conducted to identify critical epigenetic biomarkers in order to use them for early detection, and prediction of prognosis in BC patients [12].

1.2 Psychological Stress

Hans Selye first introduced the concept of stress as a biological response in 1936 [13]. Selye distinguished between acute and chronic stress [13]. Acute stress, first described by Walter Cannon in 1915, can facilitate a "fight or flight response" and aid survival. In this case, stressors trigger a cascade of hormonal changes that produce physiological changes such as increased heart rate and blood pressure [14]. This is different from chronic stress that can be detrimental to health. Selye described chronic stress as a reaction to a stressor that occurs in three stages: alarm to a stressor, followed by resistance to stressor and attempting to maintain homeostasis, and finally exhaustion in countering the stressor. These responses were termed 'general adaption syndrome' (now known in the literature as Selye's Syndrome) [13]. Stress evokes different responses in different individuals, depending on different types of stressors [15].

The human stress response is mediated by a series of events that involve the release of neurotransmitters and the secretions of different hormones controlled by the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS)[16].When stimulated by a stressor, neurons in the hypothalamus secrete two peptide hormones, Corticotropin-Releasing Hormone (CRH) and Arginine-Vasso Pressin (AVP) activating the HPA axis. CRH then travels to the anterior pituitary; stimulating secretion of the AdrenoCortico Tropic Hormone (ACTH) which is then transported through the blood stream to the adrenal cortex stimulating the secretion of corticosteroids (including cortisol in humans or corticosterone in mice).

This class of steroid hormone, glucocorticoids (GC), are considered the main player to directly impact the stress response. The HPA axis consists of a negative feedback loop, by which GCs target the hypothalamus and the interactions among the hypothalamus, pituitary gland, and adrenal gland to inhibit the secretion of CRH and ACTH (Figure 1)[16, 17]. The secretion of CRH is regulated by an intrinsic circadian rhythm operating through the hypothalamus. For cortisol, the circadian rhythm shows a peak in cortisol levels during the early hours of the day, and then declines through the rest of the day and at night. Cortisol levels then start rising again to its early morning diurnal peak (Figure 2).

Stress also triggers the sympathetic nervous system through the release adrenaline and noradrenaline. This is known as adrenergic stress. The release of adrenaline or noradrenaline, in response to stress, triggers different physiological events. Adrenaline and noradrenaline bind to adrenergic receptors α, and β. The activation of sympathetic nervous system plays a crucial role in modulating the blood flow to muscles, heart output and respiration rate, and regulating metabolic activity [18].

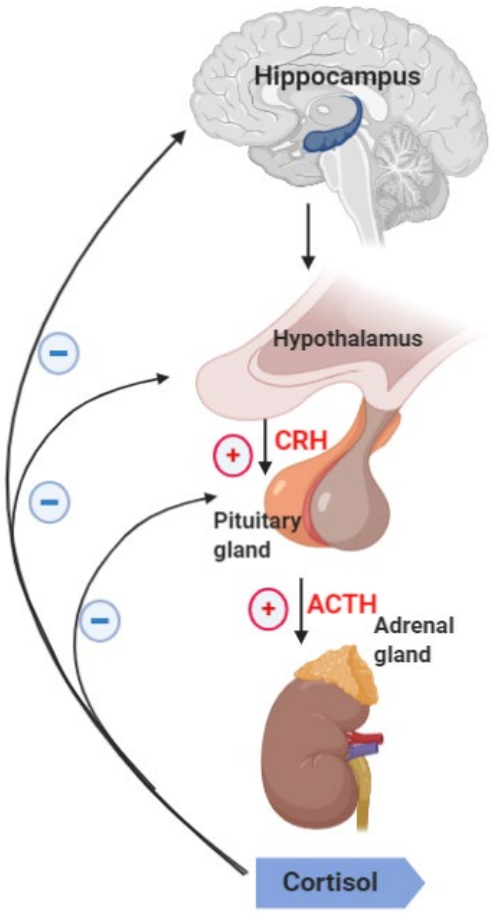


Figure 1Illustration of the HPA axis negative feedback loop describing the major components of the stress response mediated by the hypothalamic–pituitary–adrenal (HPA) axis.

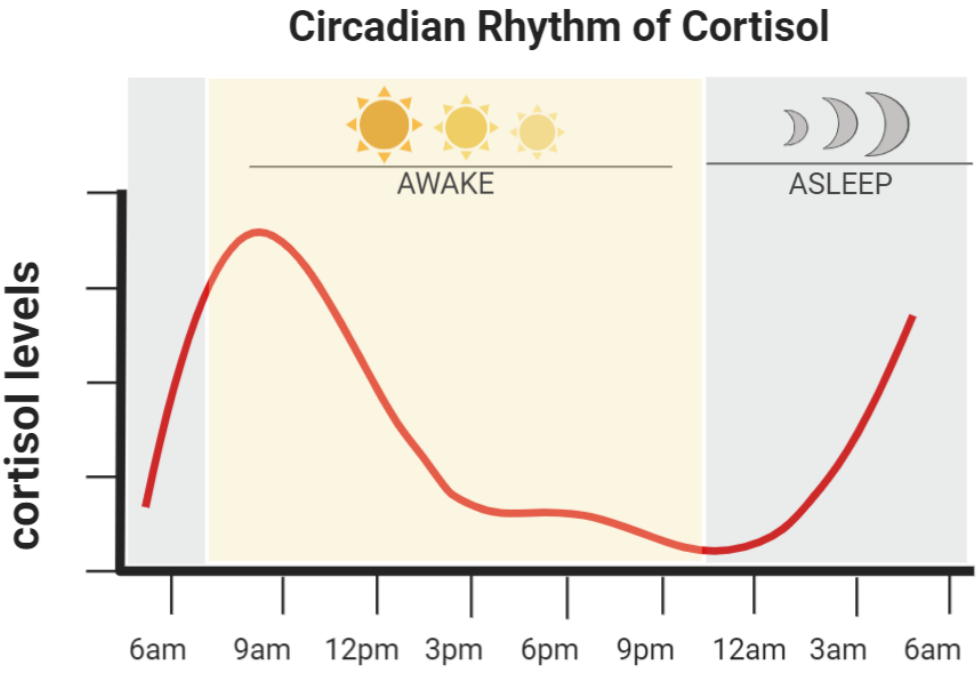


Figure 2 Circadian Rhythm of Cortisol. Cortisol rises in the early waking morning, and drops through the day, then climbs up again to its early morning diurnal peak.

1.2.1 Glucocorticoids

Glucocorticoids (GCs) are the primary stress hormones secreted from adrenal gland in a circadian manner [19]. Glucocorticoids regulate many physiological processes including metabolism regulation, cognition, skeletal growth, cardiovascular function, and immunosuppressive actions [19-21]. Glucocorticoids also play crucial role in cellular processes such as apoptosis, cell growth, proliferation, and cell differentiation [22, 23]. Due to the role of GCs in the aforementioned processes, synthesized GC's have been developed and widely utilized in general medical practice[24, 25] For example, dexamethasone is often used to reduce hypersensitivity reactions, nausea, and allergic reaction which result from chemotherapy treatment of breast cancer patients [24]. Glucocorticoids are also used to promote apoptotic cell death in malignant lymphoid cells (e.g. lymphoma). However, GCs can have adverse effects; for instance in epithelial tumours studies suggest that GC's can stimulate anti-apoptotic gene expression[24]. Also, evidence has shown that GCs mediate antagonism of therapy-induced tumour cell apoptosis[26, 27]. Therefore it is important to understand the molecular mechanisms that mediate the cellular response to GCs.

The pharmacological and physiological effects of GCs are mainly mediated by the glucocorticoid receptor (GR), which is encoded by Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*) gene [28]. The human GR is composed of 9 exons, and although the GR is derived from a single gene, multiple isoforms of GR are found, due to alternative splicing in exon 9[29]. Consequently, GR mediate different

cellular responses to GCs. The majority of research studies the GR signalling that mediates a transcriptional response when bound to GCs This is known as the "classical" signalling. However, further evidence suggests that GR can mediate a transcription-independent mechanism also known as a "non-classical" mechanism. Both mechanisms are briefly explained below:

1.2.1.1 Classic GR signalling pathway

GR is composed of three main domains: an N-terminal transactivation domain (NTD) that is encoded by exon 2, a central DNA-binding domain (DBD) that is encoded by exons 3 and 4, and a C-terminal ligand binding Domain (LBD) encoded by exons 5-9 [19, 30]. The DBD contains motifs that recognise and bind target DNA sequences called glucocorticoid response elements (GREs)[30].When GCs are not bound to their receptors, the GR resides in the cytoplasm, and forms a complex with heat shock proteins (HSPs) (HSP90, and HSP70), p23 (a chaperone of HSP90), and immunophilins of FK506 family [31]. In the presence of GCs, the GR dissociates from HSPs and other associated proteins, and translocates into the nucleus where it interacts GREs [19]. The complex of GREs with GR results in simulation or reduction of the mediated gene transcription known as the transactivation and transrepression effect retrospectively [19, 23]. Both of these effects can be regulated in two ways: either by GR tethering to DNA-bound proteins, or by direct physical interaction of the GR with other transcription factors [19]: For example, GR interacts with members of the signal transducer and activator of transcription (STAT) family enhancing the transcription of responsive genes [32]. An example of GR mediating transrepression effect is the famous mechanism by which GR

mediates the suppression of inflammation by antagonizing the activity of the transcription factors Activator Protein 1 (AP-1), and Nuclear Factor Kappa B proteins (NF-κB); GR binds directly to Jun and p65, the subunits of AP-1 and NF-κB retrospectively, inhibiting their transcriptional ability [33].

1.2.1.2 Non-classical GR signalling pathway

Studies have recently provided evidence that the GR can mediate non-genomic, transcription-independent mechanisms to stimulate cellular responses [34, 35]. These non-genomic signalling events impinge on the activity of different kinases such as, Mitogen-Activated Protein Kinases (MAPK), Protein kinase B (PKB, AKT), and Phosphoinositide 3-kinases (PI3Ks) [35, 36]. For example, the non-receptor tyrosine-protein kinase c-Src is one of the proteins associated with GR in the absence in GCs. In the presence of GCs, c-SRC dissociates from GR complex and activates a cascade of kinases leading to the inhibition of cytosolic phospholipase A2 activity [37].

The combined effects between rapid, non-genomic signalling (seconds to minutes) and classical signalling (minutes to hours) exert important effects on the physiological responses of GC signalling that may be used for therapeutic advantages [35].

1.2.1.3 GR and Nuclear Receptor Crosstalk

GR is a member of nuclear receptors (NR) and steroid receptors (SR) family along with oestrogen receptor, (ER), progesterone (PR), and androgen receptor (AR) that are ligand-inducible transcription factors[38]. Although the interplay between

these receptors is poorly understood in breast cancer, many studies investigated the cross talk between these NRs to understand their functions. For example, in a study on mouse mammary cells, the activation of GR facilitated the access of ER to specific sites by allowing an open configuration at these response elements; and the induction of ER affected chromatin structure at oestrogen-dependent GR binding site leading to a new class of GR binding elements [38-40]. This shows that the mechanism of interaction occurs in both directions, ER modulates the landscape for GR access, and GR reprograms accessibility of ER; in both ways, the co-activation of GR and ER was found to lead to the re-programming of chromatin landscape and re-arrangement of steroid receptor binding mediating gene expressions [40] . The two receptors (GR and ER) have been also reported to dictate each other's activity at an AP-1 response element in ER+ breast cancer cell lines [39]. Another example of hormonal cross talk in breast cancer is the cross talk between PR and AR in ER+ breast cancers. AR and PR are commonly expressed in ER+ subtypes of breast cancer[41, 42]. AR and PR can impact ER transcriptional activity by either directly altering ER-DNA interaction, or by sequestration of rate-limiting co-factors[41, 42]. Understanding the mechanism of crosstalk between NRs is important for the understanding of breast cancer progression.

1.2.1.4 GR and Cancer

GR is a ligand-dependent transcription factor, in the ligand bound state, the initiation/repression of gene expression seems to be tissue specific. For example, the activation of GR has been demonstrated to be pro-apoptotic in lymphoid

malignancies [43, 44], while GR activation has been found to play an anti-apoptotic in other types of cancer such as ovarian and prostate cancer [44-46]

In breast cancer, the role of GR activation in cell survival has been demonstrated to be cancer sub-type specific [39]. In ER positive breast cancer, the co-activation of GR and ER contribute to a more indolent phenotype of breast cancer [39]. In a meta-analysis, ER positive breast cancer patients, high NR3C1 mRNA expression was correlated with better prognosis in comparison to low NR3C1 mRNA expression [47]. However, in TNBC high expressions of NR3C1 mRNA were correlated with poorer prognosis [47]. In fact, in TNBC cell lines and TNBC xenografts, the activation of GR was found to inhibit apoptosis by initiating and an anti-apoptotic pathways characterised by the upregulation of serum and glucocorticoid-inducible protein kinase-1 (SGK1) and mitogen-activated protein kinase phosphatase-1 (MKP1/DUSP1)[48]. Activating GR in TNBC xenografts tumours by dexamethasone led to a significant reduction in the efficacy of treating with Paclitaxel [49, 50].

Researchers have also used GR antagonist in cancer research in efforts to antagonise the induction of genes involved in anti-apoptotic signalling pathways [49]. Mifepristone (RU-486) is a GR antagonist; that is widely studied in breast cancer research as a PR antagonist in ER+/PR+ tumours [49, 51]. Treating TNBC cell lines, with mifepristone antagonised GR-mediated upregulation of *SGK1*, and *MKP1/DUSP1,* and in TNBC xenografts, mifepristone augmented paclitaxel-induced tumour shrinkage [49]. Other non-steroidal GR selective modulators (SGRMs), such as CORT118335 and CORT108297 have been used in Cancer research. *In vivo* study demonstrated the inhibition of GR-mediated proliferation-associated

genes including FK506 Binding Protein 5 (*FKBP5), and SGK1* in response to treatment with CORT108297 in prostate cancer tissue[52].

1.2.2 Stress and Breast Cancer

As explained earlier, GC's, the primary stress hormones in humans, play a crucial role in different pathological processes [19]. This has led to the investigation of the effects of chronic exposure to stress hormones, and the biological mechanism of psychological stress on the mechanisms of cancer. Although stress has been associated with the initiation and progression of many diseases, there has been a conflict in research on whether stress "causes" or contributes to the initiation of diseases, including breast cancer, or not. However, the literature provides stronger evidence that stress can contribute to the progression of breast cancer. Below is a summary of studies that have investigated the effects of psychological stress on the initiation, progression, and response to therapy in breast cancer using*in vitro* models, *in vivo* models, and human clinical approaches. Those studies can be stratified into two categories:

1.2.2.1 Stress and breast cancer initiation

Chronic psychological stress has been reported among breast cancer patients, many of which attribute their disease to psychological stress [53]. However, proposing psychological stress as a cause of breast cancer is highly controversial in the field. Many clinical studies assess psychological stress using questionnaires completed by patients taking into account different factors including socioeconomic, access to health care, childhood history, lifestyle habits, loss of dear ones etc.[54]. A recent case-control study, that included 664 female breast cancer patients and 203 female population-based controls, found that stressful life events

increased the risk of breast cancer [55]. Another cohort study that used a questionnaire to assess stressful life events in 10,808 Finnish women over 15 years, also reported an association between stressful life events and increased risk of breast cancer during 15 years of follow-up [56]. Another case-control study in Poland also associated stressful life events with an increased risk of breast cancer incidence[54]. In addition, a case-control study that used female Invasive ductal carcinoma patients and control females matched for age and place of residence, showed an association of higher risk of breast cancer in females that described four to six major stressful life events [57]. In a cohort of Swedish women, a 2-fold increase in breast cancer risk was reported in women who described higher levels of daily stress compared to those described lower levels of stress[58].An analysis of the role of psychosocial factors in the development of breast cancer in 514 Australian women, showed that women who experienced highly stressful life events and had a lack of social and emotional support had a significant risk increase of developing breast cancer [59].

In vivo models also investigated the relationship between stress and the incidence of breast cancer. Although it is hard to assess feelings in animals, physiological stress can be induced in various ways for *in vivo* studies, such as restraint stress, immobilization of animal, forced swim test, intermittent foot-shock, cold housing, and social isolation [60]. A study used social isolation to induce stress in a rat strain that is genetically predisposed to develop mammary tumours known as the Sprague-Dawley; found that isolated animals developed mammary tumours at a higher rate compared to the control group [61].

Only few *in vitro* molecular studies invested in studying the correlating of stress with the initiation of breast cancer; and mimicked stress *in vitro* by the treatment of appropriate cell line with glucocorticoids and catecholamines. However, the interpretation of these studies are limited by the short half-life of stress hormones, and their circadian fluctuation [62]. For example, a study that used 3T3 fibroblast cells found that treatment with catecholamines resulted in long-term DNA damage, suggesting the role of stress hormones in tumour formation [62]. A different study also correlated cortisol treatment of MCF-10A cells (human mammary epithelial cells) with downregulation of Breast Cancer type1 gene*(BRCA1)* resulting in aberrant cellular proliferation, implying a potential role in breast cancer development [63].

In contrast, far more studies do not support the correlation between stress and breast cancer initiation. For example, a prospective cohort study involving 11,467 healthy women from the UK did not find correlation between social stress and breast cancer risk [64]. Another study in Finland, assessed participants of a prospective cohort study using questionnaires and found no statistically significant difference between stress of daily activities and risk of breast cancer [65]. Analysis of a study of 106,000 women in the UK used questionnaires to assess stress level of adverse life events and found no association between perceived stress levels and breast cancer risk [53]. A cohort study of 84,334 women, declared no association between stressful life events and breast cancer incidence during 7 years follow up [66]. Finally, a meta-analysis showed no significant association between stressful

life events and breast cancer risk, however, only the incidence of the death of dear ones was significantly correlated with cancer risk [67].

1.2.2.2 Stress and breast cancer progression:

More evidence supports the hypothesis that stress contributes to cancer progression, mortality, and response to therapy rather than cancer initiation. A meta-analysis of 165 studies associated stressful life experiences with decreased survival rate and increased mortality [67]. Another meta-analysis of 28 studies showed significant correlation between depressive symptoms and mortality among cancer patients [68].

Molecular studies have investigated the relation between stress and progression of cancer, some of which has been done in different types of cancers. For example in ovarian cancer, exposure to chronic stress was found to induce angiogenesis and tumour growth [69]. An *in vivo* study on melanoma, found that mice subjected to restraint stress are more prone to higher number of tumours [69]. Chronic stress has been shown to affect the immune system, supressing anti-tumorigenic activities in different cancers [70]. A study also associated high levels of stress and depression with higher levels of pro-inflammatory markers implying that the biological mechanism of stress can influence tumour growth [71].

Several molecular studies have also demonstrated the effects of psychological stress, stress hormones, and glucocorticoids on the progression of breast cancer *in vitro* and *in vivo*. For example, a recent study in 2019 used breast cancer *in vitro*and *in vivo* models to verify that stress hormones induce metastasis, increase colonization and reduce survival through increased expression of

neurotrophictyrosine kinase, receptor-related 1 (*ROR1*) [72]. Many studies explained the role of stress hormones in mediating DNA damage in breast cancer [73]. A study using *in vivo* and *in vitro* breast cancer models to study the effect of glucocorticoids on the Hippo pathway, which plays a crucial role in tumorigenesis; found that GC's promoted cancer cell survival, metastasis and chemo-resistance both *in vitro* and *in vivo* by activating TEA domain transcription factors (*TEAD4*), an upstream kinase complex of the hippo pathway.

Finally, an impressive ongoing clinical study not only assessed psychological stress using questionnaires, but also assessed physiological stress by measuring glucocorticoids in saliva samples collected at different times of the day from breast cancer patients. This study is correlating stress with the epigenetic alteration of Breast Cancer type1 gene *(BRCA)1,* as an indicator for breast cancer aggressiveness, and a stress-associated biomarker [74, 75].

1.2.2.3 Limitations of stress studies

Studies linking psychological factors and cancer incidence are prone to limitations and publication bias. In retrospective case-control studies, patients with breast cancer and their respective controls were asked to fill out questionnaires detailing their exposure to stress[76, 77]. Although 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[77]. Also, prospective studies are affected by recall bias since individuals interpret stressful events differently [77]. Hence, this type of bias makes it crucial to try to obtain multiple markers of stress.

1.3 Ageing

1.3.1 Introduction to ageing

Ageing has been defined in various ways in the literature, all in which describe the process as a gradual decline in the physiological of the body [78-80].Those time-dependent deleterious changes in many molecular and biochemical processes in cells and tissues leading to the ageing process are thought to be responsible for the increased risk of many diseases including cancer, cardiovascular diseases, and Alzheimer's [79].Ageing is an inevitable process and limits the lifespan of organisms; this has been challenging researchers to understand the processes underlying ageing over several decades [80]. Ageing can be characterised by biochemical alterations in tissue composition, progressive physiological capacity decrease, and loss of ability to respond to environmental stimuli and stressor, leading to mortality risk increase and more susceptibility to diseases [80] .

Maximum human life span has remained considerably unchanged for the past thousands years at ~125 years. However, median life expectancy has reasonably increased throughout the last 100 years by approximately 27 years [81, 82]. In order to study the basics of the ageing process, it is fundamental to understand whether ageing is a disease or a natural process; however, this is highly controversial [83]. Assuming ageing is not a disease, Hayflick estimates that human life expectancy will only increase by 15 years if the primary causes of death (i.e., cardiovascular diseases, cancer, etc.) are potentially cured [81]. Considering ageing is a major risk factor for a range of diseases including cancer, researchers are looking to classify ageing as a disease; this will accelerate research on ageing and

encourage the identification of drug treatments to attenuate the ageing processes and therefore prevent the deterioration in the functioning of the body [83].

1.3.2 Hallmarks of ageing

Ageing is a complex multifaceted process governed by different epigenetic and genetic mechanisms. Research has identified nine biological mechanisms as hallmarks of the ageing process. Those nine hallmarks can be categorised into three groups: the primary hallmarks, the antagonistic hallmarks, and the integrative hallmarks.

1.3.2.1 Primary Hallmarks

The primary hallmarks are processes that induce cellular damage at the molecular level resulting in deterioration in organ function; contributing to the ageing process. Those hallmarks include genomic instability, telomere attrition, epigenetic changes, and loss of proteostasis[79, 84].

Genomic Instability: During a life-span the DNA is subjected to many endogenous and exogenous mutagens, leading to DNA damage that includes; mutations, chromosomal breaks, and translocations, affecting gene expression and altering key biological function [85, 86]. Indeed, the accumulation of DNA damage is directly correlated with age-associated diseases [79, 87].

Telomere attrition: Telomeres play a crucial role in maintaining genomic stability, as they are repetitive nucleotides localised at the ends of chromosomes [88]. Ageing has been associated with telomere shortening, and hence associated with age-related diseases such as cancer [79, 88].

Epigenetic alteration: Epigenetic deregulation is a common feature of ageing in mammals; DNA methylation has emerged as a key epigenetic alteration contributing to the genome structure and function alterations accompanying ageing [89]. Global loss of DNA methylation is observed during ageing, along with hypermethylationof specific loci as a function of age. Since cancer is, for the most part, a disease of ageing it has been proposed that age-related epigenetic changes initiate tumourigenesis[90].

Loss of proteostasis: The homeostasis of the proteome is governed by different cellular mechanisms that regulate the turnover of human proteins known as proteostasis[91]. Ageing has been correlated with a deterioration of proteostasis[79, 91].

1.3.2.2 Antagonistic hallmarks

The human body induces certain mechanisms in response to molecular and cellular damage. These mechanisms include deregulation of nutrient sensing, mitochondrial dysfunction and cellular senescence. Although these mechanisms are critical for protecting us from disease development, they certainly aid in the ageing process [79].

For example, senescence which is a cellular process that is defined by the state of irreversible growth arrest is a process that protects against the carcinogenic transformation of cells [92]. However, increased senescence has been correlated with ageing and age-related diseases [93] . In fact, a large part of the causes of ageing is due to replicative senescence and the ability of senescent cells to alter

that function of surrounding healthy cells through the SASP (senescence associated secretory phenotype) [94].

1.3.2.3 Integrative hallmarks

These hallmarks are a result of the clinical phenotype affecting the physiological functions of organs and contributing to the clinical effect of age. These include stem cell exhaustion and altered intracellular communication [79]. Stem cell exhaustion is a mechanism in which stem cell function is lost leading to many age-related diseases such as anaemia, osteoporosis, and dysregulated intestinal function, as well as alteration in the communication between cells and different systems including the immune system [79, 84].

1.3.3 Limitation of ageing studies

Nevertheless, understanding the causes of ageing can be limited due to the multifariousness of the process. For example, most studies are done on different organisms that have shorter life span and are different to humans but yet have the similar molecular functions. Also, environmental factors affect experimental observation, and secondary effects makes explanation of primary mechanisms complicated. In addition, the lack of precise biomarkers of ageing makes this type of study difficult [95, 96].

In this project, besides considering ageing as a biological process, the psychological factors during ageing are also taken in consideration. Ageing individuals can suffer from social isolation, anxiety, and different forms of psychological stress. How

stress and ageing contribute to the progression of breast cancer is considered in this project.

1.3.4 Ageing and Breast Cancer

Breast cancer incidence increases with age, based on recent statistics from Cancer Research UK and recent epidemiological studies showed that almost 25% of newly diagnosed breast cancers in the UK occurred in patients aged 75 year and older [1]. In females, incidence rate rises steadily between the ages of 30 to 34 years, and then steeply rises between the ages of 70 to 74 years. The highest age-specific rates occur between the ages 85 and 89 years[1].

Many studies consider age a marker of prolonged carcinogen exposure, and therefore women over the age of 50 years are at high risk since approximately 80% of all breast cancers occur after that age [97, 98]. Also, the chance of developing invasive breast cancer at the age of 40 years rises gradually from less than 1.5% to over 4% by age of 70 years producing a risk of 1 in 7 women of all ages[99].

Breast cancer incidence profile increases exponentially until menopause but then slowly increases afterwards[99, 100]. Breast cancers occurring in times before menopause are usually inherited and/or a result of early life transformation affecting the immature mammary epithelium. However, breast cancers occurring after menopause are more likely to result from a prolonged exposure to stimulating susceptible epithelium that failed to age normally[99, 100]. The altered extracellular matrix and secreted products are among the stimuli thought to promote post-menopause breast tumorigenesis. Supporting the idea breast cancer biology is actually age-dependant, clinical research and biomarker studies, indicated

that breast cancer occurring after menopause tends to grow slower and is less aggressive than breast cancers occurring before menopause, even when controlled for hormone receptors, and growth factor receptor expression [99, 100]

1.4 Epigenetics

1.4.1 Introduction to Epigenetics

Our DNA is formed of two polynucleotide chains made of sequences of four bases, cytosine (C), guanine (G), thymine (T), and adenine (A). Each of the polynucleotide chains has a helical structure (a helix), and the two helices are then intertwined to give a double helix. DNA helices are wrapped around eight histone proteins forming repeated units of nucleosomes (Figure 3)[101]. The DNA and histone complex is known as chromatin, and chromatin is condensed further to form a chromosome [102, 103]. Cells within a multicellular organism contain the same DNA sequence yet have different phenotypes. Changes that alter the phenotype of cells and differentiate cells by altering gene expression without altering the DNA sequence are known as epigenetic changes[104].

The first use of the word epigenetics was introduced in the 17th century in the English dictionary; generated from the combination of the prefix “epi”,Greek for “above” or “on top of”,and genetics[105, 106]. In the 1940s Conrad Waddington defined the study of “epigenesis” as “changes of genotype that gives rise of a phenotype, during development” [107]. In the 1950s, Arthur Riggs defined epigenetics as “mitotic and mitosis heritable changes in gene function that cannot be explained by changes in DNA sequence”[105]. In the 1990s, the new consensus for the definition of epigenetics was introduced as “An epigenetic trait is a stably

heritable phenotype resulting from changes on the DNA without alterations in the DNA sequence" [107].

Epigenetic processes involve the addition or removal of epigenetic "chemical tags" on the genetic sequence, like a methyl group or an acetyl group, that are added to nucleotide bases to form combinations of differently regulated molecular signals, regulating gene expression, and leading to cellular differentiation, and phenotype changes [108].

A series of dynamic epigenetic modifications of the genome occurs during early development, and is maintained throughout life [109]. Failure to maintain epigenetic markers may result in inappropriate inhibition and activation of signalling pathways that induce diseases[110]. Different endogenous factors (e.g. hormonal control and ageing) and exogenous factors, (e.g. psychological stress), can impact the epigenetic landscape of the genome; leading to a persistent change in cell programming [104, 111]. Generally, cell-type-specific epigenetic changes are considered more stable and more pronounced than changes resulting from exogenous factors [104, 111]. Epigenetic changes are stably inherited through cell division and therefore are considered dynamic, stable, inherited mechanisms. Unlike genetic modification, epigenetic mechanisms are reversible, as they mainly consist of chemical modifications and many drugs has been developed in efforts to reverse the epigenetic alterations in cases of abnormalities [110].

Epigenetic regulation is essential during embryonic development and crucial for normal growth [112]. In 1980s, epigenetic abnormalities started to gain recognition in cancer research as evidence started emerging illustrating the involvement of

epigenetics in regulating genes involved in cancer progression and metastasis [112]. This led to the discovery of epi-drugs, which are drugs that modulate epigenetic activities in efforts to treat different types of cancer [112].

Research has identified three main epigenetic regulations that include histone modifications, methylation of DNA and abnormal expression of RNAs that are non-coding e.g. microRNAs [113].In this thesis, DNA methylation is the focus, due to the role of methylation in breast cancer and the association of DNA methylation with psychological stress, all in which will be explained in the following sections. However, we note that epigenetic modification can act independently or/ and in combination with other epigenetic mechanisms [110]

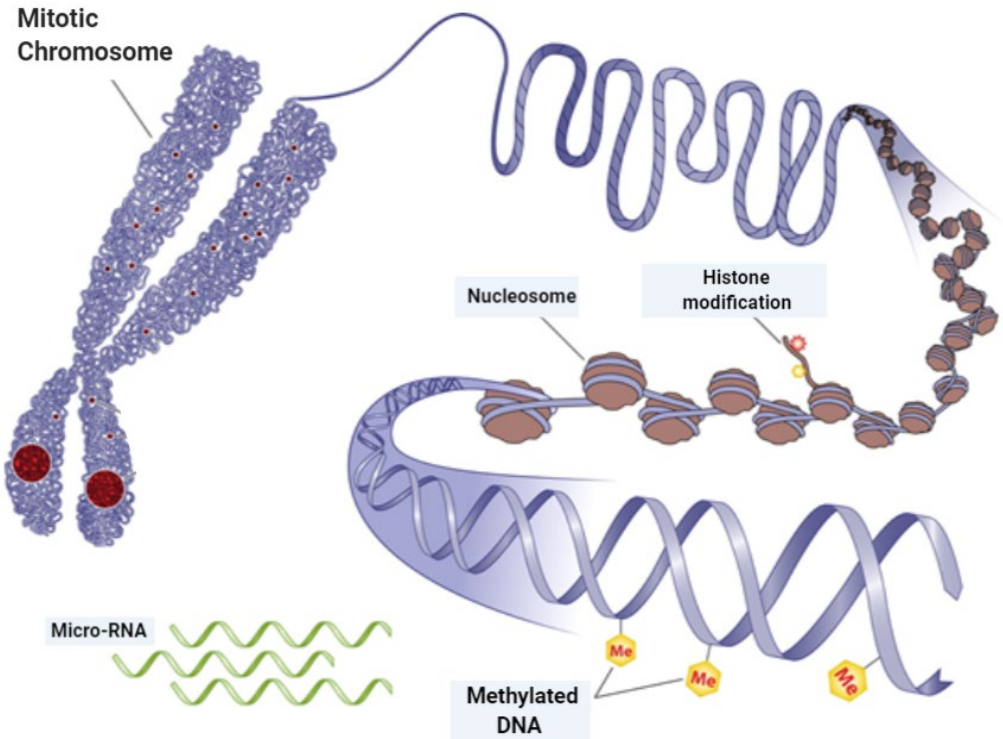


Figure 3 Epigenetic Modifications: DNA is around histone proteins to form chromosomes. Epigenetic modifications occurring on DNA strands are known as DNA methylation and epigenetics modification occurring on histones are known as histone modification. Micro-RNA also perform epigenetic modification that affects gene expressions [101]

1.4.2 DNA Methylation

Methylation is an epigenetic mechanism by which a methyl group (CH_3) is added to the 5-position of cytosine to form 5-methyl cytosine (5mC) in CpG dinucleotides (cytosine followed by a guanine and separated by a phosphate bond). A small amount of methylation can occur at CpNpG sequences, where N is either A, T, or C. However, the majority of methylation that occurs at CpG dinucleotides in the human genome makes other potential methylation sites negligible when assessing methylation levels in human DNA [114]. Moreover, non-CpG methylation is seen in embryonic stem cells, but lost in mature tissues [115]. Methylated cytosine (5mC) is estimated to comprise around 1% of the nucleic acid content in the human genome. Overall, the human genome is depleted of CpG sites that are potentially caused by a mutagenic deamination of 5mC to thymine [116]. Most CpGs on the DNA strand are methylated; and 60-90% of all CpGs are methylated in mammals [117].

The DNA methylation machinery, like all epigenetic processes, is orchestrated by three main players categorised as *writers, readers, and erasers*[118].*The writers in DNA methylation are a group of transferases that introduce new sites of methylation, and their role is crucial during embryonic development and cell differentiation*[119, 120]. *These are known as* DNA methyltransferases (*DNMTs*). *The readers in DNA methylation recognise the change made by readers and interpret the modifications introduced*[120]. Finally, *erasers; although DNA methylation can be reversed or "erased" through different de-methylation mechanisms, the main group responsible for de-methylation are* (Ten-Eleven Translocation 1,2, and 3) TETs[120]. This will be explained in detail in the following sections.

DNA methylation is catalysed by DNMTs which catalyse the deposition of a methyl group on the carbon 5 of the cytosine ring using S-adenosylmethionine as a methyl donor. Methylation can inhibit gene transcription causing gene silencing and an increase of methylation levels is known as hypermethylation. Loss of methylation can lead to an increase in gene expression, and a decrease in methylation levels is known as hypomethylation[119].

This biological process stably modifies genetic expression in cells through cellular divisions and differentiation from embryonic stem cells into specific tissues. DNA methylation plays an important role in numerous physiological processes including X chromosome inactivation, genomic imprinting, ageing, repression of repetitive elements, and common diseases such as cancer[121, 122]. Notably, depending on the underlying genetic sequences of the genes methylated and the region of alteration, DNA methylation exerts different effects on gene activities [121, 122].

1.4.2.1 CpGdinucleotides and CpG Islands

The CpG notation is interpreted as the cytosine being 5' to the guanine base[123]. Some CpGs are often grouped in clusters in the 5' promoter regions of genes, known as CpG islands. CpG islands are characterised as regions where CG are detected in more than half of its content and CpGs are detected in more than 60% of its content [124]. Within the human genome there are estimated to be approximately 27,000 CpG islands that are associated with 50-60% of genes, including housekeeping genes such as beta-actin [125, 126].

Detected ratios of CpG dinucleotides on the human genome are relatively low; however, CpG islands on long repetitive sequences are highly detected. The loci of CpGs and their methylation status have a huge impact on governing gene expression and dictating cell phenotypes. This is highly important in cancer research especially when methylation occurs at CpGs on promoter regions of genes involved in tumour suppression [127]. This will be explained more in the following section. On the other hand, CpGs at long repetitive sequences (e.g. LINE-1) are usually methylated in normal differentiated cells to conserve genomic stability and gene expression during cell division [128].Figure 4 illustrates the loci of methylation within the cancer and normal genome.

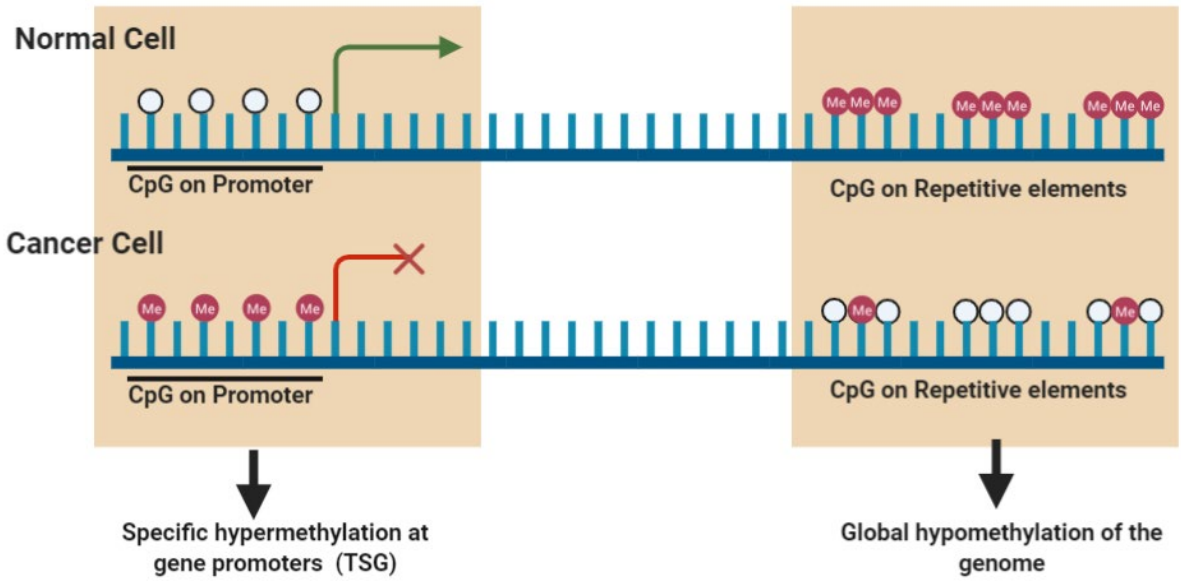


Figure 4Methylation patterns in cancer and normal genome. Promoter regions are expected to be unmethylated and active in normal cells while repetitive elements are normally methylated and active. This pattern is disrupted in cancer.

1.4.2.2 Gene Body

The gene body can be defined as the region expanding from the transcription start site to the end of the transcript[129]. Although it is still unclear how gene body methylation affects gene regulation, studies revealed the association of DNA methylation with levels of gene expression in dividing cells [121]. The bulk of the genome that includes DNA repetitive elements, such as long terminal repeats (LTR), long interspersed nuclear elements LINE, and short interspersed nuclear elements (SINE) comprise of almost half of the human DNA. These elements remain unmethylated to assure genomic stability. Loss of methylation on these elements could lead to their activation and therefore risk an insertional mutagenesis (a type of mutation) [130, 131].

1.4.2.3 DNA Methyl Transferases

DNA Methyl Transferases (*DNMTs)* catalyse the transfer of methyl group from S-adenosyl-L-methionine to cytosine residues. During DNA replication and cell division, DNA polymerase cannot distinguish between the methylated and unmethylated nucleotides; therefore *DNMT1* serves its role of copying pre-existing methylation patterns of one strand (hemi-methylated strand), to the newly synthesized strand. After DNA replication, the parental strand methylation pattern is copied to the daughter strand. This is known as maintenance methylation, and this occurs throughout the lifetime of an organism. Maintenance methylation maintains the methylation patterns set up by *de novo* methyltransferases, and therefore maintain the function of specific genes in specific cell types. Besides DNMT1's role in methylating hemi-methylated DNA, it can also repair DNA methylation [132]. DNA *de novo* methylation however, is the initiation of new methylation pattern and this mainly

occurs during early embryonic development. Dnmt3a and Dnmt3b are responsible for the *de novo* methylation of unmethylatedDNA (Figure 5) [119]. DNMTs are extensively involved, and play a crucial role in the development of stem cells and embryos. DNMTs expression is gradually reduced in cells when they reach terminal differentiation. This suggests that DNA methylation patterns in post mitotic cells are stable. Nonetheless, since epigenetics and DNA methylation are affected by environmental factors, DNMT expression can change altering DNA methylation patterns. Therefore DNMTs make attractive disease targets[133, 134].

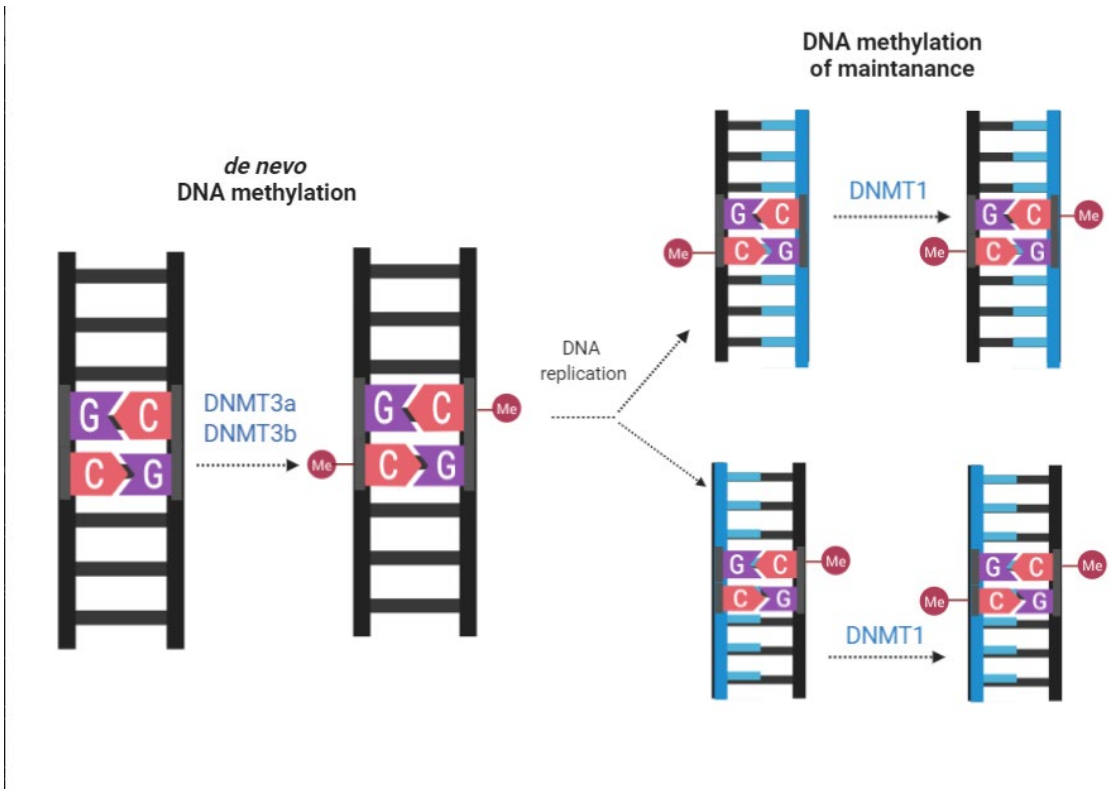


Figure 5 An illustration of DNMTs machinery, DNMT3A, and DNMT3b, are the main writers in DNA methylation process, involved in de novo methylation, while DNMT1 is the maintenance transferase, copying methylation during cell division. The methyl group on the CpGs site is illustrated by a red dot.

1.4.2.4 DNA Methylation Machinery

The binding of a methyl group to the cytosine on the promoter region can repress the transcription of genes in either one of two mechanisms. Either directly by physically impeding the binding of transcriptional proteins to the genes, or indirectly by recruiting methyl binding protein (MBDs) and other proteins of high affinity for 5mC [135]. However, the direct blocking of transcription factors through DNA methylation is rare *in vivo* [121]. The indirect mechanism is most commonly known and widely studied in the field of epigenetic inhibition of gene expression. This mechanism involves the recruitment of MBDs, the ubiquitin-like, containing PHD (Plant Homeodomain) and RING (Really Interesting New Gene) finger domains (known as UHRF proteins: Ubiquitin-like, containing PHD and RING finger domains), and the zinc-finger proteins. The recruitment of these proteins leads to chromatin remodelling[136]. DNA methylation can also enhance expression through the blockade of binding between repressor proteins and a silencer element on a promoter region of a gene[137].

1.4.2.5 DNMTs and Gene Expression

DNA methylation is an essential process for the normal control of the cell. Maintenance of methylation preserves the function of the mother cell to the daughter cell. DNA methylation is also responsible for X chromosome inactivation and genomic imprinting[138]. *De novo* methylation is crucial for cell differentiation. Therefore, during embryonic development, DNMTs are found to be extensively active; however, after cell differentiation is established, DNMTs activity is greatly reduced [121]. However, over expression of DNMTs has been recorded in multiple cancers. In fact, a study associated the over expression of DNMT1 and

DNMT3b with tumourigenesis when comparing mRNA of DNMTs in tumour and normal tissues [139]. Aberrant DNMTs expression can lead to cancer, through alterations of gene expression either by downregulating, or upregulating certain genes through hypermethylation or hypomethylation[121]:

1.4.2.5.1 DNA Hypermethylation

Hypermethylation is a term that describes the increase in methylation levels, which in cancer occurs at CpG islands of tumour suppressor promoter regions, which are protected from methylation in the normal mammalian genome. Hypermethylation of tumour suppressors causes a repression of gene transcription and potentially leads to gene silencing. The importance of CpG island hypemethylation in cancer lies in the frequency of the process and the nature of the genes involved [140]. In 1986, for example, the calcitonin gene was found to be hypermethylated and down regulated in small lung carcinoma. This discovery helped identify specific gene hypermethylation as a form of gene silencing in cancer [141].

The most commonly studied and identified epigenetic modification in cancer is the aberrant methylation of promoter regions. Hyermethylation is commonly associated with gene silencing including suspected tumour suppressor genes; e.g. *RASSF1A* (RasAssociation (RalGDS/AF-6) domain family member 1), *CDH1* (E-cadherin), and *CDKN1A* (cyclin-dependent kinase inhibitor 2A). Studies have proposed that genes can be inactivated through methylation as effectively as they are inactivated through mutations[121]. Researchershave also suggested that methylation associated silencing could be one of the cancer-predisposing hits described in Knudson's two hit hypothesis [142].

Hypermethylationmay contribute indirectly to mutations. The diversity of the genes affected by this process, the frequency of this process and the large repertoire of the carcinomas previously shown to occur on methylated promoter CpG islands all illustrates the crucial role of epigenetic alterations in propelling cancer initiation and progression [143].

1.4.2.5.2 DNA Hypomethylation

DNA hypomethylation of promoter regions seems to occur much less frequently that hypermethylation of these regions [144]. Nonetheless, in cancer, hypomethylation, or loss of methylation of promoter regions and transcriptional control sequences could lead to the activation of oncogenes. The hypomethylation of oncogenes in cancer is often associated with the global hypomethylation of the genome or satellite DNA. Another loci of hypomethylaion frequently found in cancers is the hypomethylation of the repetitive elements [145]. Activation of these repeated sequences leads to the risk of insertional mutagenesis mutation[130, 131].

The first aberrant epigenetic modification discovered in cancer was the loss of methylation in 1982; this was presented at a symposium at Johns Hopkins by Feinberg and Vogelstein who found that a significant proportion of CpG methylation was lost in every tumour type they studied compared to normal tissues [146]. This has led studies to investigate the global hypomethylation associated with different cancers including breast cancer. LINE repetitive elements are one of the most studies regions in cancer found to lose methylation and contribute to genome instability[147]. Other studies focusing on specific tumour hypomethylation found genes like melanoma antigen E (*MAGE*), urokinase-type

plasminogen activator *(uPA), and synuclein gamma gene SYNUCLEIN γ to be hypomethylated in breast cancers.* [148-150]

1.4.2.6 Demethylation

DNA methylation is a reversible mechanism. The removal of the methyl group, demethylation, is a necessary process for epigenetic modification of genes involved in cell development, cell differentiation, and many disease mechanisms including tumour progression. Demethylation process can be either passive or active, or a combination of both[120].

Passive demethylation

Passive DNA methylation occurs in dividing cells, on the newly synthesized DNA strand via *Dnmt1*. As *Dnmt1* maintains methylation through cell division, its inhibition, dysfunction, or down-regulation results in an un-methylated newly incorporated cytosine. Following several cell divisions, the overall methylation levels are reduced, and in some instances lost (Figure 6) [151].

Active demethylation

Active DNA demethylation happens through a series of chemical-enzymatic reactions to cleave the carbon-to-carbon covalent bond between methyl group and cytosine reverting 5mC to a naked cytosine. This can occur in both dividing and non-dividing cells. Although it is generally agreed upon that the 5mC is modified by deamination and/or oxidation reactions to a product that is recognised by the base excision repair (BER) pathway, the

specific enzymes involved during this process are still debated. The following mechanisms underlying this process have been suggested:

a) The first mechanism suggests creating a G/C mismatch. Modifying 5mC at the amine group results in deamination of the amine group to carbonyl group. This is aided by AID/APOBEC (Activation-Induced cytidineDeaminase/APolipoprotein B mRNA-editing enzyme complex). The 5mC is then converted into thyminecreating a mismatch of G/T that can be corrected through the BER pathway [152].
b) A second active DNA demethylation mechanism suggests the role of Tet1, Tet2, and Tet3 (ten-eleven translocation 1,2, and 3) in the addition of a hydroxyl group onto the methyl group of 5mC forming 5hmC [153, 154]. The conversion of 5hmC back into cytosine is mediated by one of two mechanisms; the first mechanism suggests the conversion of 5hmC first to 5-formyl-cytosine by oxidation and then to 5-carboxy-cytosine catalysed by Tet enzymes [155].
c) Another mechanism, involves deamination of 5hmC to 5-hydroxymethyl-uracil by AID/APOBEC (Figure 6)[156]. Also, 5hmC may regulate gene expression. In fact, the conversion of 5mC to 5hmC may impair the binding of the repressive methyl-binding protein MeCP2 [157].

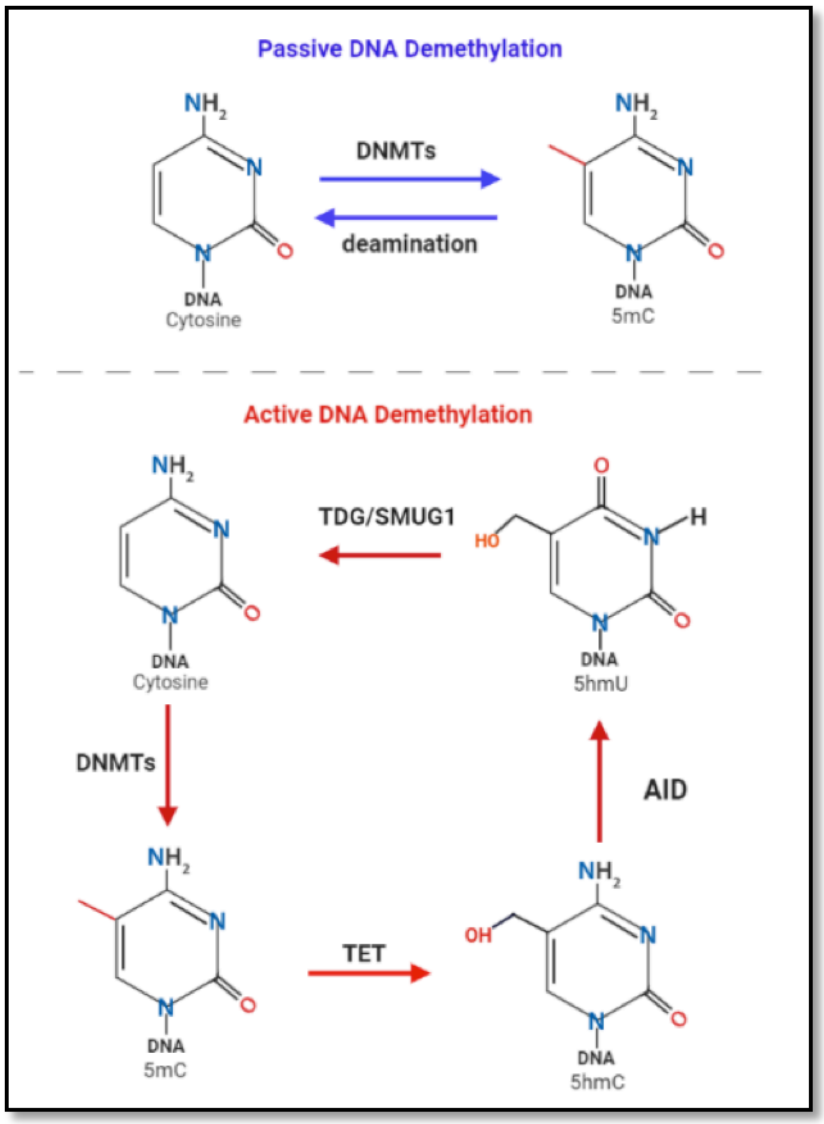


Figure 6 Passive and active de-methylation process. Passive demethylation causes loss of methylation through the absence of DNMt1. Active DNA demethylation involves enzymatic conversion to convert 5mC to naked cytosine through TET enzymes.

1.5 Epigenetics of Breast Cancer

Aberrant methylation in cancer is characterized by hypermethylation of tumour suppressors, hypomethylation of oncogenes, and global DNA hypomethylation (methylation of repetitive elements). Although in cancer research, DNA methylation is almost always considered an epigenetic alteration that inhibits gene transcription, it can also act to increase transcriptional levels. This depends on whether the methylation occurred on a negative or positive regulatory element [131].

Epigenetic modification and DNA methylation are important processes in carcinogenesis (Figure 4). For example, promoter hypermethylation of tumour suppressors may cause a loss of adhesion, lack of apoptosis, defects in DNA repair, and alteration in different functions of tumour suppressor depending on the type of gene. Loss of genomic methylation also contributes to carcinogenesis by loss of imprinting and uncontrolled overgrowth of cancer cells, inappropriate cell-type expression, and genome fragility.

The cancer genome in general is characterised by hypomethylation events on repeated regions, as well as specific hypermethylation of tumour suppressors and specific hypomethylation of oncogenes. In the later sections, more will be explained about aberrant methylation in breast cancer.

In breast cancer, the genes found to be hypermethylated are still of interest to many researchers, linking their epigenetic modification to breast cancer progression [158]. The mostly commonly reported methylated tumour suppressors in breast cancer include; Ras association (RalGDS/AF-6) domain family member 1 isoform A (RASSF1A), Breast Cancer 1

(BRCA1), cyclin dependant kinase inhibitor 2A (CDKN2A), retinoic acid receptor β (RARβ),cyclin D2 (*CCND2*), adenomatous polyposis coli (*APC*) [104, 159, 160]. Different studies have also demonstrated gene specific hypomethylation in breast cancer including interleukin 10(*IL-10*), multidrug resistance 1(*MDR1*) and Toll-like receptor 9(*TLR9*). These genes are found to be highly expressed in cancer cells that show high overall hypomethylation levels [161, 162].

The methylation pattern is currently being studied in association with theclinicopathological features including include breast cancer subtypes, age, grade, metastasis, and survival rate [163]. Different subtypes of breast cancer exhibit different gene signatures characterized by hypermethylation of certain tumour suppressers, or hypomethylation of certain oncogenes and when associated with clinicopathologic features these may have prognostic value. Methylation of genes can be crucial in determining subtypes of breast cancer and therefore sustaining the methylation pattern is important to preserve tumour phenotypes and clinical outcomes [163]. For example, *CDKN2A*, a cell cycle regulator, is one of the first tumour suppressors found to be methylated in breast cancer. However, since no evidence showed that *CDKN2A* is methylated in ductal carcinoma *in situ* (DCIS), it can be suggested that the methylation of *CDKN2A* may occur during the progression of invasive ductal carcinoma (IDC). On the other hand, *CCND2*, which is also a cell regulator gene, is found to be hypermethylated in the majority of *DCIS*, implying that methylation of this gene is an early event contributing to the initiation of breast tumourgenesis. Hypermethylation ofSecretoglobin Family 3A Member 1 (*SCGB3A1),* Retinol Binding Protein 1 *(RBP1),*Ras Association Domain Family Member 1 *(RASSF1),* Prostaglandin-Endoperoxide Synthase 2 (*PTGS2),* Proopiomelanocortin *(POMC),* Paired Box 6 (*PAX6),* Membrane

Metalloendopeptidase *(MME),* Homeobox A11 (*HOXA11),* and Fatty Acid Binding Protein 3 (*FABP3)* is commonly associated with Luminal A, Her2 + and p53 wild type breast cancer; whereas hypermethylaton of Serpin Family A Member 5 *(SERPINA5),* Thy-1 Cell Surface Antigen (*THY1),* Trefoil Factor 1 (*TFF1),* Retinoic Acid Receptor Alpha *(RARA),* Septin 5 *(SEPT5),* Mesoderm Specific Transcript *(MEST) ,* breakpoint cluster region (*BCR),* and DAB2 Interacting Protein (*DAB2IP*), is associated with basal-like HR-, and p53 mutant-tumours [163]. DNA methylation signatures and biomarkers may help better classify breast cancer into new breast cancer sub groups that are not classified by current expression subtypes revealing the genomic basis for heterogeneity of breast cancer [164]. Evidence suggests that DNA methylation signatures can also help predict prognosis and survival beyond what is currently possible [165].

Since hypermethylation is considered an early event in tumorigenesis, DNA methylation can be used as a biomarker for early detection of cancer[166]. DNA methylation can be used as a biomarker for survival and help tailor therapies for patients as DNA methylation is strongly associated with tumour progression [167, 168]. However, this is highly dependable on the tumour type and individual cases.

1.5.1 Epigenetic Therapies of Breast Cancer

DNA methylation and chromatin modification are reversible mechanisms and therefore, it is possible to design compounds to readjust alterations in gene expression to restore normal expression. Epigenetic inhibitor drugs have been approved for the treatment of haematological malignancies such as demethylatingagents 5-azacitidine (known as Vidaza) and 5-aza-2'-deoxycitidine (known as Decitabine). They work through blocking DNMTs[134].

These treatments have been shown to improve cancer management in haematological malignancies particularly[169]. Many more DNMT inhibitors are being designed and under trail such as 5-fluoro-2'-deoxycytidine[112], however, due to their common mechanism of action they tend to have an effect on the transcription of off-target, non-cancer associated genes, or can activate oncogenes [170]. The affects and benefits of epigenetic drugs on solid tumours are being assessed and are showing some signs of success in non-small cell lung carcinoma[171]. More clinical studies are needed to approve the use of epigenetic modulators in the breast cancer therapy regime.

1.6 Epigenetics and Ageing

A complex interaction among different biological affects the ageing process. The new advanced quantitative techniques in the field of epigenetics allowed an investigation of the impact of epigenetic alterations, mainly DNA methylation, on the ageing process. Studies have characterised the ageing DNA by gradual and profound demethylation of the genomic DNA as well as hypermethylation of promoter regions (non-directional pattern) (Figure 7) [172]. This phenomenon is known as "epigenetic drift". Specific DNA regions exhibit directional epigenetic changes in aged individuals implying the important role of these changes in the ageing process. Like epigenetic modifications, DNA methylation is highly susceptible and is affected by extrinsic factors including but not limited to environmental exposure, lifestyle, stress, and diet. These factors can affect the life span positively or negatively [173]. Several studies studying the "epigenetic drift" phenomenon, have demonstrated that over time, the similarity of epigenetic patterns among young individuals almost disappear. This leads to the diversity in methylome found in the elderly population.

During ageing, the continuous accumulation of epigenetic damage caused by environmental stimuli and spontaneous stochastic errors result in alteration of the DNA landscape [173].

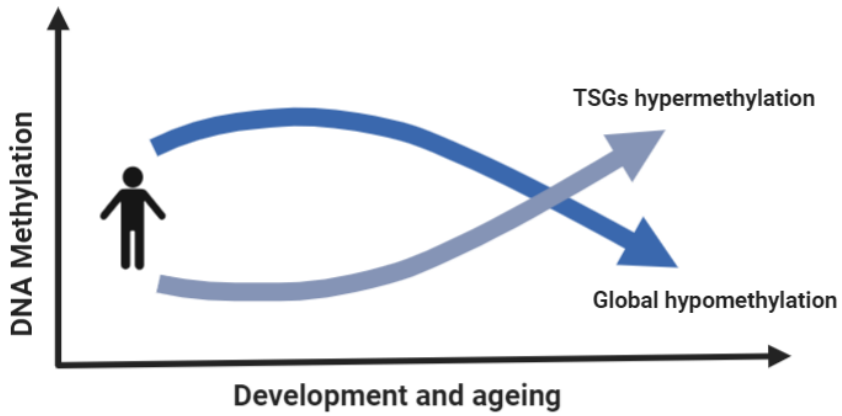


Figure 7DNA methylation changes during oncogenic development and aging. Global DNA methylation decreases with age in many tissue types, while several specific regions of the genomic DNA become hypermethylated. TSGs, tumour-suppressor genes

Global DNA hypomethylation at repetitive sequences is a prime event in ageing [173, 174]. However, the effect of hypomethylation during ageing is not the same at different repetitive elements. For instance, hypomethylation of LINE-1 repeats is not affected by ageing while Alu and HERV-K sequences tend to lose methylation at different ages [174, 175]. Specific gene promoter regions' hypomethylation, likeinterleukin 17 receptor C(*IL17RC),*andintegrin alpha L *ITGAL* loses methylation in an age-dependant manner. Loss of methylation of these genes and their transcriptional activation by demethylation has been suggested to activate autoimmune responses [176, 177]. A significant decrease in methylation of CpGs is found in the nonagenarian/centenarian genome compare to new-born DNAs [178]. These findings

suggest that age-associated hypomethylation is highly associated with transcriptional regulation, disrupting normal expression of genes and potentially leading to different diseases.

Another part of the epigenetic drift is hypermethylation. Ageing is also characterised with progressive gain of DNA methylation that leads to gene suppression and silencing, meaning that age-associated gene hypermethylation occurs at the promoter regions of particular genes. Hypermethylation often occurs at genes that are key in the process of ageing or play crucial roles in age-related phenotypes/diseases and development (protocadherins, homeobox genes) and signalling (MAPK pathways' members, ryanodine receptors) associated with cancer, longevity, and senescence [173, 179]. Specific examples include the oestrogen receptor (ESR1), RAS association domain family 1A (RASSF1A), p16/CDKN2A, suppressor of cytokine signalling 1 (SOCS1), hypermethylated in cancer d1 (HIC1), caspase-8 (CASP8), glutathione S-transferase pi (GSTP1), insulin-like growth factor II (IGF-II) and adenomatosis polyposis coli (APC). Hypermethylation of these genes has been identified a number of cancer types including breast cancer [180].

Although the use of modern methylation techniques has helped characterise hypermethylation as a prevalent event in ageing, age-related hypermethylation seems to be occurring at low magnitude compared to hypomethylation events.

1.7 Stress and Epigenetics

Studies correlating epigenetic responses to stress have mainly focused on the central systems and on epigenetic alterations of the promoter region of glucocorticoid receptor gene (NR3C1) and the resulting function and expression in the HPA axis [181].

Chronic stress has been associated with increased DNA methylation and histone acetylation of genes regulating visceral pain sensation in the peripheral nervous system of rats [182]. The same study suggested that DNMT1 is inducible in response to chronic stress conditions, indicating the potential use of DNMT1 as a potential therapeutic target for treating chronic-stress associated visceral pain [182].

Emerging studies of mind-body therapies are increasingly exploring epigenetic mechanism and gene expression changes. For example, a recent study demonstrated the effects of yoga intervention on markers of inflammation and DNA methylation in chronically stressed women and showed reduced methylation of the tumour necrosis factor (TNF) gene [183].

1.8 Glucocorticoids and Epigenetics

Studies investigating the effect of GC's on genes central to the HPA axis have demonstrated that chronic exposure leads to epigenetic modifications. For example, AtT-20, a cell line derived from a mouse pituitary tumour, incubated with dexamethasone showed a dose-dependent decrease in *DNMT1* expression in response to treatment [184]. The same study found that treating AtT-20 cells with dexamethasone resulted in a loss of methylation on FK506 Binding Protein 5 (*FKBP5)* altering its gene expression[6]Alteration of *FKBP5* resulted in enhanced expression after GR activation, leading to more GR resistance, diminished negative feedback, and a prolonged stress hormone activation after a stressor, and this was observed as anxiety-like behaviour when analysed *in vivo*[6]. Another study, correlated dexamethasone treatment of 19 individuals and observed a dynamic change at the *FKBP5* locus in human peripheral blood cells [185] . Additionally, treating rat hepatoma cells with dexamethasone led to demethylation at the tyrosine aminotransferase (*Tat*) gene [186].

Although several studies have been summarised illustrating the effects of GC's on DNA methylation in different models, little is known about the effects of cortisol on the epigenetic modification of breast cancer. Investigating how acute or chronic exposure of GC's such as cortisol contributes to the methylation patterns in breast cancer tissues with different aetiologies is highly innovative and remains to be evaluated. Therefore, this project examines the influence of stress hormones on DNA methylation in cells and animal models of breast cancer. The project also identifies the influence of changes in DNA methylation as a result of stress and ageing on the molecular biology of breast cancer.

2. Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Cell lines

2.1.1.1 Triple Negative Breast Cancer cell lines

We used the following breast epithelial cell lines;MDA-MB-231, MDA-MB-157, Hs-578T, and BT-549. The cell lines were obtained from the American Type Culture Collection (ATCC). Cells from these cell lines do not express ER, PR, or HER-2, and hence represent a triple negative breast cancer type (TNBC). All the afore-mentioned cell lines are epithelial cell breast cancer cells. MDA-MB-231 cell line is derived from adenocarcinoma. MDA-MB-157 cell line is derived from medullary carcinoma. Hs-578T and BT-549 cell lines are derived from a primary tumour of ductal carcinoma. These cells have been previously reported to express relatively high levels of the GR [187].MDA-MB-231, MDA-MB-157, and Hs-578T cells were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM/F-12 in a 1:1 ratio) with 10% foetal bovine serum (Gibco). BT-549 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) with 0.023U/ml insulin and 10% foetal bovine serum (Gibco). When treating cells with glucocorticoids, phenol red-free media and 10% charcoal stripped Foetal Bovine Serum was used (Sigma) unless stated otherwise.

2.1.1.2 ER+ Breast Cancer cell lines

The MCF-7 and T47D cell lines were also obtained from ATCC. MCF-7 is derived from adenocarcinoma retaining some of their differentiated epithelial characteristics, while T47D is derived from ductal carcinoma. Cells from MCF-7 cell line and T47D cell line express ER, PR, but not HER-2, and hence represent a luminal subtype of breast cancer. MCF-7 cells express higher levels of GR, while in comparison; T47D cells have low GR expression [187].

Cells from these cell lines were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM/F-12 (1:1)) with 10% foetal bovine serum (Gibco). When treating cells with glucocorticoids, phenol red- free media and 10% charcoal stripped foetal bovine serum was used (Sigma) instead, unless stated otherwise.

2.1.1.3 Epithelial Breast Cell line

The MCF10-A cell lines were also obtained from the ATCC. MCF10A are non-tumourigenic epithelial cell lines that are derived from fibrocystic disease. MCF10A expresses GR and responds to steroid hormone treatment (Mikosz, 2001).Cells from these cell lines were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM/F-12 (1:1)) with 10% foetal bovine serum (Gibco) and 1% HuMEC supplement (Gibco).

2.1.1.4 Mouse cell line

The murine 66CL4 cell line was a kind gift from Dr. Erica Sloan, Monash University, Australia. The 66CL4 cell line is a derivative of the 4T1 cell line, and represents ER- breast cancer type. Cells were grown in Minimum Essential Media (MEM) (Gibco) with 10% foetal bovine serum (Gibco).

2.1.2 Cell Passaging and Counting

All cell culture work was performed in a laminar flow hood under appropriate aseptic conditions. Cells were grown in T-75cm^2 filtered tissue culture flasks, and passaged when cells have reached a confluency of 70-90%. When passaging, the growth medium was discarded and cells were incubated with 2-5ml of 0.25% (v/v) trypsin-ethylene diamine Tetra acetic acid (EDTA) solution (Gibco) and incubated at 37°C for approximately 3 minutes until

the cell monolayer had detached from the flask. A 4-10 ml of the appropriate growth media is then added to deactivate the trypsin. The cell suspension was then collected and centrifuged at 500 x g for 5 minutes at 25°C. Cell pellet was re-suspended in 1 ml of fresh growth medium. Cells were then counted using a bright-line haemocytometer (Hausser Scientific, Horsham, PA, USA). The average of four different counts was used to calculate cell numbers in a 1 ml suspension. After cell counting, cell suspensions were split in the appropriate ratio or seeded into multi well cell culture plates to be used for later experiments. Cells were always incubated at 37°C and 5% atmospheric CO_2in humidified air.

2.1.3 Hormone Treatment

Prior to hormone treatment, culture media was replaced with phenol-red-free media of the appropriate type of media containing 10 % charcoal stripped media for at least 48 hours. To mimic physiologically relevant concentrations of hydrocortisone, hydrocortisone (Sigma Aldrich, UK) was diluted from stock solution concentration of 50 µM in appropriate culture medium to a final concentration of 5 µM. Growth media were replaced every 24 hrs with fresh hydrocortisone diluted in media for 20 days unless stated otherwise. RU-486 was used as an antagonist for GR to test for receptor specificity. RU-486 (Sigma, UK) was dissolved in absolute ethanol and diluted in water then in appropriate culture media to achieve a final concentration of 1µM. RU-486 was added 30 minutes prior to hydrocortisone treatment.

Dexamethasone (Dex) (Sigma Aldrich, UK) was diluted in absolute ethanol and further diluted in appropriate culture media to achieve final concentrations of 1µM, 0.5µM, and 0.25µM. Growth media were replaced every 24 hrs with Dex diluted in media.

2.2 Cell Viability

2.2.1 Principle of MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a widely used colorimetric assay for assessing cell viability through assessing cell metabolic activity. When the dye of the tetrazolium compound is reduced to formazan crystals in the mitochondria, a purple colour is produced, reflecting the number of viable cells.

2.2.2 MTT assay

Cells were plated at a density of 3000 cells/200µl in 96-well plates in hextuplicatesand incubated for 24 hours. Cells were then treated with either 0.1µM Fulvestrant (Sigma Aldrich, UK), 1µM Tamoxifen (Sigma Aldrich, UK), or/and 10nM Estradiol. After 7 days of incubation, the culture media was replaced with 200µl of 0.2 mg/ml MTT powder dissolved in the appropriate media of the cell line. Plates were incubated with MTT for 2 hours at 37°C protected from light. The MTT solution was then removed and 200 µl of dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. The absorbance was read using a spectrophotometer at OD=495 nm. Considering that the absorbance is proportional to the number of viable cells, the viability was expressed as a percentage of the control wells in each experiment.

2.3 Nucleic Acid Extraction and Quantification

2.3.1 RNA extraction from cells

RNA was extracted from cell pellets using RNeasy mini Kit (Qiagen Cat No.74104) according to the manufacturer instructions. Briefly, harvested cells were lysed using 350µl of RLT buffer and an equal volume of 70% ethanol was added to the lysate. The solution was then

transferred to spin columns provided in the kit and centrifuged for 15s at 8000 x g allowing RNA to bind to the filters in the columns. Wash buffers provided in the kit were then added to wash and purify RNA. Spin columns were centrifuged at 8000x g and RNA collected in a clean RNA/DNA-free tube and diluted in 30-50 µl of RNase-free water. RNA wasthen stored at -80°C until further use.

2.3.2 RNA extraction from Tissue

RNA was extracted from cell pellets using a spin-column method from RNeasy mini kit (Qiagen Cat no. 74104). Tissue was stored in RNA later and 30 mg of tissue was used for RNA extraction. The tissue was homogenised in buffer RLT on ice using IKA-T10 ULTRA-TURRAX homogeniser. Alternatively, tissues were physically disrupted using syringes. An equal amount of 70% ethanol was added to the lysate, and transferred to the spin-column. Wash buffers provided in the kit were then added to wash and purify RNA. Spin columns were centrifuged at 8000x g, and RNA collected in a clean RNA/DNA-free tube diluted in 30-50 µl of RNase-free water. RNA is then stored at -80°C until further use.

2.3.3 DNA extraction from cells

DNA was extracted from 1 x 10^5 cells using Gentra Puregene kit (Qiagen Cat No: 158767) according to manufacturer's manual. The protocol for 'DNA Purification from Cultured Cells' was used: Cells were trypsinized, harvested, and re-suspended in 20µl of culture medium. The cells are then lysed in cell lysis solution provided in the kit and incubated in RNase-A solution for 5 minutes at 37°C to obtain RNA-free DNA. The lysate was then vortexed for 20 seconds at high speed with protein precipitation solution and centrifuged for 1 minute at 14,000 x g. The supernatant was then transferred to 300µl of isopropanol and the mix

inverted several times until white strands are visible. The DNA was incubated in DNA hydration solution to dissolve. Samples were stored at -20°C until required.

2.3.4 Quantification of Nucleic Acids

DNA and RNA were quantified using NanoDrop 2000 spectrophotometer from NanoDrop Technologies (Thermofisher UK). This spectrophotometer uses Beer-Lambert law to correlate absorbance with RNA/DNA concentration. RNase free water and DNA hydration were used as blanks (reference for zero absorbance) when quantifying RNA and DNA respectively. The measurements were taken using 1 µl of sample loaded into the spectrophotometer and absorbance was read at 260 nm and 280 nm. Concentrations were noted in ng/µl. The 260/280 helps assess the purity of DNA and RNA preparation; DNA was considered pure when 260/280 was ≈ 1.8 and RNA considered pure when 260/280 ≈2.0.

2.3.5 DNA quality assessment using gel electrophoresis

Gel electrophoresis is a method used to check the quality and integrity of DNA by moving negatively charged nucleic acid molecules through an agarose matrix with an electronic field (electrophoresis).This separates DNA fragments according to their size, with shorter molecules moving faster and migrating further than longer ones, allowing visualisation of any fragmentation and therefore assessing the quality of DNA.

After DNA extraction, samples were mixed with 6x DNA gel loading dye (Thermofisher, UK)(1:5 ratio) and loaded to gels containing 1% agarose in 1 xTBE (Tris Borate EDTA) buffer (Biobase) and a final concentration of 0.5 µg/ml of ethidiumbromide was added to the gel. Gel Pilot 1 Kb blue was used as a molecular weight standard. The electrophoresis was

performed in 1 xTBE buffer at 120 V for 1 hour. Ethidium bromide – DNA complexes were visualized under a UV transilluminator (GelDoc XR, Bio-Rad).

2.4 Gene expression analysis

2.4.1 cDNA synthesis

cDNA was synthesised from 1µg total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosciences Cat. No. 4368814), or using QuantiTect Reverse Transcription Kit (Qiagen)as per manufacturer's instructions.The kits provide a gDNAwipe-out buffer that eliminated genomic DNA from the sample. The sample was mixed with reverse transcriptase, reverse transcription buffer and reverse transcription primer mix (all provided in the kit). The entire reaction occurred at 42°C and was inactivated at 95°C. The final reaction product was then diluted in RNA/DNA free water (1:10) for the use of PCR.

2.4.2 Primers

For SYBR green-based expression analysis of individual genes, bioinformatically-verified primers were bought from Qiagen. Primers include ESR1, DNMT1, DNMT3a, DNMT3b, PER2, and LPL, GAPDH, and β-Actin. Primers are provided in a lyophilized mix of forward and reverse primers and are reconstituted in 1.1 ml of (Tris EDTA) buffer, pH 8.0 to obtain a 10x assay solution.High efficiency of primers (>90%) is guaranteed by QIAGEN.

2.4.3 Real-time quantitative expression PCR

To assess the change in gene expression in cells, real-time PCR was performed. SYBR green-based assay was used to study gene expression from cDNA synthesized from cells, or tissue.

Work was carried out by the author of this thesis at the University of Brighton, UK and King Faisal Specialist Hospital and Research Centre (KFSHRC), Riyadh. Two different PCR machines were used with the appropriate compatible consumables. When the Viia-7 Real Time PCR System from Applied Bio systems was used at KFSHRC, master mix from RT² SYBR Green ROX qPCRMastermix (Qiagen Cat No. 330523) was prepared according to **Table 2**.The cycling conditions were as follows; 10 minutes at 95°C, 40 cycles of [15 seconds at 95° C, 35 seconds at 55°C, and 30 seconds at 72°C]. Fluorescence data collection was performed at the annealing step (55°C). Dissociation (melting) curve analysis was performed using the default-melting curve of the PCR instrument.

Table 2 PCR components mix for one reaction

Component	Volume
RT^2 SYBR Green Mastermix	12.5 µl
cDNA synthesis reaction	1 µl (100 ng)
RT^2qPCR Primer Assay (10 µM stock)	1 µl
RNase-free water	10.5 µl
Total Volume	**25 µl**

When the Rotor-Gene Q PCR instrument from Qiagen was used, master mix from Rotor-Gene SYBR Green PCR kit (Qiagen Cat No. 204074) was prepared according to Table 3. The cycling conditions were as follows; 95°C for 5 minutes, 40 cycles of [95°C for 5 seconds, and

60°C for 10 Seconds]. Fluorescence data collection was performed at the annealing step [119].

Table 3 PCR Components Mix for one reaction (Rotor -Gene)

Component Final concentration	Volume/reaction	Final concentration
2x Rotor-Gene SYBR Green PCR Master Mix	12.5 µl	1 x
Primers (QuantiTect Primer Assays x10)	2.5 µl	1 x
Template DNA or cDNA	1 µl	≤100 ng/ reaction
RNase-free water	9 µl	
Total Volume	**25 µl**	

2.4.4 Data Analysis

For all experiments, ready-to-use primer pairs with high specificity and amplification efficiency were used, and gene expression was analysed using the $\Delta\Delta C_t$ method. The cycle of threshold (C_t) values were collected from the instrument and used to analyse gene expression using the following equations:

$$\Delta C_t = C_{t(\text{gene of interest})} - C_{t(\text{housekeeping gene})}$$

$$\Delta\Delta C_t = \Delta C_{t\ (\text{gene of interest})} - \Delta C_{t\ (\text{standard control})}$$

2.4.4.1 Profiler array

Two PCR profiler arrays were bought from QIAGEN to study the expression of multiple genes. RNA was extracted and cDNA was synthesised for 500 ng of RNA as described earlier. Samples were then mixed with 1350 µl of SYBR Green (QIAGEN), and volume was adjusted with RNase-free water to a final volume of 2700 µl of PCR master mix. An equal volume of 25 µl of PCR components mix was loaded to each well containing the primers for different genes.

For Human Estrogen Receptor Signalling array, the following genes were studied (The definition of the following genes can be found in Appendix table 1):

AHR, AKAP1, APBB1, BCAR1, BCL2L1, BDNF, BMP4, BMP7, BRCA1, C3, CAV1, CCL2, CCND1, CITED2, CKB, CTGF, CTSD, CYP19A1, CYP1A1, EBAG9, EFNA5, EGR3, ERBB2, ERBB3, ESR2, FOS, FOXA1, FST, G6PD, GPER, HSP90AA1, IGF1, IGFBP4, IGFBP5, IRS1, JUNB, KLK3, L1CAM, LGALS1, LPL, LTBP1, MAFF, MED1, MMP9, MTA1, MYC, NAB2, NCOA1, NCOA2, NCOA3, NCOR1, NCOR2, NOV, NR0B1, NR0B2, NR2F6, NR3C1, NR5A2, NRIP1, NRP1, PDZK1, PELP1, PGR, PHB2, PTCH1, PTGS2, RALA, RARA, S100A6, SAFB, SNAI1, SOCS3, SPP1, TFF1, TGFA, TGFB3, THBS1, VDR, VEGFA, WISP2, WNT4, WNT5A.

For Human Epigenetic Chromatin Modification Enzymes array, the following genes were studies (The definition of the following genes can be found in Appendix table 1):

ASH1L, ATF2, AURKA, AURKB , AURKC, CARM1, CDYL, CIITA, CSRP2BP, DOT1L, DZIP3, EHMT2, ESCO1, ESCO2, HAT1, HDAC1, HDAC10, HDAC11, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, KAT2A, KAT2B, KAT5, KAT6A, KAT6B, KAT7, KAT8, KDM1A, KDM4A, KDM4C, KDM5B, KDM5C, KDM6B, MBD2, MLL, MLL3, MLL5, MYSM1, NCOA1, NCOA3 ,NCOA6 ,NEK6, NSD1, PAK1, PRMT1, PRMT2, PRMT3, PRMT5, PRMT6, PRMT7, PRMT8, RNF2, RNF20, RPS6KA3, RPS6KA5 ,SETD1A, SETD1B, SETD2, SETD3, SETD4, SETD5, SETD6, SETD7, SETD8, SETDB1, SETDB2, SMYD3, SUV39H1, SUV420H1, UBE2A, UBE2B, USP16 USP21, USP22, WHSC1.

2.5 DNA methylation profiling using MethylScreen technology

2.5.1 Principle of MethylScreen technology

The method employed by the EpiTect Methyl II PCR system is based on the detection of the remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. Enzymes are designed to digest either methylated cytosines or unmethylated cytosines, a sample with no enzymes is also used for normalisation and a sample with both digestion enzymes is also tested. DNA methylation was quantified by real time PCR using ΔCt method. The relative quantification (percentage) of methylated DNA, and unmethylated DNA were determined by comparing the results of each digested sample with the control sample that has no enzymes (Mock sample).

2.5.2 Sample preparation

An EpiTect Methyl II DNA Restriction Kit ID: 335452 wasused. Briefly, 4 μg of DNA measured by nanodrop is mixed with the restriction buffer provided in the kit. The DNA was equally distributed into 4 tubes 1) control Mo (mock) and no enzymes wereadded; 2) Ms, Enzyme A was added; enzyme A is a methylation sensitive enzyme that does not cleave methylated cytosine residues, and cleaves unmethylated cytosine residues; 3) Md, Enzyme B was added which is a methylation dependent enzyme that cleaves methylated cytosine residues and 4) Msd, both restriction enzymes were added. This helped to detect different fractions of methylated DNA. The samples were incubated in a heating block overnight at 37°C. The digestion was stopped by increasing the temperature to 65°C for 20 minutes. The samples were mixed with SYBR green and loaded into the PCR array.

2.5.3 EpiTect methyl ll PCR array

An array of 94 tumour suppressor gene promoters whose methylation pattern changes have been reported in the literature to be associated with different types of tumours. Genes included in the chosen PCR array are (The definition of these genes can be found in Appendix Table 3):

APC, BRCA1, CDH1, CDH13, CDKN2A, DAPK1, ESR1, FHIT, GSTP1, MGMT, MLH1, NEUROG1, PDLIM4, PTEN, RARB, RASSF1, RUNX3, SOCS1, TIMP3, TP73, VHL, WIF1, ABL1, AKT1, ATM, BCL2, BCR, BIRC5, BRAF, BRCA2, CADM1, CAV1, CCND1, CCND2, CD44, CDKN1A, CDKN1B, CDKN1C, CDKN2B, CDKN3, CDX2, CHFR, CTNNB1, DIRAS3, DKK3, DLC1, E2F1, EGFR, ERBB2, FAS, FOS, HIC1, HOXA1, HRAS, IGF2R, ING1, JUN, LOX, MDM2, MEN1, MYC, MYCN, NF1, NF2, NFKB1, NME1, OPCML, PRDM2, PTCH1, PTGS2, PYCARD, RB1, RET, SCGB3A1, SFN, SFRP1, SFRP2, SH3PXD2A, SLC5A8, SMAD4, SMARCB1, SPARC, TERT, TGFB1, TGFBR2, THBS1, TP53, TSC1, TSC2, VEGFAWT1, WWOX, XRCC1, ZMYND10.

2.5.4 Data Analysis

Ct values were collected and pasted to an EpiTect Methyl II PCR Array Microsoft Excel based data analysis template, which is available at: www.sabiosciences.com/dna_methylation_data_analysis.php. Results were calculated using ΔΔCt method.

2.6 Global DNA Methylation

2.6.1 LINE-1 Kit

Long Interspersed NuclearElement-1 (LINE-1) repeats elements are used as a surrogate marker for global genomic DNA methylationas it constitutes approximately 17% of the human genome, and around third of methylation incidents appears to be on repetitive

elements [128]. The percentage of methylated cytosine correlated with CpG residues in LINE-1 was determined as per manufacturer's manual. Briefly, DNA extracted from cells was fragmented by enzymatic digestion and hybridized to a biotinylated human LINE-1 consensus probe and immobilized onto a streptavidin-coated plate. Methylated fragments were detected using a 5mC antibody and a secondary antibody conjugated to horseradish peroxide (HRP). Using a microplatereader (Varioskan LUX Multimode Microplate Reader, UK) the colorimetric readout was quantified at 450 nm with an optical reference of 655 nm. A standard curve was generated using methylated and non-methylated DNA standards provided with the kit and the relative 5mC levels in each DNA sample were calculated manually on Excel.

2.7 Extraction, Quantification and Electrophoresis of Proteins

2.7.1 Protein Extraction and Quantification

MDA-MB-231, Hs-578T and MCF-7 cells were grown in T25 flasks. Cells were first washed thoroughly with cold PBS and lysed with RIPA Buffer (Sigma Aldrich, R0278) on ice for 2-5minutes. Cells were then scraped and collected in an Eppendorf tube. The lysate was then centrifuged at 13,000 x g for 20 minutes at 4°C. The supernatant was collected, protein content measured and the sample stored at -80°C until further use.

Proteins were measured using a Bradford assay. A standard curve of known concentrations of bovine serum albumin (BSA) (Sigma Aldrich) was prepared. Concentrations ranged between 0.25 – 2 mg/ml. 10 µl of each of BSA concentration was added to 200µl of Bradford reagent (Sigma) and mixed in a cuvette. Samples were also prepared in the same way; 10 µl of each sample was mixed with 200 µl of Bradford reagent. The absorbance was read at 595

nm using spectrophotometer (Nanodrop 2000c). Total protein concentrations were calculated by interpolating data from the standard curve.

2.7.2 Western Blotting

A total of 30 µg of protein was loaded into precast gels (4-20%) (Sigma), and were electrophoresed at 100V for 90 minutes. Gels were then transferred onto polyvinylidene fluoride (PVDF) membranes using dry transfer (Trans-blot turbo from Bio-Rad). Membranes were then blocked using 10% skimmed milk for 1 h, and then incubated with DNMT1, β-actin antibodies (1:1000) (Cell Signalling), and ERα antibody 1:500 (DAKO) in 5% skimmed milk overnight on a shaker at 4°C. Membranes were then washed with Tris buffered saline with Tween 20 (TBST) (Sigma Aldrich) 3 times for 10 minutes each, then incubated with appropriate secondary antibody (Anti-rabbit/ mouse 1:2000, Cell Signalling) and (Anti mouse/ goat 1:2000, Promega) in 10 % skimmed milk for 1 h on a shaker at room temperature. Membranes were then washed with TBST and rinsed with PBS. Signals were detected with Amersham ECL reagents western blotting detection reagent kit (GE Healthcare) and bands were imaged on an Image Quant LAS 400 imager (GE Healthcare). The intensities of the bands were analysed using ImageJ software.

2.8 In Vivo Study

All procedures were carried out according to U.K. Home Office regulations and the Animal Scientific Procedures Act (ASPA) 1986 (SI 2012/3039) and were approved by the University of Brighton Ethics Committee (AWERB). Animal experiments were carried out under project license (no. 70/8361) and my personal licence (No. IE8668D8E).All efforts were taken to maximize animal welfare conditions and to reduce the number of animals used in

accordance with the European Communities Council Directive (2010/63/EU).For stress studies in our laboratory, Mice were allowed to rest for 1 week after transport and then handled daily by the PI for 1 to weeks for the mice to become acclimatised the PI. The mice were housed in a designated quiet room. These procedures are in place to limit any external stressors, which may influence the data.

All mice were bought from Charles River. Upon arrival, mice were housed 5 per cage with food and water in ambient temperatures of 22°C and 12:12 hrs light: dark cycles. The number of cancer cells injected for both models is compatible with 'Guidelines for the welfare and use of animals in cancer research' British Journal of cancer (2010) where the minimum number of cells in the smallest volume is used. We have consulted the NC3Rs experimental design assistant to calculate the number of mice used for experiments. A total of 10 mice per group were used which gives >90% power to detect an effect size of 1.57 at the 5% confidence level. These calculations were performed in G*Power and based on a two-sided t-test to compare the two groups.

2.8.1 Syngeneic Mouse Model

An immune competent syngeneic model was used. Female BALB/c mice of 18 months, 9 months and 3 months, were ordered from Charles River. Mice were injected with 1x 10^4 66CL4 cells in 0.2 mL of PBS into the left fourth mammary fat pad. The majority of mice developed tumours. However, those that did not develop tumours were excluded from the study. Tumours took 2 weeks to establish tumour volumes of 50 mm^3 - 100 mm^3. Mice were weighed once every week and tumour dimensions were measured every other day using digital callipers. The tumour volume of each mouse was calculated using the equation

$$Volume\ mm^3 = L\ x\ \frac{W^2}{2}$$

in which L is the longest diameter of the tumour (length in mm), W is the shortest diameter of the tumour (width in mm). Mice were sacrificed when any parameter of the volume reached a maximum of 12 mm^3.Within each age group (18 months, 9 months and 3 months), mice were randomised into either a restraint stress (RS) or non- stress (NS) groups.

In order to stress the mice they were placed individually in ventilated 50 mL conical tubes for 2 hours daily at the same time from 10 am to 12 am. The mice were able to turn supine to prone but cannot turn head to tail. This is a well-established model of restraint stress that elevatescortisol levels [73, 188]. All primary tumours were harvested at necropsy.

2.8.2 Xenografts

An immune suppressed mouse model was used for this project. Female nude BALB/c mice were injected with $5x10^6$ MDA-MB-231 cells in 0.2 ml of PBS into the fourth left mammary fat pad. The cancer intake rate for this experiment was considerably low, only 44% of mice developed tumours (8 mice).Mice were weighed once every week and tumour dimensions were measured every other day using digital callipers. The tumour volume of each mouse was calculated as previously stated in section (2.8.1).The tumours took 8-10 weeks to establish tumour volumes of 50 mm^3 - 75 mm^3. When tumours reached ≈ 50 mm^3, tumour-bearing xenografts were randomised into stressed (RS) (n=4) and non-stressed (NS) (n=4) groups. Stressed mice were placed individually in ventilated 50 mL conical tubes for 2 hours daily at the same time from 10 am to 12 am. Mice were sacrificed when any parameter of the volume reached a maximum of 12 mm^3. One mouse from the non-stressed group died

of non-cancer related cause and was excluded from the study. All primary tumours were harvested at necropsy.

2.9 Data Analysis

GraphPad Prism was used for data analysis and generation of graphs. All experiments were carried out in biological triplicates and results are presented as a mean ±SEM unless stated otherwise. For grouped analysis two-way ANOVA was with either Bonferroni's or Dunnett's post-test. For continuous data assuming normal variance student's t-test's or one-way ANOVA was used. Details of statistical analysis are described in figure legends and results sections.

Objectives

Objective 1: Examine the influence of stress hormones on epigenetic markers in cells and animal models of breast cancer: Evaluate the impact of stress and stress hormones epigenetic modifications in breast cancer. The epigenetic effects of glucocorticoids on breast cancer cell lines are compared to the restraint stress effects in a syngeneic mouse models and a xenograft mouse model using cells with high levels of glucocorticoid receptors.

Objective 2: Characterise stress induced epigenetic modifications and their effect on the molecular biology of breast cancer at treatment. Investigate the effect of stress induced changed in epigenetic markers on the methylation status of tumour suppressors and global methylation.

Objective 3: Examine influence of age on DNA methylation: Evaluate the influence of ageing on key target genes in mouse syngeneic models of human breast cancer aged 3 months 9 months and 18 months. As age is a risk factor for breast cancer, DNA methylation profiles are investigated in aged animals following restraint stress.

3. Chapter 3: Glucocorticoids and psychological stress induce epigenetic modifications in breast cancer

3.1 Introduction

Numerousstudies suggest that exposure to different environmental factors, including psychological stress; can alter gene expression through epigenetic modifications [189-197]. DNA methylation is the best-characterized epigenetic modification, in which the DNMTs (DNMT1, DNMT3a, and DNMT3b) catalyse the addition of methyl groups onto DNA[197, 198]. There has been increasing interest in the influence of exposure to psychological stress on health and methylation-stress related studies have been the focus of many studies in brain, cord blood, and maternal blood. For example, the methylation status of the glucocorticoid receptor (GR) encoding gene, NR3C1 gene, was found to be altered in the DNA of the umbilical cord blood of stressed mothers [199, 200]. However, currently little is known of the biological significance of DNMTs in breast cancer in response to GC and/or stress.

DNMT1, the maintenance methyl transferase, binds to the newly synthesized DNA strand to catalyse the methylation of hemi-methylated DNA. This copies the methylation pattern of the original DNA strand to the new DNA strand [201]. DNMT1 also plays a major role in repairing DNA methylation [132]. Alterations in DNMT1 cause cell-type-specific changes in gene expression that can impact several pathways and cellular processes such as apoptosis, cell migration and proliferation [202]. Loss of DNMT1 results in passive DNA demethylation, leading to hypomethylation of oncogenes, global DNA hypomethylation[121]. DNMT3a and DNMT3b have similar function and structure. They introduce methylation to naked DNA and are able to methylate DNA without favouring hemimethylated DNA, hence they are called

de novo methylation transferases[121]. DNMT3a and DNMT3b differ in their expression patterns; DNMT3a is expressed relatively ubiquitously, while DNMT3b is poorly expressed in the majority of differentiated tissues [121]. However, the overexpression of DNMT1 and DNMT3b has been associated with tumourigenesis[139].

Tumourigenesis has been associated with a locus-specific hypermethylated state accompanied by broad global hypomethylation[143]. Therefore, the possible role of methyltrasferases in tumour initiation has been speculated [143, 202]. In other words, cancer is characterized by tumour suppressor promoter region hypermethylation, oncogene hypomethylation and global DNA hypomethylation.

Regions that are susceptible to hypermethylation are the CpG islands that extend on the promoters of tumour suppressor genes involved in cell cycle regulation, DNA repair, drug resistance, differentiation, apoptosis, angiogenesis and metastasis [203]. Methylation of tumour suppressors leads to their inactivation in cancer, therefore, hypermethylation has been considered a mechanism of gene silencing of tumour suppressors besides mutational silencing [204]. Alongside the role of hypermethylation in gene inactivation, hypermethylation also contributes to cancer progression due to its potential role in the induction of mutations particularly in the coding regions of genes [205].

Hypomethylation has been found on some oncogenes sequences and retrotransposons in the genome of cancers [205]. However, hypomethylation of promoter regions of oncogenes tend to be less common than hypermethylation of CpG-islands spanning promoter regions. Nonetheless, several cancer-associated hypomethylation can occur on different gene regions, including transcription control sequences [206]. The hypomethylation of cancer-

linked genes is associated with decreases in overall genomic methylation [207]. Hypomethylation of highly repeated DNA sequences, comprising the majority of retrotransposition activity in humans, is the main reason behind global DNA hypomethylation found in cancer[208, 209]. The most abundant retrotransposons in the human genome are LINE elements. Loss of methylation in LINE and their transcriptional activation in cancer has been associated with mutational alteration, and disruption of nearby genes, and chromosome instability [210].

In this chapter, the effect of stress on DNA methylation in breast cancer is investigated by assessing the expression of DNMT's and other epigenetic markers in response to exposure to the stress hormone, cortisol. The effect of cortisol on DNMTs was studied *in vitro* by treating different breast cancer cell lines with physiologically relevant doses of cortisol. The effect of stress on DNMTs was assessed *in vivo* using a xenograft of TNBC model. The effect of cortisol treatment in altering methylation patterns of key tumour suppressor genes, and global methylation patterns was then further investigated.

3.2 *In Vitro* Results

3.2.1 The effect of cortisol on DNA Methyl Transferases and other epigenetic markers in BC cell lines

To investigate the epigenetic effects of cortisol exposure on breast cancer, different breast cancer cell lines were treated with physiological concentrations of cortisol (5µM)[211] every 24 hours for 20 days. Total RNA was then extracted from treated cells (cort) and untreated cells (unstim), cDNA was synthesised and the expression of *DNMT*s were evaluated through qRT-PCR. Results are expressed as relative quantifications to untreated cells (unstim) and β-actin was used as an endogenous control for standardization. MCF-7 cells were used to represent an ER positive subtype of breast cancer. MDA-MB-231, Hs-578T, and Bt-549 cells were used to represent TNBC. MCF-7, MDA-MB-231, Hs-578T, and Bt-549 cells were selected due to their relatively high glucocorticoid receptor (GR) expression [187]. Experiments were performed in biological triplicates and technical duplicates and a t-test was used to calculate statistical significance between treated and untreated cells.

MDA-MB-231 cells were first treated with cortisol for 30 minutes, 24 hours, and 7 days but there were no significant effects of *DNMT1* (Appendix Figure 1) MDA-MB-231 cells treated with cortisol resulted in a significant decrease of *DNMT1*expression (*P*=0.0307). The expression of *DNMT3a* and *DNMT*3b were not significantly changed in response to cortisol treatment (Figure 8). Hs-578T cells treated with cortisol resulted in a significant decrease of *DNMT1* and *DNMT3b* (*P*=0.0429, and *P*=0.0060 respectively); whereas *DNMT3a* was significantly increased in response to cortisol treatment (*P*=0.0244) (Figure 8).

BT-549 cells treated with cortisol resulted in a significant decrease of *DNMT1* (*P*= 0.0051). The expression of *DNMT3a* and *DNMT3b* were not significantly changed in response to cortisol treatment (Figure 8).

However, treating MCF-7 cells with cortisol resulted in a significant increase of *DNMT1* (*P*= 0.0355) (Figure 8). The mRNA expression *of DNMT3a, and DNMT3b* were not significantly different to untreated cells after treatment with cortisol.

In summary, TNBC cell lines MDA-MB-231, Hs-578T, and BT-549 treated with cortisol for 20 days resulted in a significant decrease of *DNMT1* expression, while treating an ER+ cell line MCF-7 resulted in a significant increase of *DNMT1* expression.

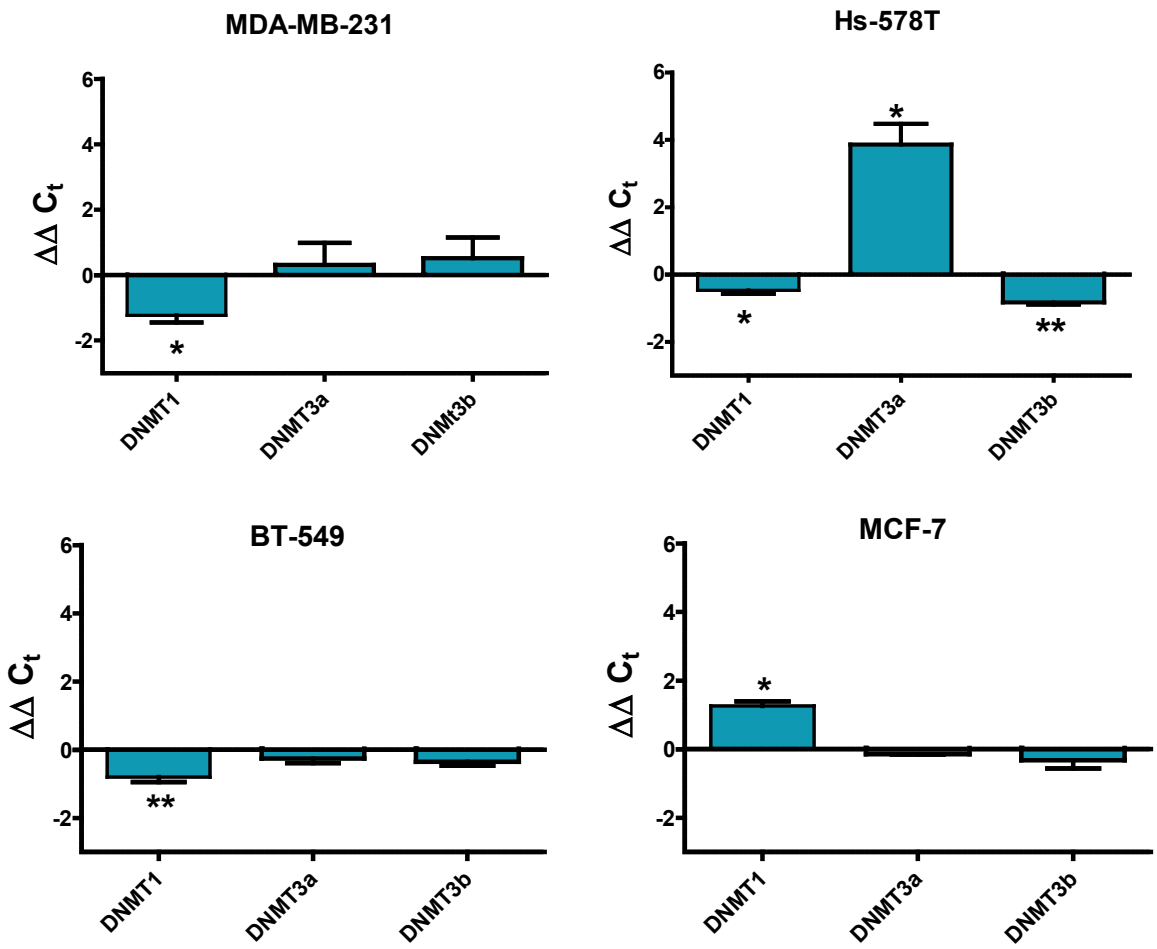


Figure 8*DNMTs* mRNA expressions in response to cortisol treatment in MDA-MB-231, Hs-578T, BT-549, and MCF-7 cells. MDA-MB-231, Hs-578T, BT-549, and MCF-7 cell lines were treated with 5µM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1, *DNMT*3a, and *DNMT*3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

To control for hormone specificity, cells from both MDA-MB-231 and MCF-7 cell lines were treated with the GR antagonist mifepristone known as (RU-486). RU-486 is added at a concentration of 1 µM daily, 30 minutes prior to the addition of 5µM cortisol for 20 days. Total RNA was extracted from treated cells (cort), cells treated with RU-486 followed by cortisol (RU-486), and untreated cells (unstim) to evaluate the expressions of *DNMTs*using qRT-PCR as explained for Figure 8. In MDA-MB-231 cells the cortisol-evoked decrease in *DNMT*1 was blocked by RU-486, as was the cortisol-evoked increase in *DNMT*1 expression in in MCF-7 cells (Figure 9).

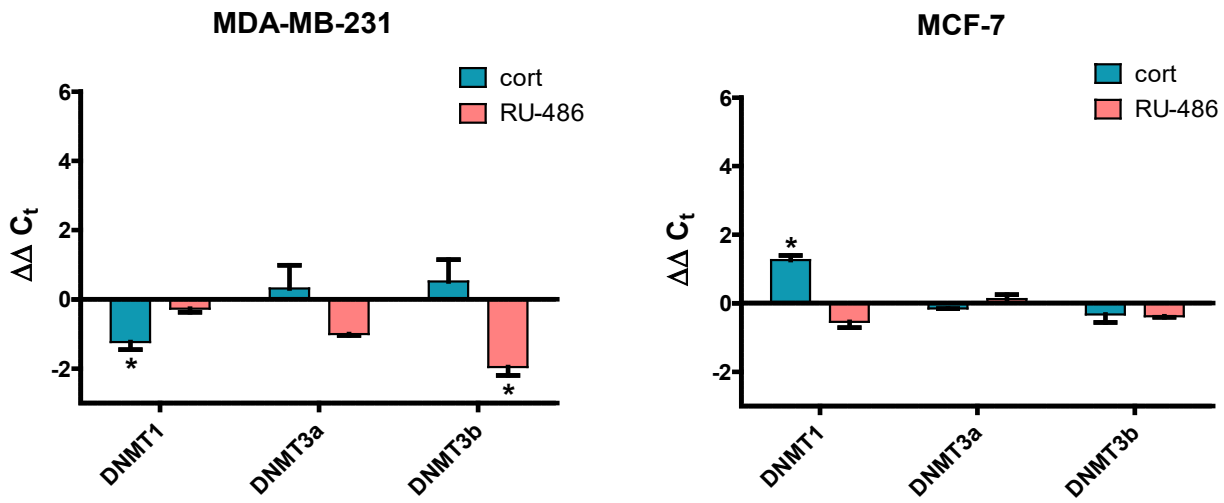


Figure 9 The changes in *DNMTs* expressions in MDA-MB-231 and MCF-7 cell lines in response to treatment with GR antagonist Mifepristone RU-486. MDA-MB-231, MCF-7 cell lines were treated with 5µM of cortisol for 20 days with and without RU-486. RU-486 was added 30 minutes prior to the addition of cortisol at a concentration of 1µM. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1, *DNMT*3a, and *DNMT*3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

To fully determine if these GC effects on *DNMTs* were mediated by the GR, an ER^{+}T-47D cells (low GR expressing cells) [187]were treated with 5μM cortisol every 24 hours for 20 days. *DNMT*s expressions were determined as previously described. *DNMT1, DNMT3a and DNMT3b* expression did not change in cells treated with cortisol compared to controls (Figure 10).

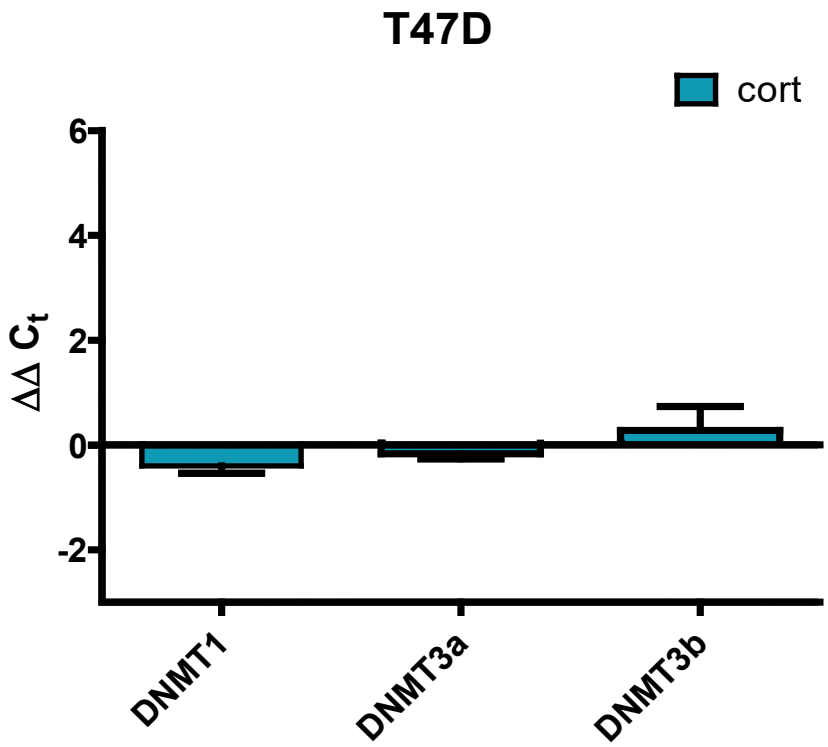


Figure 10*DNMT*1, *DNMT*3a and *DNMT*3b mRNA expression in response to cortisol treatment in T47D cells: T47D cells were treated with 5uM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1, *DNMT*3a, and *DNMT*3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (un-treated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).

A mouse cell line was used to evaluate the effect of cortisol on the expression of *DNMT*s. However, since the direction of change in *DNMT*s expression in response to cortisol treatments appears to be dependent on whether the cell lines express the ER based on results described above; we initially analysed the cells for the ER, as thereare discrepancies in the literature with some authors suggesting that 66Cl4 cells are ER- and others ER+ [212, 213]. In our laboratory 66cl4 cells did not express ER as shown by immunofluorescence in (Appendix Figure 3). To examine the effect of cortisol on 66cl4 cells,qRT-PCR analysis was performed. Cells were treated with 5µM of cortisol every 24 hours for 20 days. RNA was then extracted from cortisol treated cells (cort) and untreated cells (unstim), to determine *DNMTs* expressions as previously described. The expression of *DNMT1, DNMT3a* and *DNMT3b* was found to be significantly decreased after 20 days exposure to cortisol ($P < 0.05$) (Figure 11).

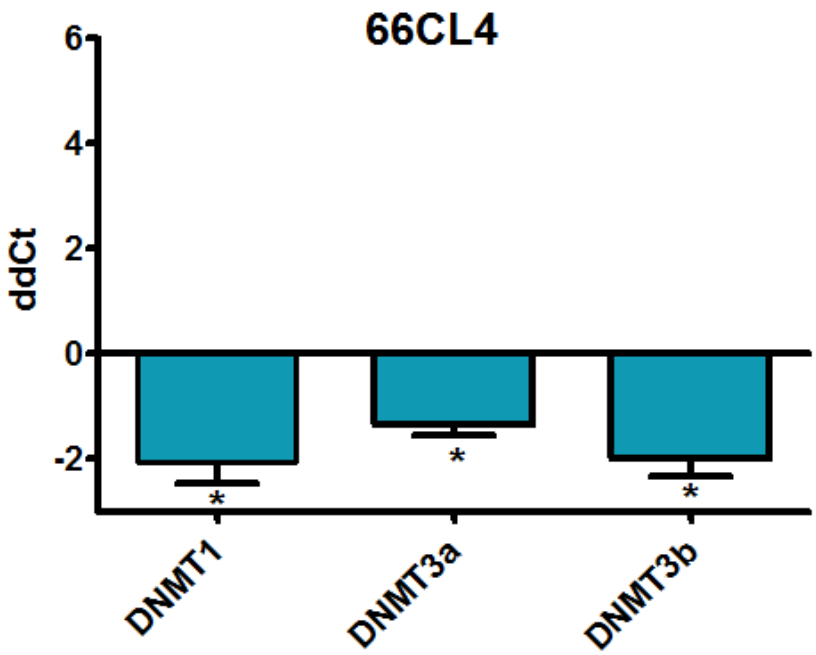


Figure 11DNMTs expression in 66cl4 cells in response to cortisol treatment Expression of DNMT1, DNMT3a, and DNMT3b decrease significantly in response to cortisol treatment for 20 days. 66cl-4 cell lines were treated with 5µM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate DNMT1, DNMT3a, and DNMT3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

3.2.2 The effect of cortisol on other epigenetic markers in MDA-MB-231 TNBC cells

To determinethe effect of cortisol treatment on epigenetic chromatin modification enzymes, MDA-MB-231 cell lines were treated daily with 5µM of cortisol for 20 days. RNA was then extracted and cDNA was synthesised. The template cDNA was then used to study the mRNA expression of different genes in RT² Profiler PCR Array. The array profiles 84 key genes encoding for enzymes involved in DNA and histone modification. A significant decrease in the expression of Histone Deacetylases*HDAC*1, *HDAC*3, *HDAC*5, *HDAC*6, *HDAC*7, *HDAC*8, *HDAC*9, *HDAC*10, *HDAC*11, (P = 0.0089 , 0.0044, < 0.0001, < 0.0001, < 0.0001, 0.0001, < 0.0001, 0.0011, and 0.0009 respectively) and a significantupregulation in Methyl-CpG Binding Domain 2 gene (MBD2) (*P= 0.0021*) was found in treated cells compared to control untreated cells (Figure 12).

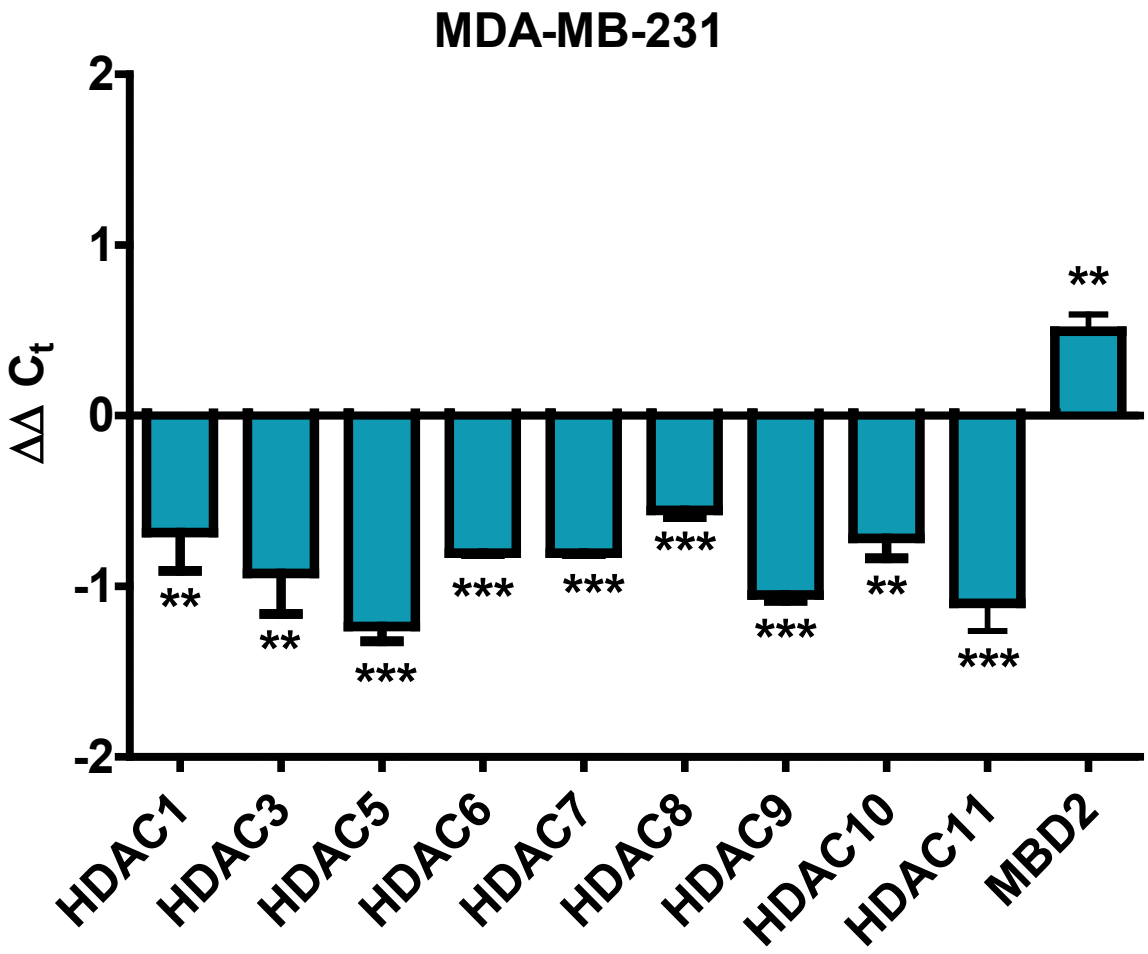


Figure 12 Changes in Epigenetic chromatin modification enzymes in MDA-MB-231 in response to cortisol treatment. MDA-MB-231 cells were treated daily with 5µM of cortisol for 20 days. RNA was then extracted from cells and cDNA was synthesised. cDNA template was then used to analyse the expression of a panel of epigenetic chromatin modification enzymes using RT^2 profiler array from QIAGEN through qRT-PCR. The array contains ready prepared primers of genes of interest. Results were then analysed using the ΔΔCt method with normalisation to β-Actin. Gene expression is presented as a relative quantification in comparison to untreated cells. Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells). * represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$) Results shown are a representation of 2 biological replicates.

3.2.3 The effect of cortisol on the methylation of tumour suppressor genes in BC cell lines

The finding that the change in *DNMT*s expression, particularly *DNMT*1, and the *HDAC*sin response to cortisol suggests changes in the epigenome of cell lines. Further studies were carried out to determine the effect of the changed*DNMT*son the methylationpattern of the most widely studied tumour suppressor genes. MDA-MB-231 and MCF-7 cell lines were treated daily with 5µM cortisol for 20 days. DNA was extracted from MCF-7 and MDA-MB-231 cells, and the quality and integrity of DNA was verified using gel electrophoresis. DNA was then subjected to a methylation dependent and/or methylation sensitive restriction enzyme which digests methylated and/or un-methylated DNA respectively. A mock DNA sample was also included in the PCR run, which is a sample that has not been subjected to any enzymatic digestion. After digestion, the remaining input of DNA was analysed using qRT-PCR on an array containing primers for promoter regions of 94 tumour suppressor genes. The method employed by the EpiTect Methyl II PCR System is based on detection of remaining input DNA after digesting methylated cytosines, and/or unmethylatedcytosines using ΔCt method and normalising against the mock sample. Experiments were performed in biological triplicates, changes in methylation percentage between treated and untreated samples were considered for statistical analysis to compare treated and untreated samples. When analysing PCR results of cortisol treated MCF-7 cells, change in methylationwas detected in the following tumour suppressor genes: *APC, DAPK1, RARB, BCR, CDKN1C,* and *DIRAS3* (Figure 13), However, significant increases in methylation were only detected in *RARB* ($P < 0.001$), *BCR* ($P < 0.001$), *CDKN1C* ($P = 0.024$) implying potential silencing or down

regulation of the expression of these key genes. On the other hand, analysis of PCR results of cortisol treated MDA-MB-231 cells showed a decrease in methylation levels of the following tumour suppressor genes D*APK1, ESR1, MGMT, ABL1, AKT1, BIRC5, CDKN1A, ING1, MDM2, NF2, PYCARD, and TERT* (Figure 14). However, significant changes were only detected for *ESR1* ($P < 0.001$), *ABL1* ($P < 0.05$), *AKT1* ($P < 0.01$), and *BIRC5* ($P < 0.01$) suggesting a potential activation of these tumour suppressors.

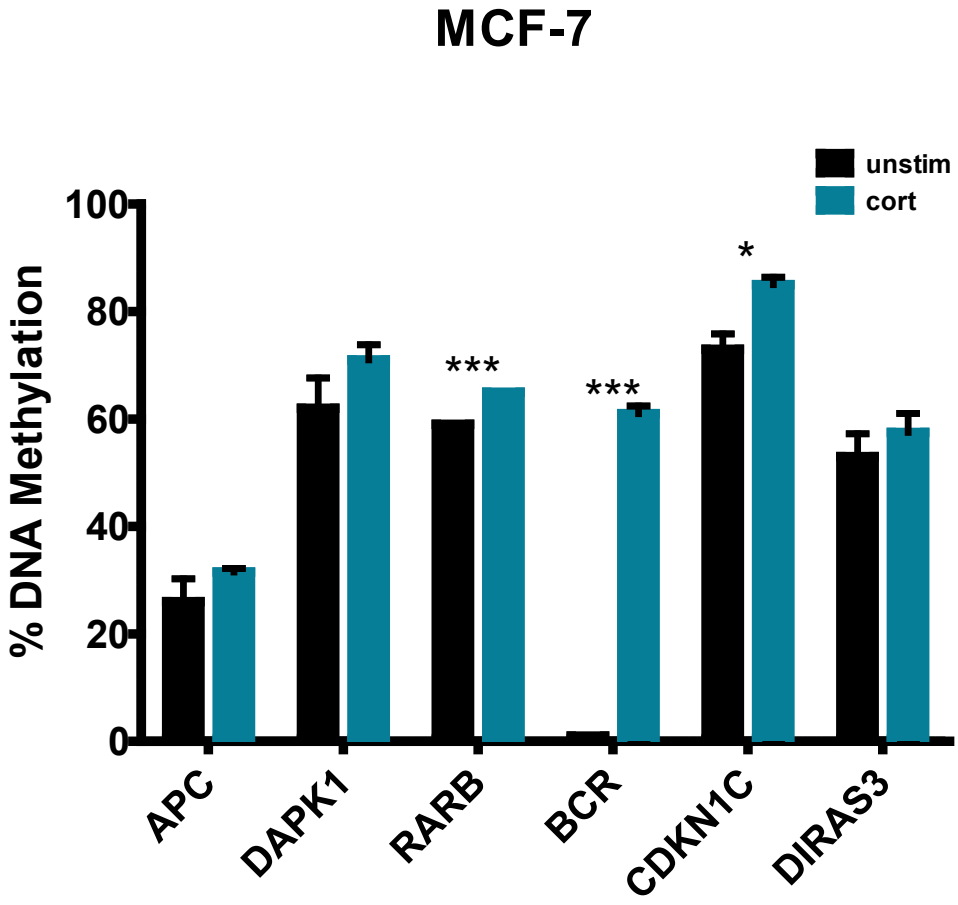


Figure 13Methylation levels of APC, DAPK1, RARB, BCR, CDKN1C, and DIRAS3 in response to 20 days of cortisol treatment. MCF-7 cells were treated daily with 5μM of cortisol for 20 days. DNA was then extracted, and subjected to a methylation dependent and/or methylation sensitive restriction enzyme, which digests methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR on an array containing primers for promoter regions of 94 tumour suppressor genes. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. 4% change in methylation percentage between treated and untreated samples was considered for multiple t-test with Bonferroni correction to compare untreated (unstim) and treated (cort) samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

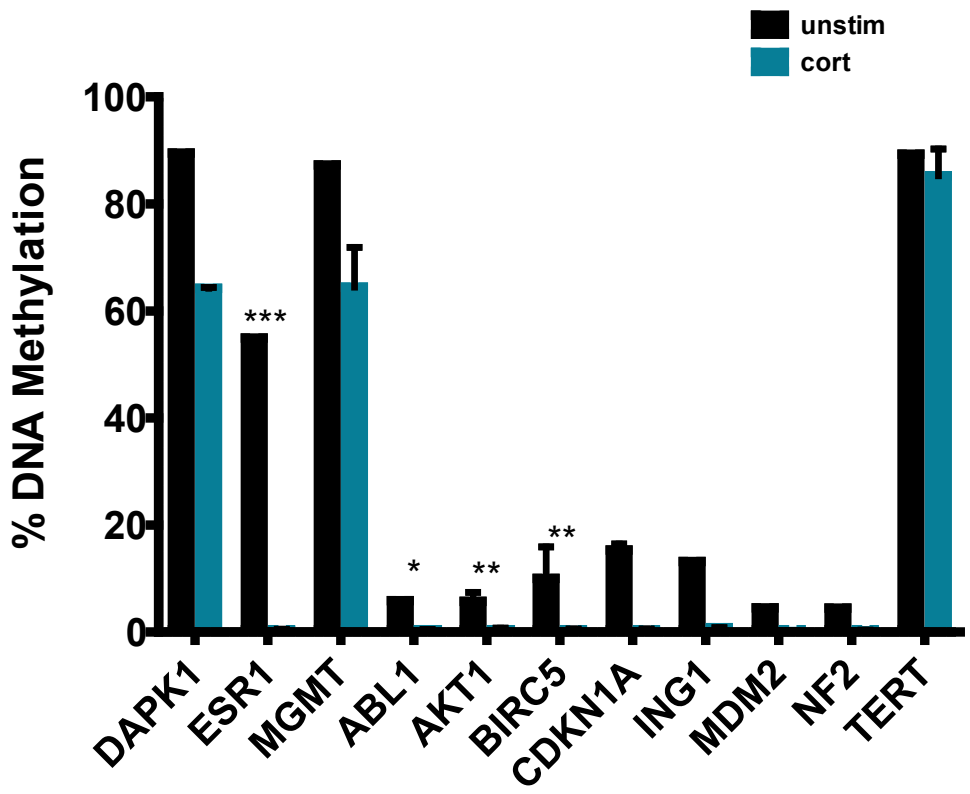


Figure 14Methylation levels of DAPK1, ESR1, MGMT, ABL1, AKT1, BIRC5, CDKN1A, ING1, MDM2, NF2, and TERT. MDA-MB-231 cells were treated daily with 5μM of cortisol for 20 days. DNA was then extracted, and subjected to a methylation dependent and/or methylation sensitive restriction enzyme which digests methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR on an array containing primers for promoter regions of 94 tumour suppressor genes. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. 4% change in methylation percentage between treated and untreated samples was considered for multiple t-test Bonferroni correction to compare untreated (unstim) and treated (cort) samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.2.4 The effect of cortisol on global DNA methylationin BC cell lines

To investigate the effect of cortisol of global DNA patterns, the change in the percentage of methylated cytosines on the repetitive element LINE-1 was assessed. MDA-MB-231, BT-549 and MCF-7 cells were treated daily with 5µM of cortisol for 20 days. DNA was then extracted from treated cells (cort) and untreated cells (unstim). The percentage of methylated cytosines on the extracted DNA was assessed using a LINE-1 ELISA kit. Since LINE-1 comprises for a bulk of human genome (17% - 20%) and around third of methylation incidents appears to be on repetitive elements, LINE-1 can serve as a surrogate marker for global DNA methylation[128].Using an ELISA method described earlier in chapter 2 to compare the differences in LINE-1 methylation between treated cortisol treated cells and untreated cells. MDA-MB-231 cells treated with cortisol resulted in a significant decrease (P= 0.05) in the percentage of methylated cytosine found in LINE-1 in comparison to un-treated cells(Figure 15). BT-549 cells treated with cortisol have also resulted in a decrease in the percentage of methylate cytosine on LINE-1. However, the decrease was not significant. On the other hand, a higher percentage of methylated cytosine was detected in cortisol treated MCF-7 (cort) in comparison to untreated cells (unstim). However, the increase was not significant.

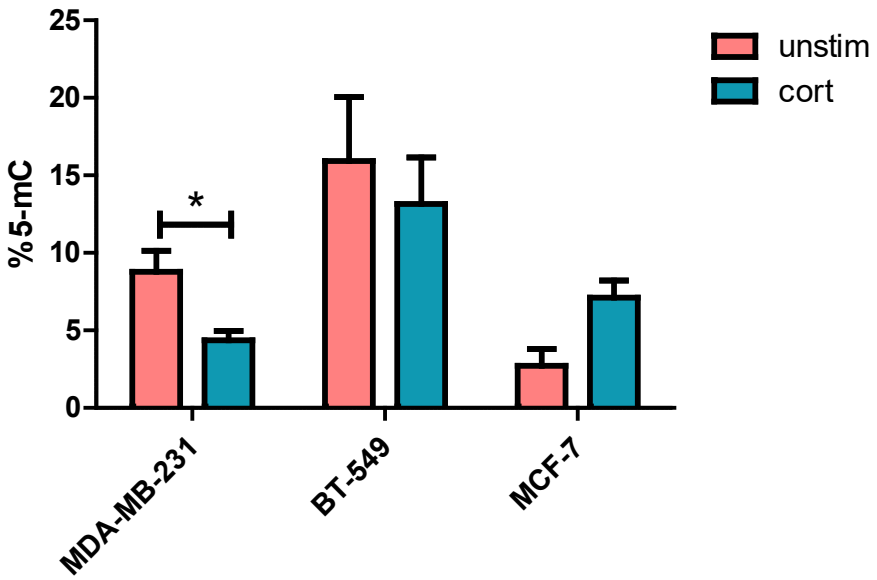


Figure 15 The percentage of methylated cytosine correlated with detectable CpG residues in LINE-1 in MDA-MB-231, BT-549, and MCF-7 cells in response to 20 days of treatment with cortisol. MDA-MB-231, BT-549, and MCF-7 cells were treated daily with 5µM of cortisol for 20 days. DNA was then extracted from untreated cells (unstim) and cells treated with cortisol (cort). The 5-methylcytosine (5-mC) was then detected and quantified using an ELISA based method. Results are presented as a percentage of 5-mC associated with the detectable CpG residues found on Long Interspersed Nucleotide Element 1 (LINE-1) repeats. Analysis were performed on OD values using t-test to compare untreated (unstim) and treated (cort) samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

3.2.5 The effect of Dexamethasone on the DNA methyltransferase in TNBC cell lines

To evaluate if the changes in *DNMT*s expressions can be altered using other GCs, breast cancer cell lines were treated with Dexamethasone (Dex) a synthetic GC that is more potent that hydrocortisone[214]. TNBC cell lines MDA-MB-231, and Hs-578Twere treated with 1µM of Dex for 24 hours. These cell lines have been verified to express GR [187]. Total RNA was then extracted from treated cells (Dex) and untreated cells (unstim), and was used to evaluate *DNMT*s expression. The expression of *DNMT*1 in MDA-MB-231 cells was significantly decreased in response to 24 hours of Dex treatment (*P*= 0.0458), while the expression of *DNMT*3a, and *DNMT*3b were not changed. In Hs-578T cells, the expression of *DNMT*1, *DNMT*3a and *DNMT*3b was found to be significantly decreasedafter 24 hours of exposure to Dex (*P*=0.0429, 0.0244, 0.0060respectively) (Figure 16)

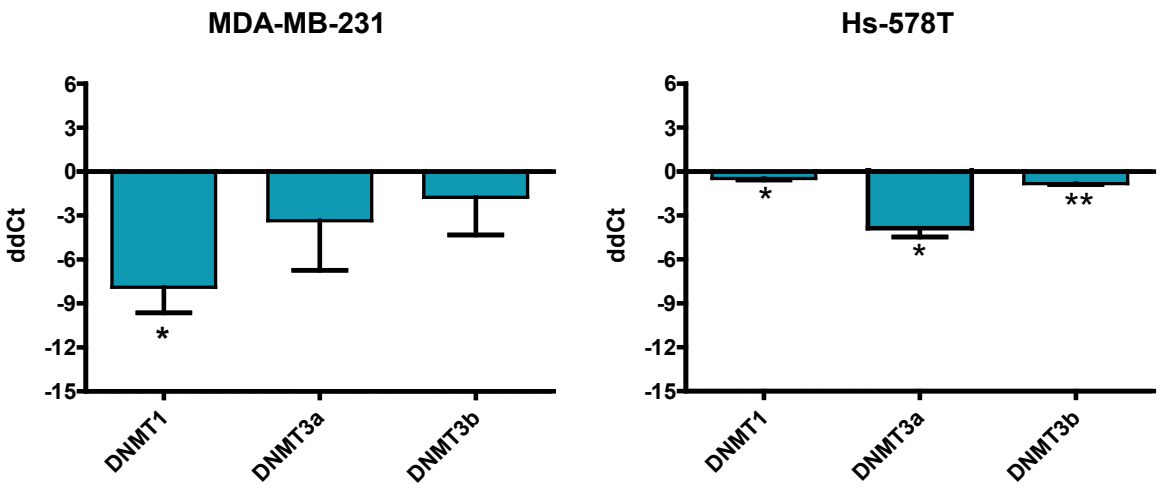


Figure 16*DNMT*1 mRNA expression in response to dexamethasone treatment in MDA-MB-231 and Hs-578T TNBC cells. Breast cancer cells from MDA-MB-231 and Hs-578T cell lines were treated with 1µM of cortisol for 24 hours. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1, *DNMT*3a, and *DNMT*3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).mRNA expression of *DNMT*1 was significantly decreased in Dex treated MDA-MB-231 and Hs-578T cells in comparison to untreated cells. mRNA expression of *DNMT*3a and *DNMT*3b were significantly decreased in Dex treated Hs-578T cells in comparison to untreated cells.

To control for hormone specificity, MDA-MB-231 cell lines were treated with the GR antagonist (RU-486). RU-486was added at a concentration of 1 μM for 30 minutes prior to the addition of 1μM Dex for 24 hours. Total RNA was then extracted from treated cells (Dex), cells treated followed by RU-486 (RU-486+Dex) and untreated cells (unstim), cDNA was synthesised and the expressions of *DNMTs* were evaluated through qRT-PCR. Results are expressed as relative quantifications of untreated cells (unstim), and β-Actin was used as an endogenous control for standardization. Experiments were performed in biological triplicates and technical duplicates, and a t-test was used to calculate statistical significance between treated and untreated cells. In MDA-MB-231 cells, the decrease in *DNMT1* was abrogated when blocking the GR with RU-486 (Figure 17).

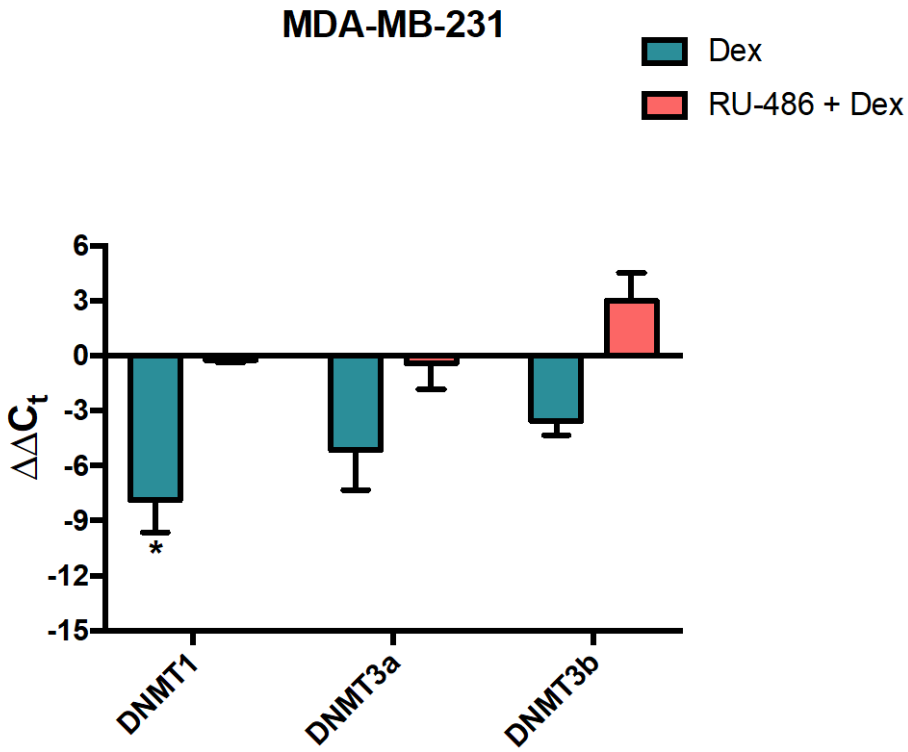


Figure 17The change in *DNMT*1 expression in MDA-MB-231 cell line was abrogated when treated with GR antagonist Mifepristone RU-486. Breast cancer cells from MDA-MB-231 cell line were treated with 1µM of Dexamethasone (Dex) for 24 hours days with and without RU-486. RU-486 was added 30 minutes prior to the addition of dexamethasone at a concentration of 1µM. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1, *DNMT*3a, and *DNMT*3b mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells).* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

3.3 *In Vivo* Results: MDA-MB-231 Xenograft model

3.3.1 The effect of psychological stress (restraint stress) on *DNMT*s expression

To assess the effects of psychological stress on the expressions of *DNMT*s, a stressed MDA-MB-231 xenograft model was developedas described in Chapter 2 section 2.8.2. Female nude mice were subcutaneously injected MDA-MB-231 cells, and tumour-bearing xenografts were randomised into restraint stress group (n=4) and non-stress group (n=3). Tumour volumes were monitored from injection day to the end point of the study, and tumours' weights were measured at necropsy. There were no significant differences in tumour volumes or tumour weights between the control and the restraint stress (RS) groups (Figure 18). At the end of the study, RNA was extracted from harvested tumours, to analyse the expression of *DNMTs* using qRT-PCR.Results were normalised to control group (non-stress group) and β-Actin was used as an endogenous control for standardization. There was a significant increase in *DNMT1* expression in tumours isolated from stressed mice but no significant difference in *DNMT3a*, and *DNMT3b* expression in comparison to the non-stressed group (Figure 19).

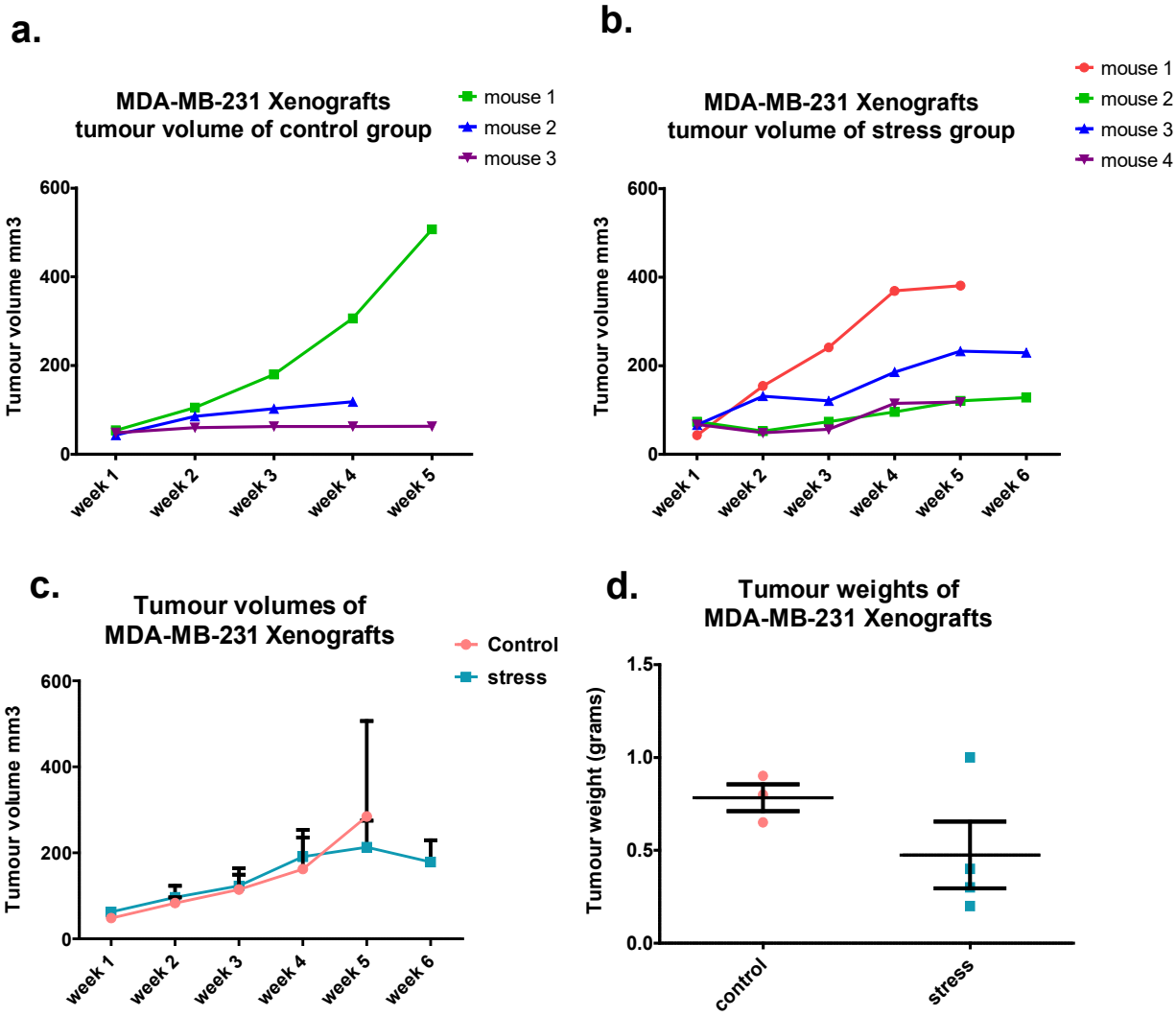


Figure 18 Tumour volumes and weights of MDA-MB-231 xenografts. Female nude mice were subcutaneously injected with $5x10^6$ MDA-MB-231 cells in 0.2 ml. When tumour volumes reached 50 mm^3, tumour bearingxenografts were randomised into restraint stress group (n=4) and non-stress group (n=3). Stressed mice were placed individually in ventilated tubes where they can turn supine to prone but cannot turn head to tail. Mice were sacrificed when either parameter of the volume reached a maximum of 12 mm. Tumour volumes' progression is monitored throughout the study, and tumours weights were measured upon necropsy. There were no significant differences in tumour volumes or tumour weights between the control and the restrain stress (RS) group.

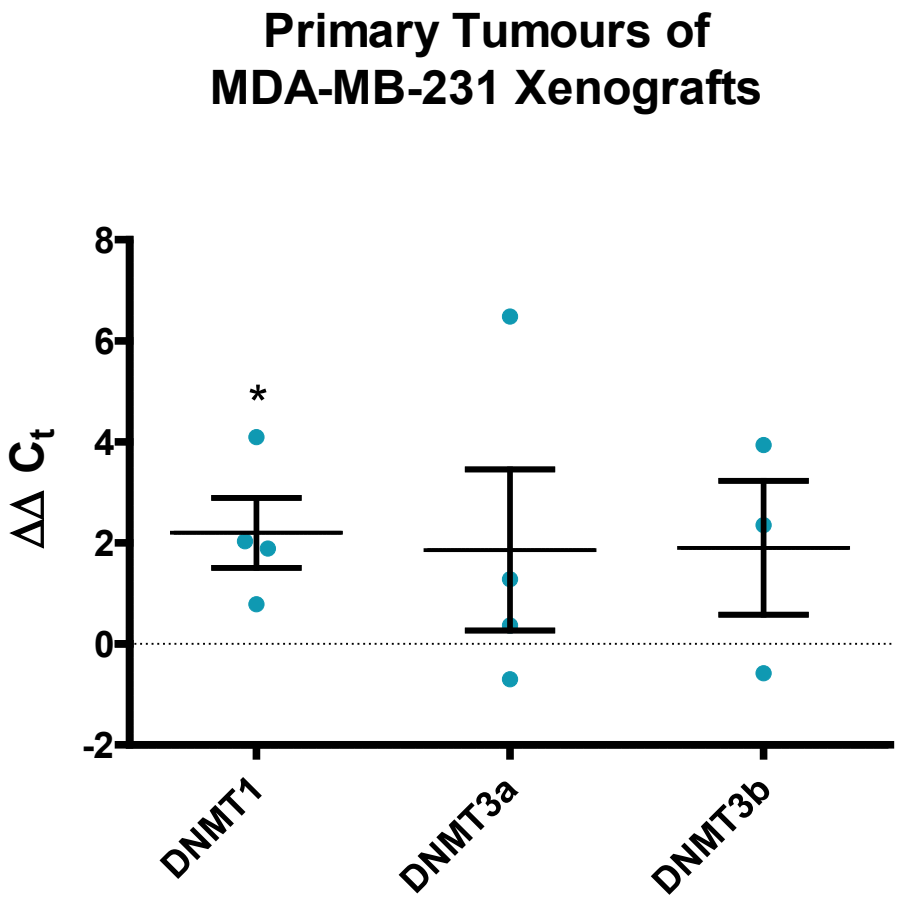


Figure 19*DNMT*1 mRNA expression in increased by stress in MDA-MB-231 xenograft model. Female nude mice were subcutaneously injected with $5x10^6$ MDA-MB-231 cells in 0.2 ml PBS. When the tumour volumes reached 50 mm^3, tumour bearingxenografts were randomised into restraint stress group and non-stress group. Stressed mice were placed individually in ventilated tubes where they could turn supine to prone but could not turn head to tail. At the end of the study, RNA was extracted from harvested tumours, cDNA was synthesised and *DNMT*s expressions were analysed using qRT-PCR. β-Actin was used as an endogenous control. Results were normalised to control group (non-stress group) and are presented as relative quantification calculated using the ΔΔCt method. Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (non-stressed group). * represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

3.3.2 The effect of restraint stress on global DNA methylation

To assess the effects of psychological stress on global DNA methylation, DNA was extracted from harvested primary tumours from the mice described above, and used to assess the percentage of methylated cytosine on LINE-1 using LINE-1 ELISA kit. The relative 5mC levels in DNA samples were measured using an ELISA method described earlier; methylated fragments were detected using a 5mC antibody and a secondary antibody conjugated to HRP. The colorimetric readout is quantified at 450 nm with an optical reference of 655 nm. Although the difference in means of the methylated cytosine between the control and stress group was not statistically significant, data indicate that the percentage of methylated cytosine on LINE-1 in the majority of mice in the stress group is less compared to the control group, but this change is not significant (Figure 20).

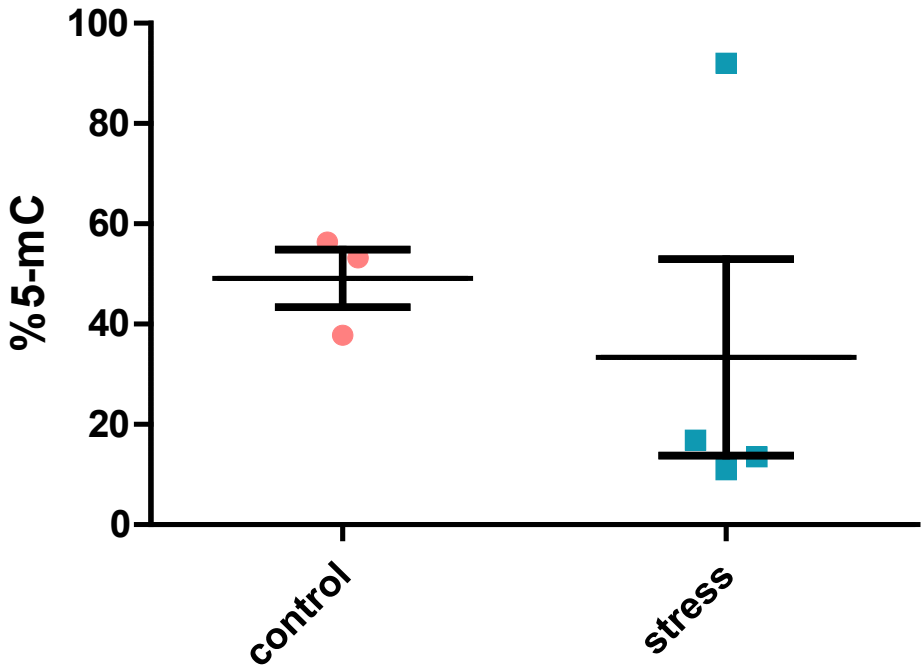


Figure 20 The percentage of methylated cytosine correlated with detectable CpG residues in LINE-1 between control stressed and non-stressed (control) mice. The MDA-MB-231 xenograft model was developed. Female nude mice were subcutaneously injected with $5x10^6$ MDA-MB-231 cells /0.2 ml PBS. When tumour volumes reached 50 mm3, tumour bearingxenografts were randomised into restraint stress group (n=4) and non-stress group (control) (n=3). At the end of the study, DNA was extracted from primary tumours**.** The 5-methylcytosine (5-mC) was then detected and quantified using an ELISA based method. Results are presented as a percentage of 5-mC measured relative to the total cytosine content of Long Interspersed Nucleotide Element 1 (LINE-1) repeats. The colorimetric readout is quantified at 450 nm with an optical reference of 655 nm. Analysis was performed using a Mann-Whitney test on OD values to compare non-stress (control) and stress group.

3.4 Discussion

Exposure of cells to cortisol for 20 days resulted in an alteration of DNA methylation patterns. These alterations are characterised by changes of methylation levels on promoter regions of tumour suppressor genes, as well as changes of global methylation hypothesising that cortisol leads to epigenetic modificationthrough the alteration of gene expression of key epigenetic markers. Treating breast cancer cells with dexamethasone, a synthesised GC, also resulted in changes of *DNMT*s expressions. In addition, the *In vivo*study has also suggested an alteration in *DNMT*s expression in response to stress along with changes of global methylation.

Cortisol resulted in a significant decrease in*DNMT*1 expression in MDA-MB-231 and H758T cells, and a loss of global methylation characterised by loss of methylation on LINE-1 in MDA-MB-231 cells. There was also a loss of methylation on the promoter region of tumour suppressor genes in response to cortisol in MDA-MB-231 cells. These tumour suppressor genes included: *DAPK1*, which is a key modulator of apoptosis and programmed cell death[215]; *ESR1*, the gene encoding for oestrogen receptor alpha; *MGMT*, which plays a crucial role is cancer development and DNA repair [216];*ABL1* that is involved in cellular differentiation, cell division and cell adhesion [217];*AKT1* is a key player in cell division, cell differentiation and apoptosis [218], and *BIRC5* which is a negative regulator of apoptosis [219].Combining these results together, the loss of methylation on the aforementioned genes, as well as the global loss of methylation may be due to the decreased expression of *DNMT*1. *DNMT*1 plays an important role in passive de-methylation, and hence the

downregulation of *DNMT*1 can lead to loss of methylation causing de-methylation of tumour suppressors, or global genome hypomethylation. Although loss of methylation on tumour suppressor genes can lead to their re-expression, it is unknown if the changes in methylation levels caused by cortisol treatment are sufficient to restore their function. Furthermore, although loss of methylation on tumour suppressors might be favourable for cell regulation, global loss of methylation, on the other hand, is correlated with genomic instability and can cause deleterious mutations [220]. A rather interesting gene that has been found to lose methylation on the promoter region was the ESR1 gene. The loss of methylation on ESR1 may allow the re-expression of ER in TNBC a possibility that will be examined in the next chapter.

Although the majority of the tumour suppressor genes from the array showed no significant changes in methylation levels in response to cortisol treatment, it is not known why certain genes lost methylation on their promoter region when treating MDA-MB-231 cell line with cortisol. For example, the tumour suppressor gene B-cell lymphoma 2 (BCL2) remained methylated after treating MDA-MB-231 cells with cortisol. The BCL2 is an apoptosis regulator gene that has been found to be regulated by oestrogen in a study related to chemotherapy resistance [221]. The study demonstrated that the loss of methylation on ESR1 and re-expression of ER upon the use of the de-methylation agent (5-5- aza-2'-deoxycytidine) in KPL-4 breast cancer cell line, oestrogen induced an up-regulation of Bcl-2 mRNA [221]. However, in this chapter we showed that the methylation levels of BCL2 remained unchanged despite the loss of methylation on ESR1 promoter region. Studies have shown that in different breast cancer cell lines or tumour specimens, altered expression of host genes has been associated with aberrant DNA methylation, including Breast Cancer

gene 1 (BRCA1), Wilms Tumour 1 gene (WT1), Cyclin Dependent Kinase Inhibitor 2A (CDKN2A, p16), and Ras Association Domain Family Member 1 gene (RASSF1) [222-224]. In this study, the methylation patterns of these genes were not affected by the treatment of cortisol in MDA-MB-231 cell lines. However, the reason why certain genes did not show any changes in methylation levels in response to cortisol is yet to be investigated.

MCF-7 cells treated with cortisol resulted in an increase in global DNA methylation characterised by increased percentage of methylated cytosine on LINE-1 repetitive elements. Cortisol exposure in MCF-7 cells lead to the hypermethylation of promoter regions of key tumour suppressors including *APC, DAPK1, RARB, BCR, CDKN1C, and DIRAS. APC* plays its tumour suppressor role through the Wnt signal transduction pathway [215]. *DAPK1* is a key modulator of apoptosis and programmed cell death [215]. *RARP* is a nuclear transcriptional regulator and is found to play a role in reducing cell migration in breast cancer [215]. *CDKN1C* is negative regulator of cell proliferation and is found to be associated with a number of human cancers including breast cancer [225]. *DIRAS* is a member of ras superfamily and has been found to be downregulated in 70% of invasive breast cancers[166]. These changes can be correlated with the significant increase of *DNMT*1 expression, along with the increased global methylation, which can be potentially causing transcriptional silencing of transposons, which is crucial to the maintenance of a genome.

The contradicting results between ER+ MCF-7 cells and TNBC and MDA-MB-231 cells could be due to the ER expression; but this remains to be evaluated.

Cortisol did not induce any significant differences in *DNMT*1, *DNMT*3a, and *DNMT*3b expression in T47D cells. This finding is likely due to the fact that T47D cells express low

levels of the GR [187]. This further supports the hypothesis that the change of *DNMT*s expression in other cell lines described earlier is specifically due to cortisol exposure. The result from blocking GR with antagonist (RU-486) in different cell lines has also confirms that the changes in the expression of the *DNMT*s are due to the activation of GR by cortisol. In conclusion, cortisol plays a role in the expression of the *DNMT*s which in turn alters the epigenetic landscape of breast cancer cells.

In xenografts model developed*,*no significant difference in volumes of primary tumours between control and stress groups was observed; however, this is a common observation in stress studies[73, 226]. *DNMT1* expression was altered in mouse tumours that have been subjected to stress. *In vivo* results also demonstrated less of global methylation in stressed mice in comparison to non-stressed mice.

Abnormal DNA methylation is thought to be a major event in the progression of tumours characterized by extensive genome hypomethylation leading to chromosomal instability and localised DNA hypermethylation, which is crucial in tumourigenesis, leading to tumour suppressor silencing [227]. DNA methylation is catalysed by *DNMT*1, the methylation maintenance gene, and *DNMT*3a, and *DNMT*3b,which are the*de novo* methylation genes. However, *DNMT*1 also plays a role in *de novo* methylation, and in passive demethylation; downregulation of*DNMT*1 can lead to loss of methylation causing de-methylation of tumour suppressor, or global genome hypomethylation[228, 229]. Understanding the changes in tumour suppressors and methylation alterations will not only result in better therapeutic strategies by targeting specific genes, but will also lead to potential detection of early diagnosis biomarkers, considering that DNA methylation is assumed to be an early event of tumour formation [230-232].

During breast cancer tumorigenesis, dysregulated expression of *DNMT*1, *DNMT*3a, and *DNMT*3b may therefore be of importance in dysregulating gene expression, and especially that of tumour suppressor genes [233]. In line with studies that demonstrate how stress dysregulate*DNMT*s expressions in the brain, the results of this chapter show that exposure to cortisol does indeed dysregulate expression of *DNMT*s in different ways depending on the type of breast cancer. Dysregulation of *DNMT*s also led to alteration in methylation of tumour suppressor genes. The results translate to suggest that exposure to stress hormones contributes to the tumourigenesisof breast cancer in an epigenetic manner. However, the mechanism by which stress, and glucocorticoid hormones, induces the changes of *DNMT*s expression, *HDAC*s expression, and methylation patters in breast cancer needs to be elucidated.

4. Chapter 4: The Effect of cortisol and psychological stress on the methylation status of the oestrogen receptor in triple negative breast cancer

4.1 Introduction

Breast cancer is molecularly classified based on three main biomarkers: oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [234]. Approximately 75% of breast tumours are ER positive, known as Luminal A, and Luminal B breast cancer[235, 236]. Breast cancers that lack ER, PR, and HER2 are known as Triple Negative Breast Cancer (TNBC), and they account for 15% to 20% of breast cancer tumours [234, 237]. This molecular characterisation is crucial at diagnosis as it dictates the type of the therapy the patient receives, and plays a role in predicting clinical outcomes. Patients with tumours expressing ER (Luminal A, B) are candidates for endocrine therapy [238, 239], while patients with tumours lacking ER expression or are TNBC are unresponsive to endocrine therapy, as these therapies are molecularly targeted therapies that works by modulating or antagonising ER, and thus inhibiting the growth of the tumour [238, 240-242].

Steroid receptors including ER, PR, GR, and androgen receptor (AR) have been shown to play an important role in breast cancer progression [38]. Although the role of ER and PR in breast cancer is relatively well established, the orchestrated functional interactions of these nuclear receptors have been only recently recognised [38, 40]. Increasing evidence is demonstrating the role of GR in the interaction with nuclear receptors in breast cancer progression. For example, the activation of PR, AR, and GR leads to the modification of ER-mediated gene expression in ER+ breast cancer [38].

Oestrogen is a key modulator for the development of normal and malignant breast tissues. The ER signalling pathway is activated by the binding of its ligands, oestrogen or estradiol

(E2), to two receptors ERα and ERβ [243]. However, in clinical settings only ERα, encoded by *ESR1*, is being targeted in breast cancer as its protein level has been found to be significantly higher in tumours in comparison to normal tissues [244]. Oestrogens mediate two signalling pathways; genomic and non-genomic. The classic genomic pathway requires the binding of oestrogen to its receptor. The receptor is then dimerised and translocates into the nucleus binding to specific chromatin regions known as oestrogen response elements (ERE) of oestrogen responsive genes [238, 245]. This genomic pathway takes hours to days to alter the transcription of ER targeted genes [246]. The major role of ER as a transcriptional factor mediating genes involved in cellular growth and proliferation made ER signalling pathway a major therapeutic target for tumours expressing ER [246, 247]. The ER receptor can be targeted directly by either partial or pure antagonists such as tamoxifen (TAM) and fulvestrant (Fulv), or indirectly by aromatase inhibitors [236, 243, 247, 248].

The transcriptional activity of ERα is activated by two functional domains AF1 and AF2. AF1 acts independently from oestrogen, while AF2 requires the binding of oestrogen for activation. A well-known selective oestrogen receptor modulator (SERM) and a partial oestrogen antagonist in breast cancer is tamoxifen (TAM). Tamoxifen can compete with oestrogen at the receptor site blocking oestrogen mediated activity of AF2 and inhibiting cellular growth [236, 248-250]. Fulvestrant, on the other hand, forms complexes when binding to ER disturbing both AF1 and AF2 associated transcriptional activity [238, 250, 251]. However, fulvestrant induced confirmations are not stable resulting in ER downregulation; hence fulvestrant is a selective oestrogen receptor down-regulator (SERD) [251]. Unlike tamoxifen, fulvestrant is a pure ER antagonist with no reported partial agonistic activity [250-252].

Breast cancer tumours lacking ERα expression are known to be unresponsive to hormonal treatment [236]. This loss of expression can be attributed to genetic and epigenetic causes [238]. However, compelling evidence from the literature suggests that it's the epigenetic changes which are the main cause behind the ER silencing [134, 238, 245, 253-255]. The loss of ERα in ER- negative breast cancer cell lines has been associated with the downregulation of *ESR1* mRNA expression, which can be a result of methylation of CpG islands on the promoter region and exon 1 of *ESR1*[238, 245]. The fact that the methylation process is mainly governed by the DNMT family of enzymes[134] and that the maintenance methyltransferase DNMT1 plays a role in demethylation[256], directed studies into investigating the role of DNMT1 in ER silencing. Furthermore, *DNMT1* mRNA and protein levels were found to be elevated in ER-negative cell lines when compared to ER-positive cell lines [257-259]. However, methylation is not the only mechanism governing ERα silencing; studies have shown that histone deacetylation plays a role [255, 260]. Histone deacetylases (HDACs) are known to regulate gene expression, and were a target for assessing ER silencing in breast cancer. HDAC1, HDAC2, and HDAC6 are associated with the regulation of ERα [245, 261, 262]. In an ER-positive cell line, the over expression of HDAC1 resulted in a loss of ERα [263]. Besides DNMTs and HDACs, components of corepressor complexes like CpG binding domain proteins (MBDs) play a crucial role in the epigenetic process in general and ER silencing in breast cancer. ChIP analysis has detected MBD1, and MBD2 at the promoter region of ERα in ER-negative breast cancer cell lines, while this was not found in ER-positive breast cancer cell lines, implying the involvement of MBDs in the silencing of ERα [264]. Taken together, the repressing transcription mechanism of ERα in breast cancer is

orchestrated either solely by DNMTs, or by a combination of DNMTs and HDACs, and corepressor complex elements.

Data suggesting that ER is silenced epigenetically led to investigations into the possibility of re-expressing ER and restoring its functionality and hormonal sensitivity to provide a therapeutic target for ER-negative breast cancer. Different approaches have been used to facilitate the re-expression of ER; such as DNMT inhibitors, HDAC inhibitors, and a combination of both. One study demonstrated that treatment of ER-negative breast cancer cells with a demethylating agent and the DNMT1 inhibitor, 5-aza-2'deoxycytidine (5-aza-dC), led to the re-expression of ER mRNA and functional ER proteins, by specifically inhibiting DNMT1 expression [133, 134, 255]. The group also used a combination of (5-aza-dC) and the HDAC specific inhibitor,trichostatin A (TSA), for an enhanced effect of ER re-expression in TNBC cells [255]. Another study demonstrated the re-expression of ER-α by TSA in a cell line that had lost ERα expression [263].

The findings in Chapter 3 illustrating the decrease in *DNMT1* in TNBC in combination with the results from the tumour suppressor array showing loss of methylation on the promoter region of *ESR1* gene, led to the hypothesis that long term exposure to cortisol can lead to the re-expression of ERα in TNBC. In this chapter, we investigate the methylation status of a panel of ER-negative breast cancer cell lines, along with the mRNA expression of *ESR1* in response to long term exposure to cortisol. Further studies were conducted to test whether the activation of HPA axis and the release of cortisol in response to psychological stress would have a similar effect on the methylation and expression status of ER. This was investigated by subjecting a TNBC MDA-MB-231 xenograft model to restraint stress.

4.2 Results

4.2.1 Cortisol induces a loss of methylation and the upregulation of ESR1 leading to re-expression of ER in triple negative breast cancer cell lines

In the previous chapter, we found that 20 days exposure to cortisol resulted in a decrease of methylation of *ESR1*. To further investigate that loss of methylation of *ESR1* in TNBC cells in response to long exposure to cortisol, different TNBC cells including MDA-MB-231, MDA-MB-157, Hs-578T, and BT-549 were treated with 5µM cortisol for 20 days. MCF-7, an ER+ cell line was also treated with 5µM of cortisol for 20 days and served as a control. DNA was extracted from control untreated cells (unstim) and cells treated with cortisol (cort). An equal amount of DNA products were subjected to digestion by four restriction enzymes. The first digest has no enzymes added and contains the whole input of the genomic DNA of the sample. The second digest is the methylation-sensitive restriction digest and it contains methylated DNA sequences. The third digest is the methylation-dependent restriction digest, and it contains unmethylated DNA sequences. The fourth digest is a combination of methylation-sensitive and methylation dependent digests, and was used to control for background and determine the success of the digestion process. The product of each digest was used for qRT-PCR runs. We determined a loss of methylation of *ESR1* in TNBC cells treated with cortisol compared to unstimulated cells (Figure 21). The percentage of *ESR1* methylation was significantly decreased in MDA-MB-231 (p = 0.0027), Hs 578T (p = 0.0001), BT-549 (p= 0.0016), and MDA-MB-157 (p= 0.0313). The ER+ MCF-7 cells showed no change in *ESR1* methylation in response to cortisol treatment compared to untreated cells.

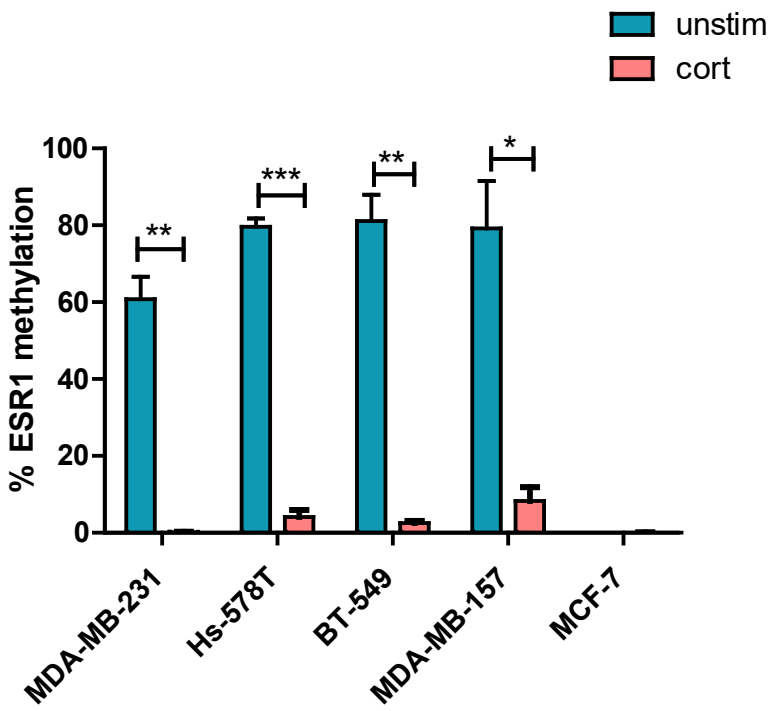


Figure 21*ESR1*promoter methylation in response to cortisol treatment in breast cancer cell lines. A panel of breast cancer cells were treated with 5µM of cortisol for 20 days. MDA-MB-231, Hs-578T, BT-549, and MDA-MB-157 represent TNBC. MCF-7 represents an ER+ cell line. DNA was extracted from untreated control cells (unstim) and cortisol treated cells (cort). DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes which digests methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for *ESR1* to study the change of the methylation status on the promoter region of *ESR1*. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. 4% change in methylation percentage between cortisol treated and untreated samples was considered for T-test to compare untreated (unstim) and treated (cort) samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Results showed significant loss of methylation on the promoter region of *ESR1* in in treated cells of triple negative breast cancer cell lines. Results showed no significant changes in MCF-7 cell lines in response to cortisol treatment.

To study whether the loss of methylation of *ESR1* found in TNBC in response to cortisol treatment is sufficient to upregulate the *ESR1* mRNA expression, MDA-MB-231, Hs-578T and BT-549 cell lines were treated with 5µM of cortisol for 20 days. RNA was then extracted from treated and untreated cells and cDNA was synthesized and subjected to qRT-PCR. *ESR1* mRNA expression was quantified using the ΔΔCt method. Results in Figure 22 demonstrate a significant increase in *ESR1* expression in MDA-MB-231, Hs-578T and BT-549 cell lines in response to cortisol treatment.

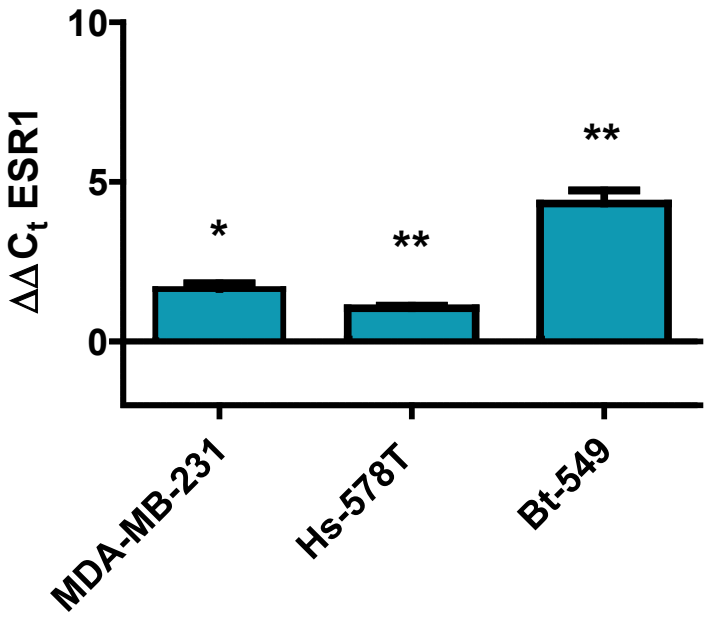


Figure 22 Gene expression analysis of *ESR1 in TNBC* cell lines in response to cortisol treatment. MDA-MB-231, Hs-578T, and BT-549 cell lines were treated with 5µM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate *ESR1* mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (un-treated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells). * represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

We next investigated whether the loss of methylation was translated into ER protein expression in TNBC by Western blot analysis. MCF-7, an ER+ cell line was used as positive control for ER expression. MDA-MB-231 and Hs-578T cells were treated with 5µM of cortisol for 20 days. Protein was extracted from whole cell lysate of control untreated cells (unstim) and cortisol treated cells (cort) from both cell lines. Equal amounts of protein were used for Western blot analysis. Results in Figure 23 demonstrate the re-expression of ER protein in both TNBC cells in response to 20 days of treatment with cortisol (Figure 23 a, b, and c).

Based on previous findings from chapter 3, it was suspected that the loss of methylation of *ESR1* is due to the decreased expression of DNMT1. We therefore analysed the protein expression of DNMT1 using western blot analysis in MDA-MB-231 and Hs-578T treated with cortisol for 20 days. However, there were no significant changes in DNMT1 protein expression in response to cortisol treatment in both cell lines (Figure 23 a, d, and e).

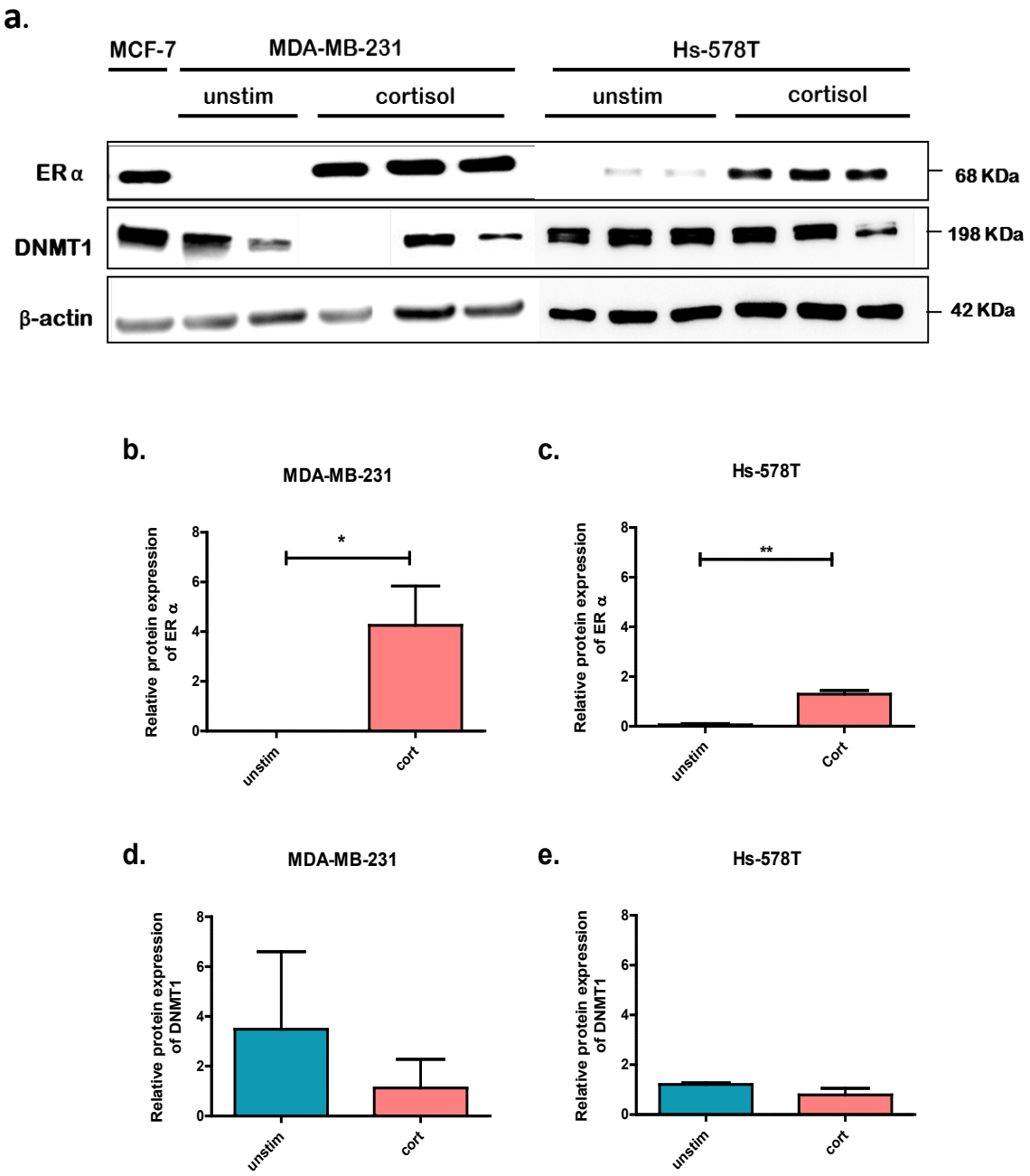


Figure 23 Effects of long term cortisol exposure on ER protein expression in MDA-MB-231 and Hs-578T cells. TNBC MDA-MB-231 and Hs-578T were treated with 5 µM of cortisol for 20 days. MCF-7 was used a positive control in this experiment. Whole cell lysate was prepared from control untreated cells (unstim) and treated cells (cort). A. Western blot analysis was performed using antibodies against ER, DNMT1, and βActin (βActin was used as endogenous control of expression). B and C Represent quantification of ER α expression from corresponding western blot performed using imagej software. Results demonstrate higher expression of ERα in cells treated with cortisol (cort) in MDA-MB-231 and Hs-578T respectively in comparison to control untreated cells (unstim). D and E Represent quantification of ER α expression from corresponding western blot performed using imagej software. There were no significant changes in DNMT1 protein expressions in response to cortisol treatment in both cell lines. T-test was used to compare protein expression between untreated (unstim) and treated (cort) samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

4.2.2 The effect of cortisol on the functionality of the re-expressed ER in TNBC

The function of the re-expressed ER in TNBC was analysed by testing the ability of the re-expressed ER protein to respond to an ER antagonists. MDA-MB-231 and BT-549 cells were treated with 5µM of cortisol for 20 days. Untreated control cells (unstim) and cells treated with cortisol (cort) were then plated into a 96-well plate. Both groups of cells were subjected to either one or a combination of the following ER antagonists: 1 µM of Fulvestrant (Fulv) and/or 1µM of Tamoxifen (Tam). Cells were also treated with Estradiol (E2) at a concentration of 10 nM to test the response of cells to an ER agonist. The concentrations of treatments used were optimisedby treating MCF-7 (an ER+ cell line) cells with different doses of Tam, Fulv, and E2(Appendix Figure 4). MDA-MB-231 and BT-549 cells were incubated with each treatment for 7 days, and an MTT assay was then preformed to determine cell viability. The viability of cortisol-treated MDA-MB-231, and BT-549 cells was significantly decreased in response to fulvestrant. This suggests that the re-expressed ER previously described is actually active, functional and facilitates an oestrogen response to Fulvestrant (Figure 24)

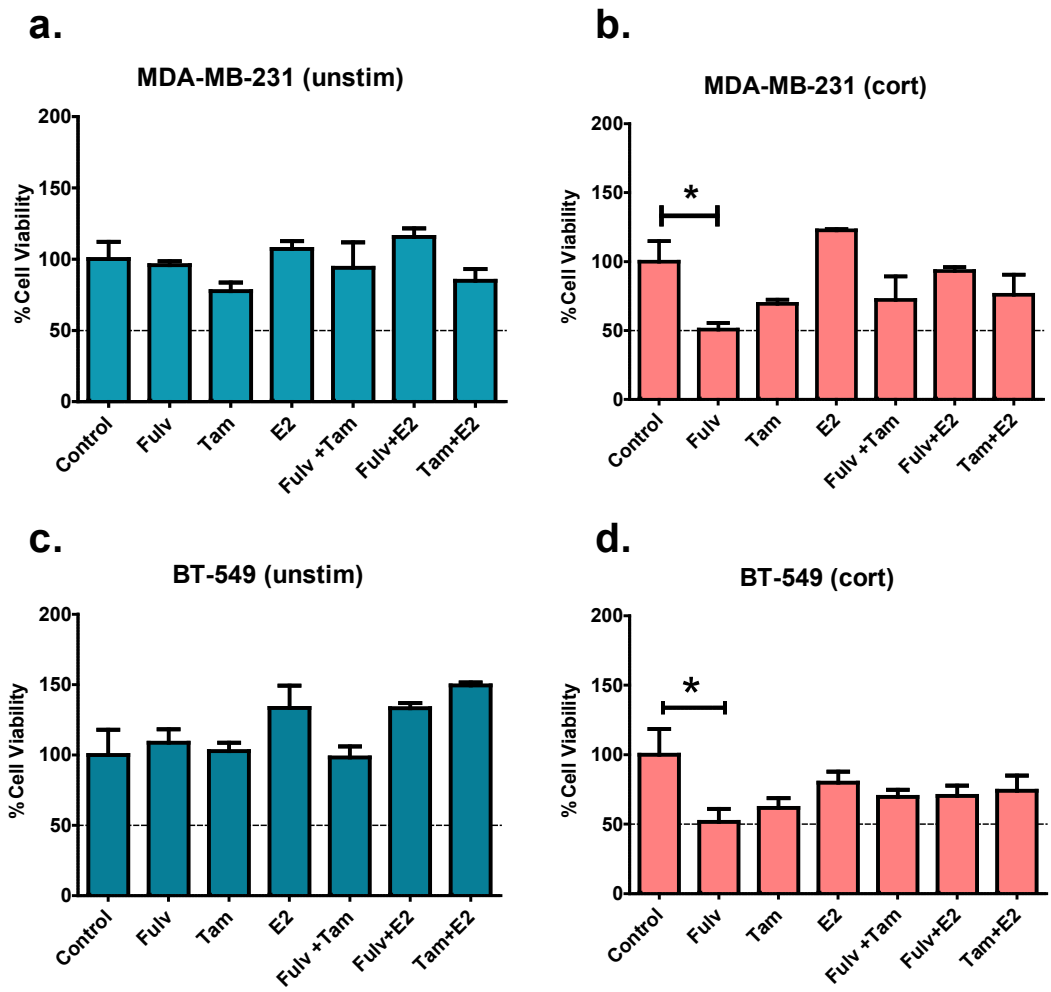


Figure 24 MTT results demonstrating sensitivity of TNBC to Tamoxifen and Fulvestrant after 20 days of treating with cortisol. MDA-MB-231 and Bt-549 triple negative breast cancer cell lines were treated with 5µM cortisol for 20 days (cort). 3000 cells/ well of control untreated cells (untim) and cortisol treated cells (cort) of both cellswere plated in a 96-well plate and treated with either one or combination of the following: 1µM of Fulvestrant (Fulv), 1µM of Tamoxifen (Tam), and 10 nM of Estradiol (E_2). **A**. Control MDA-MB-231 (unstim) cells shows no change in cell viability is response to treatment with Fulv, Tam, E_2, and combination of treatment. **B**. Cell viability of MDA-MB-231 treated with cortisol significantly decreases in response to treatment with Fulvestrant and Tamoxifen. Cell viability significantly increases in response to E_2. **C**. Control BT-549 (unstim) cells shows no change in cell viability is response to treatment with Fulv, Tam, E_2, and combination of treatment. **D**. Cell viability of BT-549 treated with cortisol significantly decreases in response to treatment with Fulvestrant and Tamoxifen. Cell viability significantly increases in response to E_2. Mean ±SEM is presented and one-way ANOVA followed by Dunnett correction was used to compare all conditions to control.* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

4.2.3 Downstream effect of ER re-expression

To determine if cortisol could alter downstream ER target genes, a PCR array was used to profile 84 key ER target genes. MDA-MB-231 cells were left untreated (control), treated with 5 µM of cortisol for 20 days, or treated with 5µM of cortisol for 20 days followed by 4 days of treatment with 10nM of E2. RNA was then extracted and cDNA was synthesised. The template cDNA was then used to study the mRNA expression of 84 different genes in RT² Profiler PCR Array for Human Estrogen Receptor Signalling genes. Analysis of profiler array data was performed using Qiagen software using ΔΔCt method. In cells treated with cortisol for 20 days, results demonstrated downregulation of more than 2 fold in the following genes *APBB1, BCAR1, BDNF, BMP4, C3, CCND1, CKB, CTGF, ERBB3, FOS, FST, HSP90AA1, IRS1, L1CAM, NCOR1,* and *PELP1*. There was also an increase of more than 2 fold changes in the following genes; *NR0B1, LTBP1, SNAI1, TGFB3, WISP2, WNT4,* and *WNT5A* in cells treated with cortisol for 20 days (Figure 25). In cells treated with 20 days of cortisol followed by 4 days of E2 treatment a downregulation of more than 2 fold changes was demonstrated in *EGR3, ERBB3, FOS, FST, GPER1, IGFBR3, KLK3, L1CAM, MMP9, PELP1,* and *VEGFA*, and an increase of more than 2 fold in *SNAI1, WNT4,WNT5A,* and *LPL* (Figure 26)

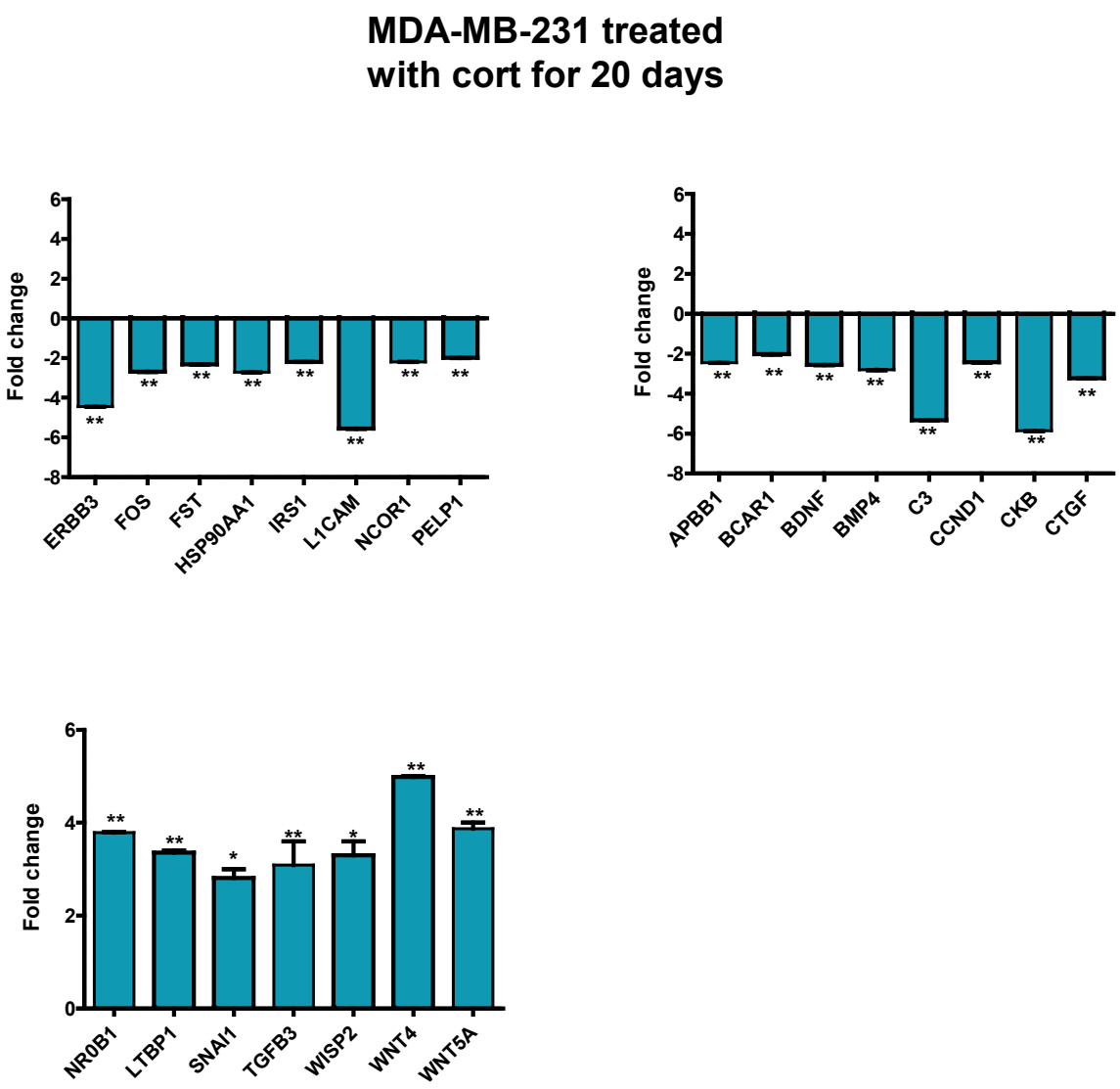


Figure 25 Gene expression analysis of oestrogen receptor signalling genes using RT^2 PCR profiler. MDA-MB-231 cells were grown in phenol red free media and charcoal stripped FBS; cells were then treated with 5µM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate mRNA expression of 84 genes involved in oestrogen receptor signalling genes using RT^2 PCR profiler array. Results are presented as fold changes calculated using the ΔΔCt method normalised to control cells (un-treated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells). * represents significant difference (* p<0.05, ** p<0.01, ***p<0.001)

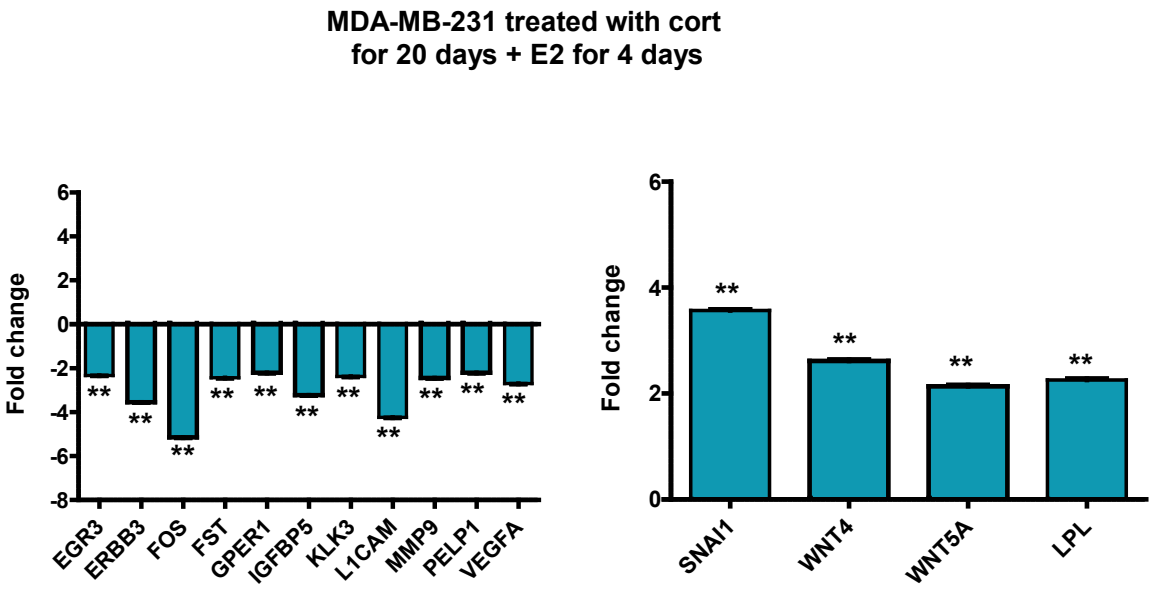


Figure 26 Gene expression analysis of oestrogen receptor signalling genes using RT^2 PCR profiler. MDA-MB-231 Cells were grown in phenol red free media and charcoal stripped FBS; cells were then treated with 5µM of cortisol for 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate mRNA expression of 84 genes involved in oestrogen receptor signalling genes using RT^2 PCR profiler array. Results are presented as fold changes calculated using the ΔΔCt method normalised to control cells (un-treated). Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (untreated cells). * represents significant difference (* p<0.05, ** p<0.01, ***p<0.001)

4.2.4 The effect of dexamethasone on the ER status in MDA-MB-231

To evaluate if the changes in *ESR1* promoter methylation pattern are altered using other GCs, MDA-MB-231 breast cancer cell lines were treated with Dexamethasone (Dex) a synthetic GC that is more potent that hydrocortisone. Cells were treated with 0.25 µM, 0.5µM, and 1µM of Dex for 24 hours. DNA was extracted from control untreated cells (unstim) and cells treated with Dexamethasone (Dex). An equal amount of DNA products were subjected to digestions with different digestion enzymes as described in chapter 2. We determined a loss of methylation of *ESR1* in TNBC treated with Dex at a concentration of 1µM compared to unstimulated cells.

To control for hormone specificity, MDA-MB-231 cells were treated with the GR antagonist mifepristone (RU-486). RU-486 was added at a concentration of 1 µM for 30 minutes prior to the addition of 1µM cortisol for 24 hours. DNA was then extracted from control (unstim) cells, Dex treated cells with or without RU-486. DNA was then subjected to enzymatic digestion as described earlier, and the product of the digestion was used for qRT-PCR run. The loss of methylation found on the promoter region of *ESR1* was abrogated when GR was blocked with RU-486 (Figure 27).

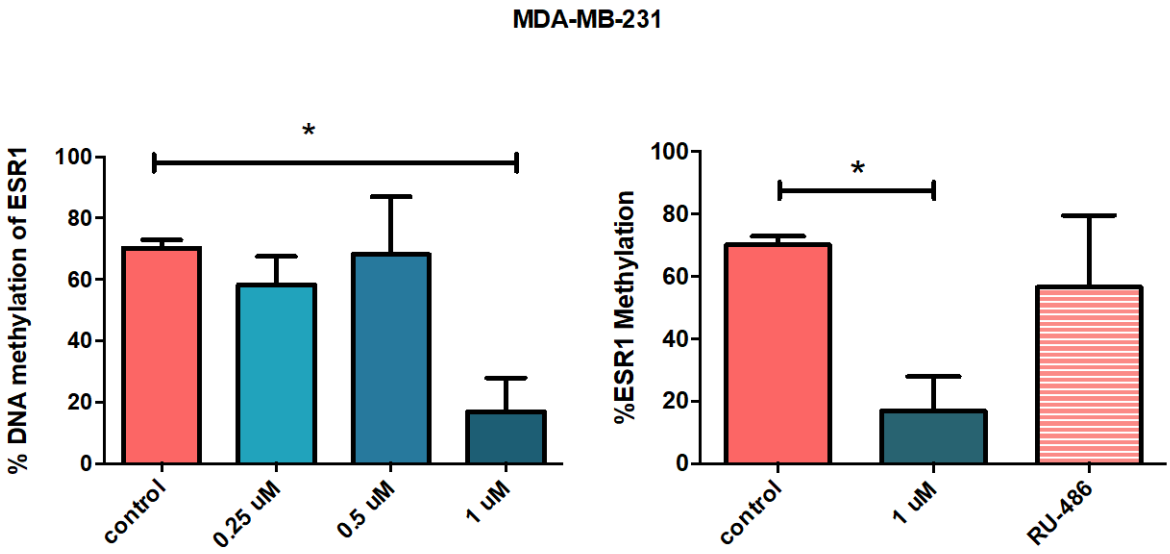


Figure 27 Loss methylation of the *ESR1*promoter in response to dexamethasone treatment, this loss was abrogated when blocking with GR antagonist Mifeprestone (RU-486). MDA-MB-231 breast cancer cells were treated with 0.25µM, 0.5 µM, and 1µM of Dexl for 24hours. In a separate experiment MDA-MB-231 cells were treated with 1µM of Dex for 24hours and compared to MDA-MB231 cells treated with 1µM of GR antagonist (RU-486) 30 minutes prior to the addition of Dex. DNA was extracted from untreated control cells and Dex treated cells. DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for *ESR1* to study the change of the methylation status on the promoter region of *ESR1*. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. T-test to compare untreated (control) and treated samples.* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

4.2.5 The effects of psychological stress on the methylation pattern of ER and its expression in a xenograft mouse model

To assess the effect of psychological stress on the methylation pattern of *ESR1*, the MDA-MB-231 xenograft model that was described in chapter 3 was used. Nude mice were injected with $5x10^6$ MDA-MB-231 cells into the fourth left mammary fat pad of mice. When the tumour volume reached 50- 100 mm^3, tumour bearing mice were randomised into a control group (n=3) and restraint stress group (RS) (n=4). Mice were subjected to 4 weeks of dailyrestraint stress. Restraint stress model was used for this investigation due to evidence suggesting that restraint stress elevates cortisol level[73]. At the end of the study, primary tumours were harvested and DNA was extracted from tumours. DNA products were then analysed using a restriction enzyme-based method followed by qRT-PCR. EpiTect Methyl II PCR primer for *ESR1* was used to study the change of the methylation status on the promoter region of *ESR1*. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. Although there was a decrease in the average methylation percentage in RS group compared to the control group this was not significant. However, the presented trend indicates that psychological stress exerts an effect on the methylation status of *ESR1* in the RS group compared to control group (Figure 28). To investigate whether the loss of methylation on the promoter region of *ESR1* was sufficient to upregulate the expression of *ESR1*, total RNA was extracted from the same tumours and cDNA was synthesized. mRNA expression of *ESR1* was assessed using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method.

ESR1 mRNA expression was significantly higher in RS group compared to control group (p=0.0451) (Figure 29). Taken together, we have determined that the percentage of methylation of *ESR1* in each mouse is negatively correlated with the expression of *ESR1* (Figure 30).

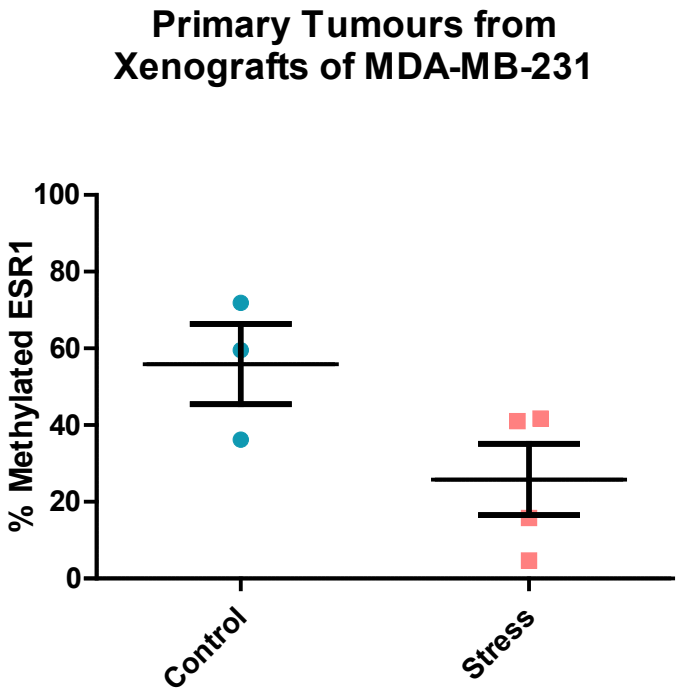


Figure 28 Methylation p of *ESR1* in primary tumours of MDA-MB-231 derived xenografts. Nude mice were injected with $5x10^6$ MDA-MB-231 into the left mammary fat pad. When the tumour volume reached 50- 100 mm^3volume, tumour bearing mice were randomised into a control group and restraint stress group. At the end of the study, primary tumours were harvested**.** DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes which digests methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for *ESR1* to study the change of the methylation status on the promoter region of *ESR1*. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. Results are presented as ±SEM and changes in methylation percentage between control and RS groups were analysed using t-test. Although the decrease in the average of methylation percentage in stress group compared to the control group is not significant, the presented trend indicates that psychological stress exerts an effect on the methylation status in RS group compared to the control group.

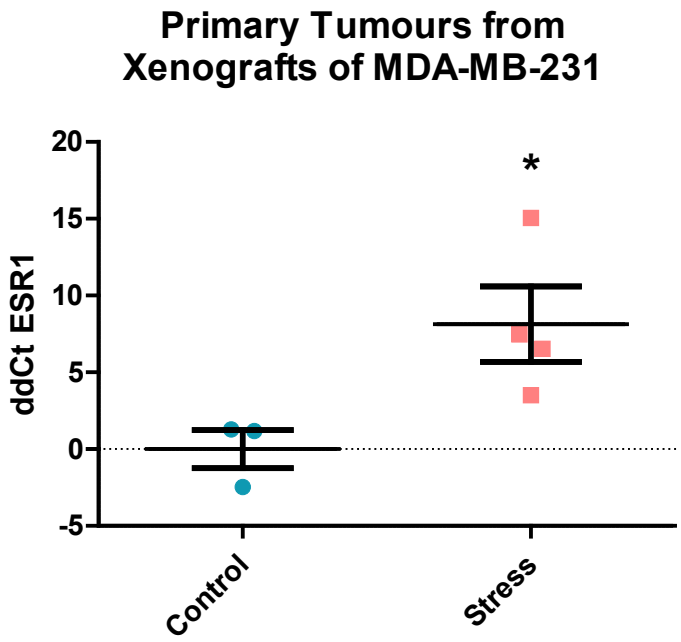
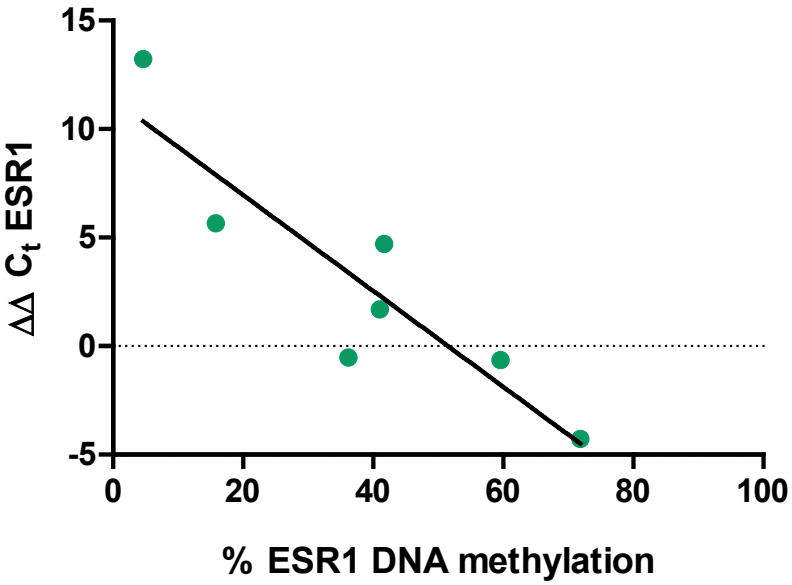


Figure 29 Gene expression analysis of *ESR1* in the primary tumours of MDA-MB-231 derived xenografts. Nude mice were injected with $5x10^6$ MDA-MB-231 into the left of mice. When tumour volume reached 50- 100 mm^3volume, tumour bearing mice were randomised into a control group and restraint stress group. At the end of the study, primary tumours were harvested, total RNA was extracted, and cDNA was synthesised to evaluate *ESR1* mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control group Mean ± SEM expressed and one sample t-test was used to compare the mean ±SEM significance to a hypothetical value of 0. * represents significant difference (*p<0.05, ** p<0.01, ***p<0.001). The mean *ESR1* relative expression was significantly increased in stress group (*p*=0.0451)



Pearson r	**-0.9009**
95% confidence interval	**-0.9854 to -0.4595**
P value (two-tailed)	**0.0056**
P value summary	**
Is the correlation significant? (alpha=0.05)	**Yes**
R square	**0.8116**

Figure 30 Negative correlation between the percentage of methylation of the promoter region of *ESR1* and the gene expression of *ESR1*. Nude mice were injected with $5x10^6$ MDA-MB-231 into the left of mice. When tumour volume reached 50- 100 mm^3volume, tumour bearing mice were randomised into a control group and restraint stress group (RS). At the end of the study, primary tumours were harvested, total RNA was extracted, and cDNA was synthesised to evaluate *ESR1* mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. DNA was also extracted from harvested tumours, and used to evaluate the DNA methylation of *ESR1* using qRT-PCR. Results demonstrated significant negative correlation between the percentage of methylation of the promoter region of *ESR1* and the gene expression of *ESR1*. Pearson r test was performed p =0.0056 and R square value = 0.8116

4.3 Discussion

The expression of ER is primarily governed by epigenetic mechanisms that are mainly regulated by *DNMTs* and *HDACs*[238, 264]. Previous findings demonstrated that long term exposure to cortisol lead to the down regulation of *DNMT1*, and *HDAC1*. The fact that the inhibition of HDAC results in a chromatin modification that favours active chromatin and promotes gene reactivation through degradation of *DNMT1*, and the role of *DNMT1*downregulation in demethylation has led to investigate whether long term exposure to cortisol could re-express ER and restore sensitivity to breast cancer endocrine therapy.

Our findings demonstrated that exposing a panel of TNBCcells to cortisol for 20 days causes a significant loss of methylation and a significant increase in mRNA *ESR1* expression, and upregulation of ERα protein. Cellular proliferation was also significantly reduced in response to endocrine therapy treatment, implying the restoration of sensitivity to endocrine treatment. Although cells were grown in charcoal stripped FBS and phenol red-free media, it is suspected that media used might have traces of hormone content [265].

Although initially, the re-expression of the functional ER was hypothesised to be attributed to the down regulation of *DNMT1*, western blots did not show consistent loss of DNMT1 protein in TNBC cells treated with cortisol. Therefore, future work will explore whether DNMT1 activity is inhibited by cortisol in TNBC cells and whether this change in activity can account for re-expression of ER.

The downstream effect of ER targeted genes was also explored. The data suggest that cortisol can regulate ER response genes; resulting in a less aggressive or basal-like phenotype. For example, the upregulation of Proline-, glutamic acid-and leucine-rich

protein1 (*PELP1*), an oestrogen receptor coactivator and proto-oncogene, has been reported to be associated with poor prognosis in breast cancer patients [266], and with resistance to endocrine therapy *in vitro*[267]. However, cortisol downregulated the expression of *PELP1* suggesting a favourable phenotype. The over expression of ErB-B2 receptor tyrosine kinase 3 (*ERBB3*) has been associated with malignant phenotypes as it plays a crucial role in cell proliferation and migration [268, 269]. Results demonstrated that cortisol treatment led to the downregulation of *ERBB3*. Our results are also in line with a study that demonstrated the degradation of *ERBB3* in response to E2 treatment in ER+ MCF-7 cells [270]. The overexpression of the cell adhesion molecule *(L1CAM*)has been correlated with tumour aggressiveness and poor prognosis in patients and promotion of breast cancer motility *in vitro* [271, 272]. L1CAM wasdownregulated in response to cortisol treatment, suggesting a favourable phenotype and less aggressiveness. This suggestion can be also supported by a study that demonstrated the over-expression of L1CAM promoted invasion and migration of TNBC cells, and*L1CAM* was therefore regarded as a driver of tumour progression [273]. Another study found *L1CAM* to be overexpressed in fulvestrant resistant MCF-7 cells; the results of this study demonstrated a negative correlation between L1CAM and ER status [274]. The inhibition of the Snail Family transcriptional repressor1 (*SNAI1*) was reported in literature to restore sensitivity to tamoxifen in oestrogen- hyposensitive MCF-7 cells [275]. This explains our findings showing that cortisol treated TNBC MDA-MB-231 cells demonstrated a partial and non-significant response to tamoxifen, and PCR arrays demonstrated an upregulation of *SNAI1* in cortisol treated MDA-MB-231 cells. This is in line with results of a study demonstrating the downregulation of several genes related to EMT including *SNAI1* upon the co-activation of ER and GR in ER+ BC [39]. However, other studies

have reported a higher expression of *SNAI1* in patients with complete pathological response to therapy, postulating a protective and anti-tumorigenic role for *SNAI1* in breast cancer [276]. Another study used integrated analysis to demonstrated that the regulation of miR-204, -200c, -34a, and -10 plays a role in increasing the survival rate of invasive breast cancerby up-regulating *SNAI1* and other genes [277]. Thus the demonstrated increase in *SNAI1* expression in response to cortisol treatment could be protective. We also found that *WNT4* was increased in cortisol treated cells. others have shown that an induced overexpression of *WNT5Ain vitro* led to tumour suppressive responses characterised by impaired migration and invasion [277]. Investigators have also correlated reduced expression of *WNT5A* to loss of ER and early relapse in invasive ductal carcinoma patients [278, 279]. In line with these studies, the significant increase in *WNT5A* mRNA expression in MDA-MB-231 TNBC cells further supports the suggestion of less aggressive cancer cell phenotype.

Results also demonstrated downregulation of *CCND1* in MDA-MB-231 in response to cortisol treatment. This is in line with a study found *CCND1* to be downregulated in ER+ breast cancer upon the co-activation of GR and ER compared to ER activation alone [38].

We have also explored the effects of dexamethasone on the methylation and expression of ER. Indeed, treating with dexamethasone resulted in loss of methylation on the promoter region of *ESR1* in a dose-dependent manner; and this loss of methylation was abrogated by blocking the GR with mifepristone (RU-486); implying that the change in methylation on the promoter region of *ESR1* is GR mediated. The release of cortisol in response to psychological stress has also led to the loss of methylation of the promoter region of *ESR1*. This was demonstrated *in vivo* by subjectingTNBC xenografts to restraint stress[73]. Although not all

mice lost the methylation of the promoter region of *ESR1*, this could be due to the resilience of some animals to stress in comparison to other specimens in the study. The loss of methylation found in mice was negatively correlated with the mRNA expression of *ESR1.*

In conclusion, we have demonstrated that exposure to glucocorticoid hormones could potentially activate ER in ER-negative breast cancer and restore sensitivity to endocrine therapy. Although it is possible that the epigenetic change could be due to the alteration of *DNMT1* and *HDAC1* expression, the detailed mechanism is yet to be investigated. However, these results are of great clinical benefit to patients with hormone-resistant triple negative breast cancer.

There has been increasing evidence of epidemiological correlation between psychological stress and the molecular effects of stress hormones and glucocorticoids on cancer cells. However, although many studies correlated stress to worse prognosis and associated stress hormones with tumorigenesis, it is worth remembering that stress can facilitate a “fight or flight response” and aid survival depending on the duration of stress. Our results suggest that psychological stress might potentially be altering the phenotype ofbreast cancer in some patients. However, this is not necessarily a bad or harmful effect; our findings imply that stress can play a protective role in this case. Restoring the ER expression along with its functionality and sensitivity to endocrine therapy might potentially be a new therapeutic approach to target triple negative breast cancer. Although, there was diminished enthusiasm for re-expressing ER in TNBC due to unsuccessful clinical trials our findings suggest that this is still worth pursuing.

5. Chapter 5: The effect of age and psychological stress on the epigenetics of breast cancer

5.1 Introduction

Around 80% of breast cancers in the UK arise in women over the age of 50 with highest risks in people age 90+ [1, 280]. However, breast cancer is the most frequent cancer among women under the age of 40 [281]. Although TNBC can occur at any age, it is more likely to be diagnosed in younger patients, under the age of 40 [282-285]. In TNBC patients, increased age has been inversely correlated with tumour grade, and positively correlated with disease free survival. Hence, patients diagnosed with TNBC under the age of 40 have been reported to have poorer prognosis [284, 286]. However, the biological differences between triple negative tumours developed in younger patient and triple negative tumours developed in older patients remain elusive and unclear [286].

Epigenetic alterations mediate the interaction between the environment and the genome during the entire lifetime of organism. Studies have shown that epigenetic modifications e.g. DNA methylation play crucial roles in the ageing process [173]. Therefore, understanding the mechanism governing these events is essential to understand the molecular mechanisms that contribute to age-associated physiological deterioration and disease [173, 287].

Recent studies have demonstrated that methylation patterns clearly change throughout the lifespan of species. Like cancer, age is correlated with steady but profound changes in DNA methylation[288]. This directional change characterized by global hypomethylation and promoter region hypermethylation in aged individuals is known as “epigenetic drift “[289].

Although, the mechanisms responsible for the epigenetic drift during ageing are still not clearly understood, *DNMT*s remain the main contributor to epigenetic modifications altering protein levels and enzymatic activities by changing methylation patterns [287, 290]. Many studies have associated the decrease of *DNMT*1 with ageing. For example, investigators used a *DNMT1* $^{+/-}$ mouse model and found that a significant decrease in DNA methylation level in the brain cortex and hippocampus affected cognitive functions in an age-dependent manner[291]. This decrease in *DNMT1* expression reported may be the cause behind the loss of global, and loci specific methylation during ageing [292]. Besides the loss of methylation globally and on promoter regions, hypermethylation on promoter regions of specific genes has been observed during ageing [292, 293]. For example, the Period Circadian Regulator gene *PER2* plays a crucial role in regulating the circadian rhythm in humans, this is particularly important in this project as the disruption in circadian rhythm has been associated with the development of breast cancer [294-297]. Therefore, *PER2*plays a crucial role in stress responses as the rhythmic expression contributes to the regulation of the HPA excitability and the regulation of cortisol diurnal rhythm[298]. However, the molecular changes of circadian genes including *PER2*are yet to be understood [294]. Nevertheless, the promoter region of (*PER2*) is found to be subjected to epigenetic modification and hypermethylation during ageing [299]. *PER2*has also been found to be deregulated epigenetically in many cancers including breast cancers lacking oestrogen receptor and prostate cancer [297, 300].

Another gene, Lipoprotein lipase (*LPL*) was found to decrease with age through hypomethylation, but found to be hypermethylated in cancers such as prostate cancer [301-303]. However, in TNBC LPL was found to be highly expressed as a result of epigenetic

reprograming [301-303]. *LPL* has been reported to play a role in the carcinogenesis process in humans as it can modify the apoptosis pathway [303].

The glucocorticoid receptor gene *NR3C1* is also of a great interest as it plays a role in both stress and ageing process; one study demonstrated that trauma exposure at different ages exert different effects on the methylation status of NR3C1 [304].

Finally, although DNA methylation is the most widely studied mechanism in correlation to ageing, histone modifications also play a crucial role in the ageing process [298]. Aberrant expressions of HDACs have been reported to be involved in the alteration of lifespan in different organisms, and HDACs inhibitor were proposed as drug class to fight ageing related conditions [305, 306]. However, less is known about the role of histone modifications and HDACs in the ageing process[307].

In this chapter, we explore the epigenetic alterations affected by ageing and stress. An *in vivo* study has been designed with 3 age groups of syngeneic BALB/C female mice. All mice were injected with 66cl4 cellsinto the mammary fat pad as described in chapter 2. Within each age group (3 months, 9 months, and 18 months), mice were randomised into restraint stress group and a control non-stress group. Upon necropsy, primary tumours were analysed and the differences in *DNMTs* expression among age and stress groups was determined. This chapter aims to investigate how different age groups react to stress and how that affects tumour progression and response to therapy. The methylation status on *NR3C1, LPL*, and *PER2* and the expression of *NR3C1*and *HDACs* were analysed using PCR.

5.2 Results

5.2.1 Tumour weights, volumes, and metastasis status

In order to study the effect of stress on the expression of DNMTs, female BALB/c mice of 18 months, 9 months and 3 months were injected with 1x 10^4 66CL4 cells. During the development of tumours, mice were monitored and their tumour size was measured with a calliper every other day. During the ageing process, some mice died due to non-cancer related reasons. When tumour volumes reached 50- 100 $mm^{3,}$ mice were randomised into either restraint stress (RS), and non- stress (NS) groups within each age group as shown in table below (Table 4).

Table 4 In vivo groups and number of mice

	NS	RS
3 months	9 mice	9 mice
9 months	7 mice	8 mice
18 months	4 mice	8 mice

Mice were subjected to restraint stress for 2 hours daily at the same time from 10 am to 12 am, by placing mice individually in adequately ventilated 50 ml tubes. This is a well-established model known to elevate cortisol levels of mice[188, 308]. When mice were sacrificed blood was collected from mice, for later analysis of cortisol. Primary tumours,

lungs, ovaries and kidneys were also collected. Primary tumours were weighed. Upon necropsy, organs that were suspected to have potential metastasis were noted.

The primary tumour weights were significantly lower in mice aged 18 months in both stressed and non-stressed groups compared to mice aged either 3 months or 9 months (Figure 31a). The number of organs showing metastatic lesions in 18 month old stressed mice was significantly highercompared to the number of affected organs in 9 months old stressed mic; however, the number of affected organs was significantly higher compared to non-stressed mice (Figure 31b, c). This shows a clear trend for a lower primary tumour weight in the old animals and more metastases regardless of whether the animals were stressed or not (Figure 31). There was no significant difference in primary tumour volume among the groups (Figure 31d, e, f).

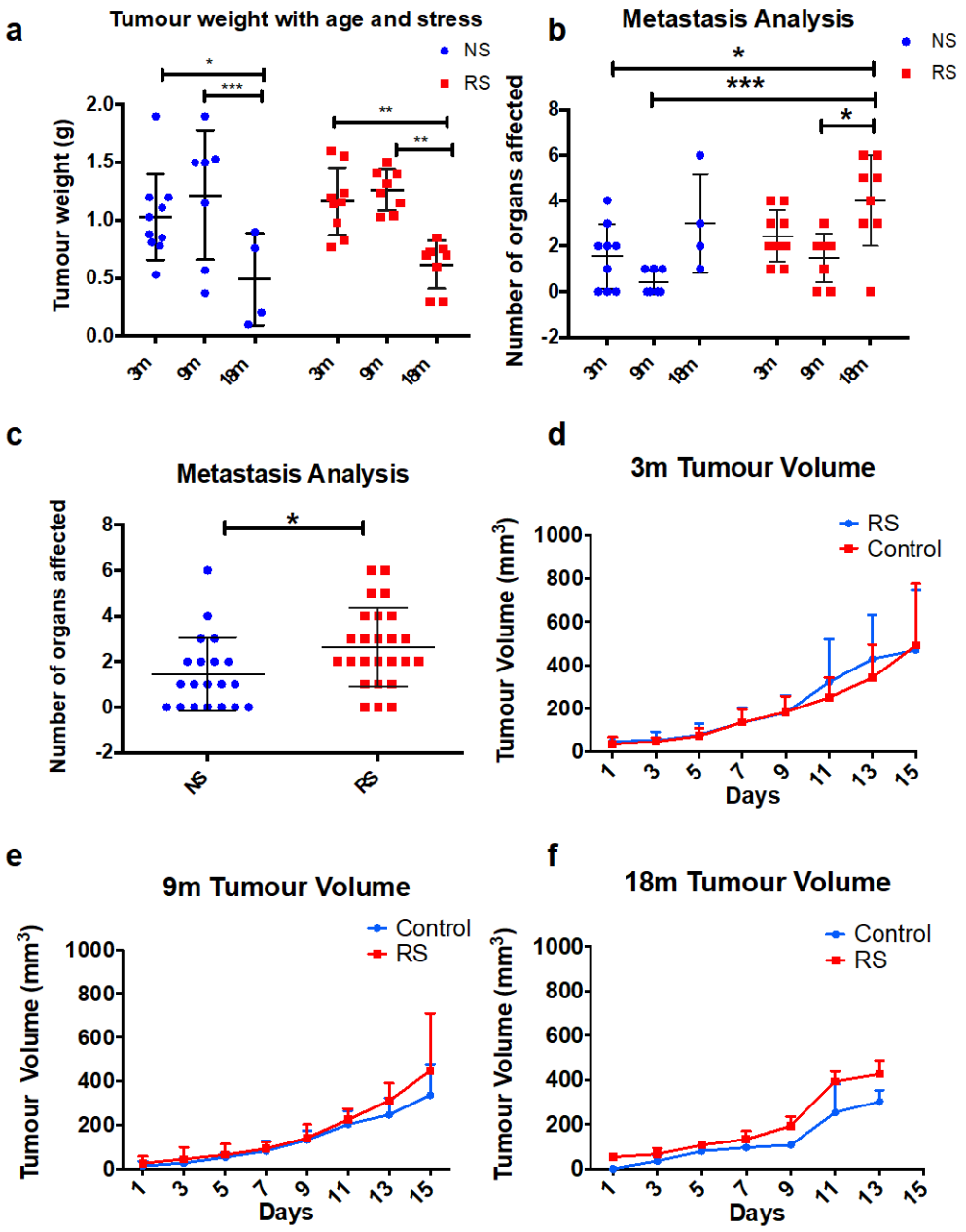
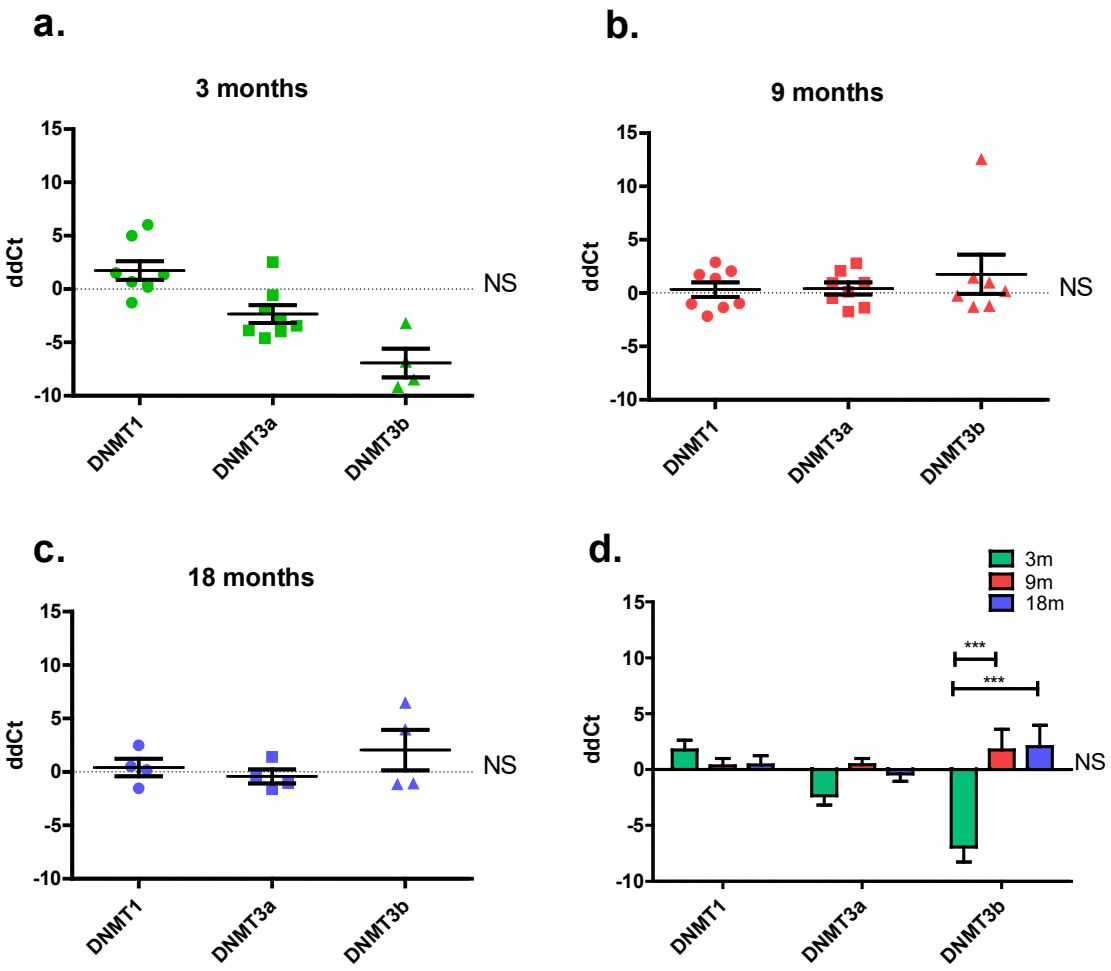


Figure 31Tumour weights, volumes, and metastasis status After injecting BALB/c mice with 66CL4 breast cancer cell line, tumours were weighed in non-stressed control groups (NS) and restraint stressed groups (RS) of different ages (3 months, 9 months, and 18 months). a. Significant decrease in the tumour weights of the 18-months old non-stressed (NS) mice, compared to 3-months old and 9-months old (NS) and a significant decrease in the tumour weights of 18-months old stressed mice compared to 3-months mice and 9-months mice of the same group of stressed mice (RS). b. Significant increase of number of organs of potential metastasis in the stressed group of mice (RS) compared to non-stressed mice (NS). d,e,f illustrates no significant difference in primary tumour volume among the groups. One-way ANOVA with Bonferroni post-test was used to compare among all age groups of different genes * represents significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.2.2 DNMT expression

All primary tumours were harvested at necropsy. Total RNA was extracted from the tumours, cDNA was synthesised and the expression of *DNMT*s were analysed using qRT-PCR. *GAPDH* was used as an endogenous control for standardisation as it is considered a stable housekeeping gene in ageing studies [309, 310]. Expressions of genes are presented as relative quantification calculated using the ΔΔCt method. Results were normalised to control group (non-stress group) of the same age group, and analysed by comparing the mean significance to a hypothetical value of 0 (non-stressed mice) (Figure 32 a, b, and c).

There was nosignificant change in *DNMT1, DNMT3a*, and *DNMT3b* expression between stressed and non-stressed groups (Figure 32d). A two-way ANOVA was used to analyse the data, and analysis showed a significant effectin age variation among genes (P = 0.0233). Post hoc analysis showed this change to be due to decrease in the expression of *DNMT3b* expression in mice of 3 months old groups in comparison to mice of 9 and 18 months old. There was also a significant interaction of the genes with age (P = 0.004).



Two-way ANOVA			
Source of Variation	**P value**	**P value summary**	**Significant?**
Interaction	0.0004	***	Yes
Age	0.0007	***	Yes
Gene	0.112	ns	No

Figure 32DNMTs expression in primary tumours of 66CL4 syngeneic mouse model. A syngeneic mouse model of female BALB/c was developed using 66CL4 cells. The model contained mice of three different age groups (3 months, 9 months, and 18 months). All mice were subcutaneously injected with $1x10^4$ 66cl4 cells. When tumour volumes reached 50 mm^3, mice were randomised into restraint stress (RS) and non-stress groups (NS), within each age group. Stressed mice were placed individually in ventilated tubes where they could turn supine to prone but cannot turn head to tail. At the end of the study, RNA was extracted from harvested tumours, cDNA was synthesised and DNMTs expressions were analysed using qRT-PCR. GAPDH was used as an endogenous control. Data from stressed mice were normalised to control group (non-stress group) from the same age group. Expressions of genes are presented as relative quantification calculated using the ΔΔCt method. Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (non-stressed mice). A Two-way ANOVA with Bonferroni post-test was used to compare among all age groups of different genes * represents significant difference(* p<0.05, ** p<0.01, ***p<0.001)

5.2.3 HDAC1 expression

In order to study the effect of stress on the expression of *HDAC1*, the syngeneic mouse model developed from 66cl4 cell line, was subjected to restraint stress as described earlier in this chapter. Total RNA was extracted from harvested tumours, cDNA was synthesised and HDAC1 expressions were analysed using qRT-PCR. GAPDH was used as an endogenous control for standardisation. Results were normalised to control group (non-stress group) of the same age group. Expression of *HDAC1* is presented as relative quantification calculated using the ΔΔCt method. There was no significant effect of stress on HDAC1 expression (Figure 33).

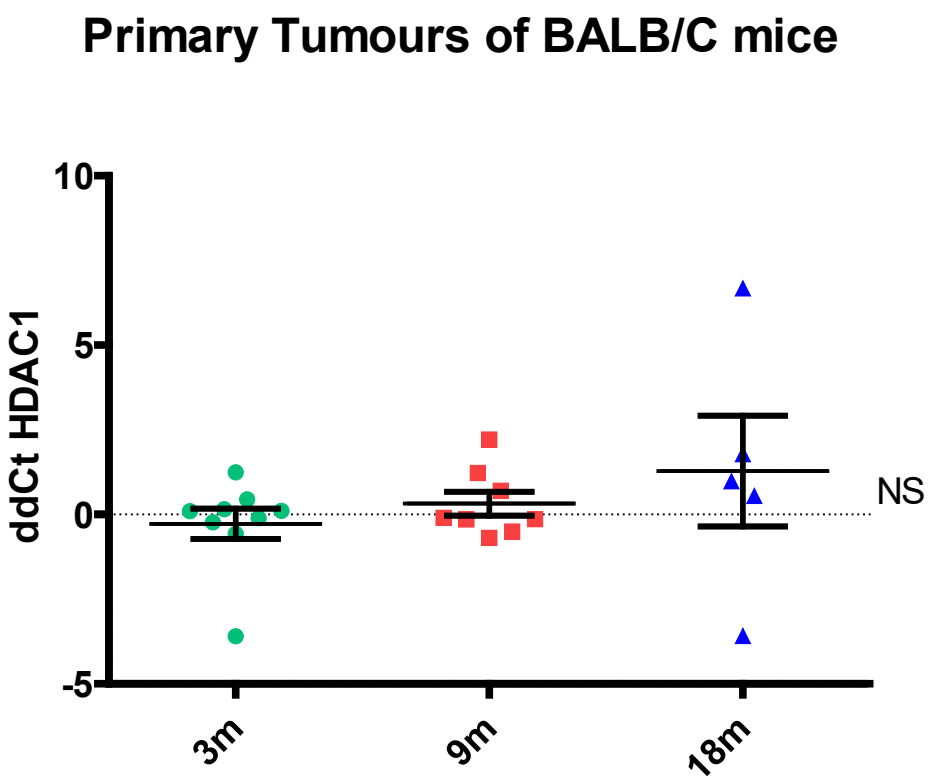


Figure 33 HDAC1 expression in primary tumours of 66CL4 syngeneic mouse model. A syngeneic mouse model of female BALB/c was developed using 66CL4 cells. The model contained three different age groups (3 months, 9 months, and 18 months). All mice were subcutaneously injected with $1x10^4$ 66cl4 cells. When tumour volumes reached 50 mm^3, mice were randomised into restraint stress (RS) and non-stress groups (NS), within each age group. Stressed mice were placed individually in ventilated tubes where they can turn supine to prone but cannot turn head to tail. At the end of the study, RNA was extracted from harvested tumours, cDNA was synthesised and HDAC1 expression was analysed using qRT-PCR. GAPDH was used as an endogenous control. Results were normalised to control group (non-stress group) from the same age group. Gene expressionis presented as relative quantification calculated using the ΔΔCt method. Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (non-stressed mice). One-way ANOVA with Dunn's post-test was used to compare among all age groups * represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

5.2.4 GR methylation status and expression

To assess the effect of stress and age on the methylation status and expression of Glucocorticoid Receptor (GR) primary tumours, the afore-mentioned syngeneic model was used. DNA was extracted from primary tumours, and DNA products were then subjected to enzyme digestion for PCR analysis. Although there was a trend for an increase in methylation in 18 month old mice; which was inhibited by stress, there were no significant differences in the methylation status between stress and non-stress groups nor among the different age groups (Figure 34, a).

RNA was also extracted from harvested tumours, cDNA was synthesised to study GR expression using qRT-PCR. GAPDH was used as an endogenous control. Results were normalised to control group (non-stress group). There was a significant decrease in GR expression in stressed mice of 9 months compared to non-stressed mice of the same age group and when compared to 3 months stressed group (Figure 34, b).

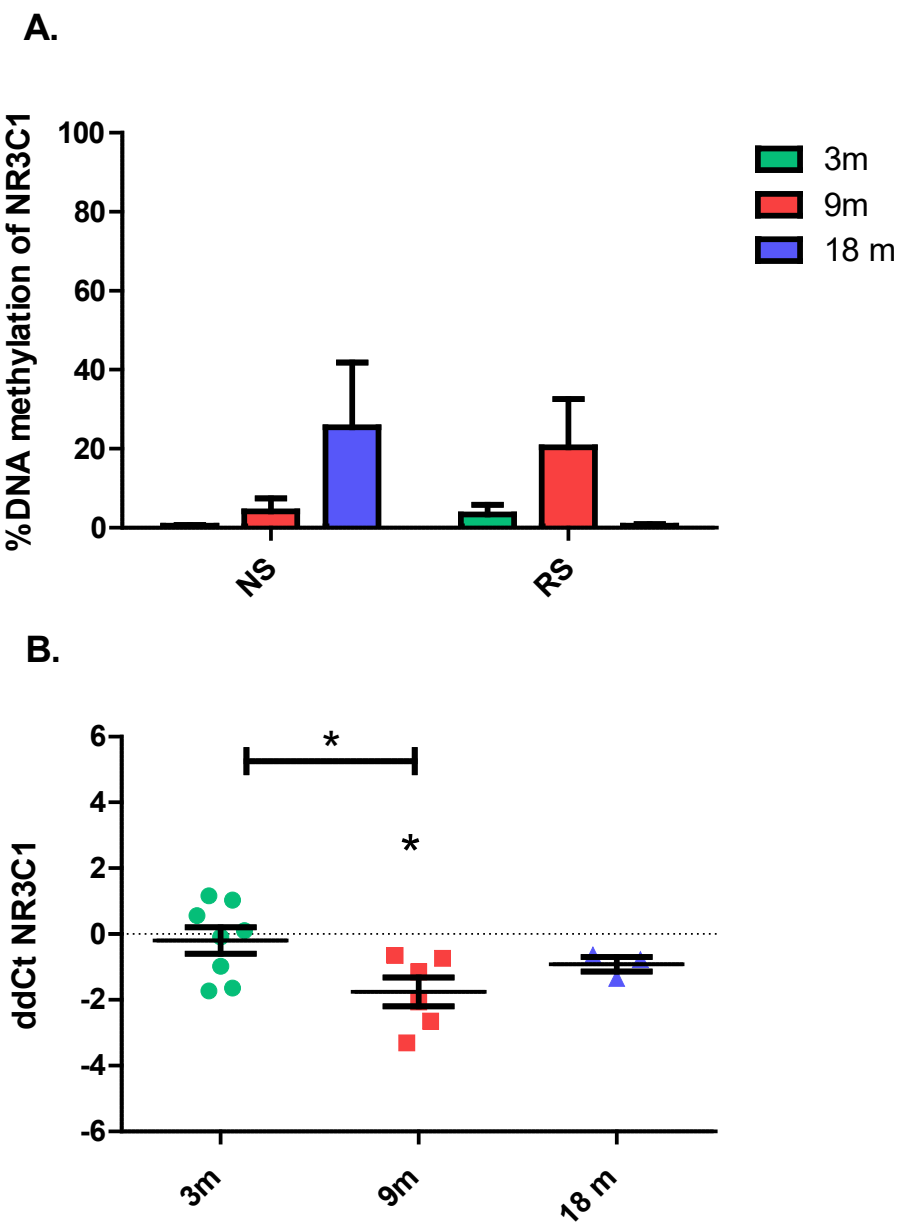


Figure 34*NR3C1* methylation status and expression in primary tumours of 66Cl4 syngeneic mouse model. A syngeneic mouse model of female BALB/c was developed by injecting $1x10^4$ 66cl4 cells subcutaneously. The model contained three different age groups (3 months, 9 months, and 18 months). When tumour volumes reached 50 mm^3, mice were randomised into restraint stress (RS) and non-stress groups (NS), within each age group. At the end of the study, DNA and RNA were extracted from primary tumours. **A.** DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes which digests methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for GR to study the change of the methylation status on the promoter region. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method. Mean± SEM expressed, and two-way ANOVA was used to compare among groups. **B**.RNA was also extracted from harvested tumours, cDNA was synthesised and NR3C1 expression was analysed using qRT-PCR. GAPDH was used as an endogenous control. Results were normalised to control group (non-stress group) from the same age group. Gene expression is presented as relative quantification calculated using the ΔΔCt method. Mean ± SEM expressed and one sample t-test was used to compare the mean significance to a hypothetical value of 0 (non-stressed mice), and one-way ANOVA with Dunn's post-test was used to compare among all age groups * represents significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.2.5 LPL methylation Status

To assess the effect of stress and age on the methylation status and expression of *LPL*, DNA was extracted from primary tumours of mice described above and DNA products were then subjected to enzyme digestion for PCR analysis. There were no significant differences between stress and non-stress groups or among the different age groups (Figure 35).

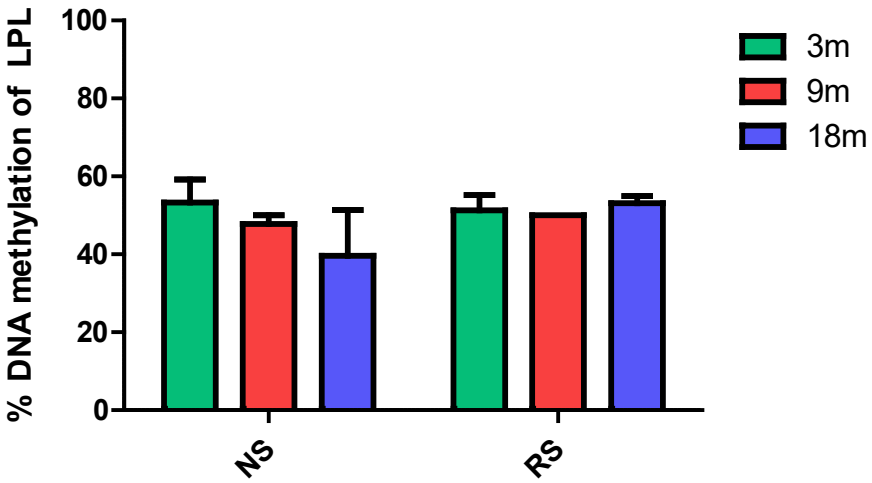


Figure 35LPL methylation status and expression in primary tumours of 66Cl4 syngeneic mouse model. A syngeneic mouse model of female BLAB/c was developed by injecting $1x10^4$ 66cl4 cells in 0.05 ml 66CL4 cells subcutaneously. The model contained three different age groups (3 months, 9 months, and 18 months). When tumour volumes reached 50 mm^3, mice were randomised into restraint stress (RS) and non-stress groups (NS), within each age group. At the end of the study, DNA was extracted from primary tumours, and DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes which digest methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for LPL to study the change of the methylation status on the promoter region. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔCt method.Mean± SEM expressed, and two-way ANOVA was used to compare among groups.

5.2.6 PER2 Methylation Status

To assess the effect of stress and age on the methylation status and expression of PER2 primary tumours from the previously described syngeneic model was used. DNA was extracted from primary tumours, and DNA products were then subjected to enzyme digestion for PCR analysis. Although we detected an average of 30% DNA methylation of PER2 in control 3 months old mice and nothing in any other groups; there were no significant differences between stress and non-stress groups or among the different age groups (Figure 36).

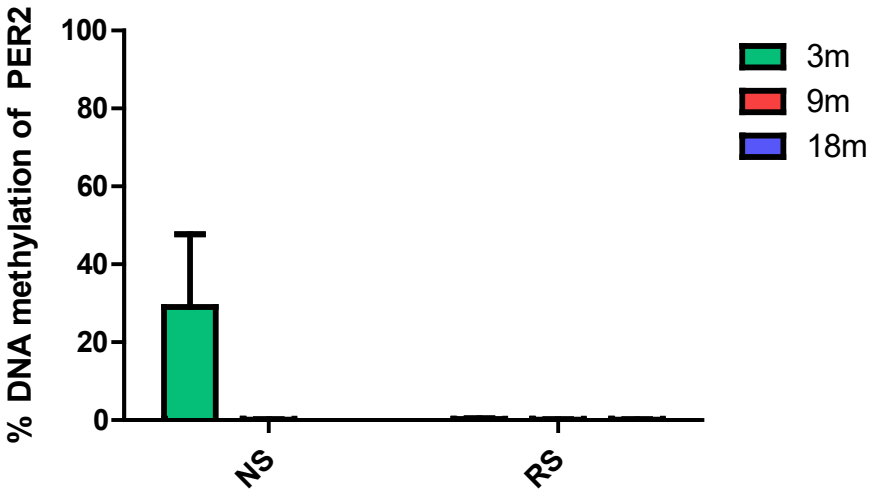


Figure 36PER2 methylation status and expression in primary tumours of 66Cl4 syngeneic mouse model. A syngeneic mouse model of female BALB/c was developed by injecting $1x10^4$ 66cl4 cells in 0.05 ml 66CL4 cells subcutaneously. The model contained three different age groups (3 months, 9 months, and 18 months). When tumour volumes reached 50 mm^3, mice were randomised into restraint stress (RS) and non-stress groups (NS), within each age group. At the end of the study, DNA was extracted from primary tumours, and DNA products were then subjected to methylation dependent and/or methylation sensitive restriction enzymes which digest methylated and/or un-methylated DNA respectively. The remaining input of DNA was analysed using qRT-PCR and EpiTect Methyl II PCR primer for PER2 to study the change of the methylation status on the promoter region. Results are expressed as a percentage of methylated DNA in comparison to the amount of un-digested DNA of the same sample using the ΔΔCt method

5.3 Discussion

Analysing the tumour development in the developed of primary tumours of syngeneic mouse model, no significant difference in volumes of primary tumours between control and stress groups was observed; however, this is a common observation in stress studies [73, 226]. Lower primary tumour weight and a higher number of organs with metastasis was found in stressed older animals. In fact, there is a clear trend for less primary tumour weight in the old animals and more metastases regardless of whether the animals were stressed or not. The reason behind that could that aged mice have a blunted response to stress as suggested in the literature, and some of our unpublished data supports this[311]. The findings are inline with evidence in literature demonstrating that stress, and age can promote metastasis. For example, a study using ovarian cancer mouse model demonstrated that the aged host is highly susceptible to metastasis [312]. A study using breast cancer PDX models found that the increase in stress hormones resulted in activating GR at distant metastasis sites, and increased colonization [72]. Another study found that stress induced tumour cell migration as well as invasion in pancreatic mouse model [313].

Results have shown a decrease in DNMTs expression in mouse mammary tumour tissues with ageing. This correlates to findings in the literature associating loss of DNMTs expressions with age in different tissues including the brain cortex and hippocampus [89, 292]. Given that the analysis and genetic study was done on the primary tumours and not the normal healthy aged tissue, the unchanged expressions of the genes analysed (*DNMT1, DNMT3a, DNMT3b , NR3C1, and HDACs*) might have been different if the mammary fat pad

of the mice was analysed and compared to tumour tissues. However, the quality of nucleic acids extracted from fat pads was not good enough to perform gene expression analysis.

Also, the tumours in the syngeneic mouse model were derived from a cell line which its cells have bypassed the normal ageing process (replicative senescence) and therefore the ageing-related results in this chapter might have been masked.

Finally, the methylation status of LPL, PER2, was found to remain unchanged in the syngeneic model design.

In conclusion, this chapter suggested correlation between ageing and stress and a decrease in *DNMT*s expressions. However, more investigation is required to understand the epigenetic mechanism in ageing stressed mice and its effect on progression of breast cancer.

6. Chapter 6: Conclusion and Discussion

6.1 Conclusion

The main aim of this project is to evaluate the impact of acute and chronic psychological stressors, through release of stress hormones, on DNA methylation in breast cancer. The project also aimed to examine the influence of ageing on the epigenetic regulation in breast cancer. This project focused on triple negative breast cancer (TNBC) as it is one of the most aggressive types of breast cancer, has poorer prognosis, and cannot be targeted by endocrine therapy [234].

In this thesis, it was shown that psychological stress and cortisol can affect DNA methylation in TNBC. More specifically, cortisol induced epigenetic changes characterised by a decrease in *DNMT1* expression, loss of methylation on key tumour suppressor genes, *DAPK1, ESR1, MGMT, ABL1, AKT1, BIRC5, CDKN1A, ING1, MDM2, NF2,* and *TERT*and loss of methylation on the repetitive element LINE-1 that has been used as a surrogate marker for global methylation. One of our main finings was that following cortisol treatment, the oestrogen receptor gene ESR1 was found to lose methylation in TNBC cells; where this gene is usually epigenetically silenced [134, 238]. Since loss of methylation commonly leads to re-expression of the gene, further investigation proved the re-expression of functional ER in TNBC cells in response to cortisol treatment. Furthermore, a TNBC xenograft study was designedto confirm the hypothesis that stress hormones could re express the ER in mammary tumours in response to a restraint stressor. The xenograft model verified the loss of methylation on *ESR1* promoter, and subsequent increase in ESR1 expression in primary tumours in mice subjected to restraint stress.

With regards to the influence of ageing, older mice exhibited a decrease in *DNMT1* in breast cancer tumours with age increase from 3-18 months. However we did not observe any significant effects of psychological stress on *DNMT1, DNMT3a, DNMT3b, HDACs, LPL, and PER2*in the tissue of breast primary tumours of the syngeneic mouse model.

Results demonstrating decrease in *DNTM1* in breast cancer is in line with the literature; for example, a recent study investigated the expression of *DNMT1* in chickens in response to nutritional stress and acute or chronic immobilization stress in the anterior pituitary[314]. Their results showed a decrease in *DNMT1* expression in response to chronic stress leading to passive demethylation in the hepatic GR expression [314]. Another study demonstrated a dose-dependent decrease in *DNMT1* in cells derived from mouse pituitary tumours in response to 5 days of treatment with dexamethasone [184]. This project contributes to the research by linking stress to epigenetics, and glucocorticoids to DNMTs, and demonstrated the loss of *DNMT1* expression in response to treatment with cortisol and dexamethasone in TNBC cell lines.

For many years, researchersattempted to usean epigenetic treatment approach to target TNBC in order to restore ER expression and gain sensitivity to endocrine therapy. One study showed that the use of demethylation agent (5-aza-dC) and HDAC inhibitor (Trichostatin-A) could successfully restore a functional ER in TNBC cells[245]. Many studies have also used HDAC inhibitors to supress aberrant HDAC activity restoring ER expression and tamoxifen sensitivity in TNBC cells[261, 315-318]. However, efforts to use epigenetic approaches to target breast cancer are under investigation and yet to be approved [319, 320] . The promising results presented in this project demonstrating the loss of methylation on *ESR1*and the restoration of ER function in response to cortisolsuggest that efforts are worth

being invested in this area. This is particularly important due to the knowledge that patients with breast cancer experience high levels of stress [321].

Although chapter 1 summarised a number of studies demonstrating the negative effect of stress, and glucocorticoids on the progression of breast cancer, the results presented regarding the re-expression of functional ER and the loss of methylation on tumour suppressors suggest that stress can play a protective mechanism, by altering the epigenetic landscape of an aggressive breast tumour, to potentially a tumour that is sensitive to endocrine therapy. This epigenetic alteration was achieved without the use of an epigenetic inhibitor, suggesting the role of glucocorticoids in epigenetic modifications.

In regards to the ageing, our main conclusion was that age is indeed an important factor affecting *DNMTs* expression in breast cancer. The drop in *DNMTs* expression found in older mice (Figure 32, d) is in line with finding in literature. A study investigating the role of ageing in females on *DNMT1* and *DNMT3b* expression found the level of *DNMT1* gradually dropped up to the age of 64; however, the expression of *DNMT3B* decreased linearly with increased age [287]. This conclusion contributes to this research field as it helps reveal the molecular and epigenetic mechanisms contributing to age-associated breast cancer.

6.2 Limitations of the study

6.2.1 Limitation of *in vitro* models

Cell culture is a well-established and excessively used method in cancer research[322]. Although it provides a well-controlled environment to study a relatively well characterised cell line, cell culture has many limitations. First, although cell culture helps simplify data analysis, results obtained from one cell line is not necessarily a representation of other cell lines. Hence, a panel of TNBC cell lines were assessed in this project.

Second, cell culture has become the gold standard method to study cancer cells in controlled and defined conditions; however, these conditions are not representative of the natural environment of cancer or tumour growth. Cancer is a complex disease that is tightly connected to other systems in the body, such as the immune system and endocrine system, and tumour growth is also highly affected my its micro environment and different growth factors all by which are not fully presented in a cell culture model. For example, the aim of this project was to study the molecular changes of cancer cells in response to psychological stress; to investigate this aim *in vitro*, cortisol was chosen to treat breast cancer cells as it is the primary stress hormone released in the body. However, psychological stress is a complicated process that involves the activation of HPA axis and the autonomous nervous system releasing different catecholamines and glucocorticoids. Therefore, *in vitro* results were reported as effects of cortisol, while the effect of psychological stress was evaluated using *in vivo* models. Also, cancer cell lines may not be an ideal representation of tumours; when screening 51 different breast cancer cell lines to compare them to breast cancer primary tumours, profound differences were detected between cell lines and the primary

tumours of the same subtype. However, the majority of the genetic aberrations were similar in cell lines and tumour samples from patients of the same breast cancer type [323, 324].

Another disadvantage of the use of cell culture that has been repeatedly reported in the field is cross contamination. Research has reported cross contamination of cell lines, beside genotypic and phenotypic changes in the same cell lines among different laboratories [323]. To overcome this, a new panel of TNBC cell lines was bought from ATCC, and handled according to standards of operation ensuring cell lines are handled one at a time, and early passages used.

Therefore, key findings in this project were verified in *in vivo* studies to investigate the stress effects on breast cancer tumours.

6.2.2 Limitation of *in vivo models*

Translating *in vitro* results into *in vivo* models is critical in cancer research. A variety of different *in vivo* models are currently available and widely used in cancer research. However, an adequate evaluation is necessary to ensure the ultimate design of the model to answer the relevant experimental question regarding initiation, progression, genetic changes, metastasis, and response to therapy in cancer studies.

A very widely used *in vivo* model is the syngeneic mouse model. This model is very useful; strain-matched cells provide a more global model of breast cancer in the presence of tumour-host microenvironment interactions, and the host's immune system. It also has the advantage of high and rapid intake rates of cancer cells providing high experimental throughput. However, cells used in our model represent later stages of the disease, and were highly metastatic to specific routes. For this project's syngeneic mouse model, BALB/C

mice were subcutaneously injected with 66CL4 cells, a metastatic derivative of 4T1 cell line. This was chosen to best represent a breast cancer model to study the effects of stress and ageing in an immunocompetent model.

Human derived breast cancer xenografts are also a useful model in studying cancer genetics. In this project, a triple negative breast cancer (MDA-MB-231 cells) xenograft model was developed; this allowed us to study a specific subtype of breast cancer and subject the mice to psychological stress. However, mice used for this model are athymic, and therefore, the effect of the immune system on tumour growth and progression is not considered in such models. Yet, this *in vivo* model served our aim in studying the effect of psychological stress on the expression of oestrogen receptor in a well-defined cell line known to lack ER. This model also suffers from a great disadvantage, which is the low intake rate of cancer cell line; only 40% of mice injected for this project developed tumours. This influenced our power and would need to be repeated with double the n.

In regards to psychological stress induction, there are different protocols applied in the field to study psychological stress *invivo* such as; cold housing, restraint stress, and intermittent foot-shock. All in which are reported to mimic psychological chronic stress when performed on repeated basis, and trigger the HPA axis [60]. For this project, restraint stress was used as a chronic stressor; this is based on evidence showing that chronic stress increases corticosterone plasma levels and induce tumour progression in mouse models [187, 188, 308]. Mice were subjected to repeated daily restraint stress for approximately two weeks,

Finally, conducting this project at the University of Brighton, all procedures were carried out according to U.K. Home Office regulations and the Animal Scientific Procedures Act, all

efforts were taken to maximize animal welfare conditions and to reduce the number of animals used in accordance with the European Communities Council Directive, this had an effect on the number of animals used for this projects, and hence the analysis of results.

6.3 Future Prospective

The project has explored the effects psychological stress and stress hormones exerts on the epigenetics of breast cancer. Changes in DNMTs, the main enzymes catalysing the methylation process, in response to cortisol treatment have been investigated in this project. However, the mechanism by which cortisol affects the expression of methylation enzymes remains to be evaluated. The re-expression of functional ER *invitro*, and loss of methylation on the promoter region of the *ESR1* gene that encodes for ER have been shown *invivo*. The lack of ER in TNBC cells lines and in many ER- tumours is known to be due to methylation and histone modification[238]. Although methylation of the promoter region of ESR1 has been shown in this project, and although it has been proposed that the loss of methylation could be through passive methylation (i.e. the decrease in *DNMT1*), the enzyme activity of DNMT1 and other enzymes involved in the mechanism of ER activation and silencing are yet to be investigated.

The lack of hormone receptors such as ER in TNBC is one of the greatest challenges in treatment approaches of TNBC. Since cancer cells of TNBC do not express ER, patients diagnosed with this type of BC do not respond to endocrine therapy. Although results have successfully shown the loss of methylation on the promoter region of *ESR1* in response to psychological stress *in vivo*, the functionality of theER receptor has been tested *in vitro*only and not *in vivo*. It is essential to examine the response of re-expressed ER *in vivo* to currently used oestrogen regulators and modulators such as Tamoxifen, and Fulvestrant. This would have a great impact on the treatment and therapy of TNBC.

The role of GR in breast cancer therapy is important because many patients are treated with Dexamethasone as part of their treatment regimen to reduce side effects of treatments. Dexamethasone is a glucocorticoid that is more potent than cortisol and also acts by binding to GR[214], however, the effect of dexamethasone on the ER functionality must be assessed. Dexamethasone treatment led to the loss of methylation of the promoter region of *ESR1*; however, this has been only shown *invitro*. *In vitro* data represent a first glimpse in understanding the role of dexamethasone treatment and its association in changing cancer cells phenotype.

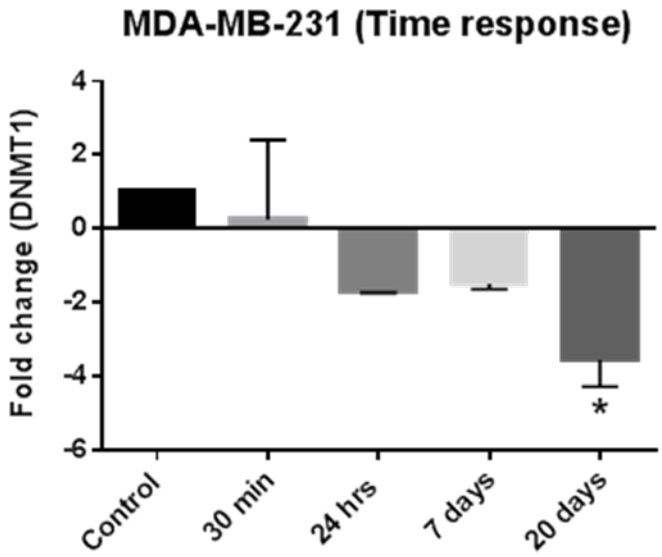
Finally, more verification is essential towards understanding epigenetic inhibition role of stress and glucocorticoids in breast cancer.

6.4 Novel contribution to knowledge

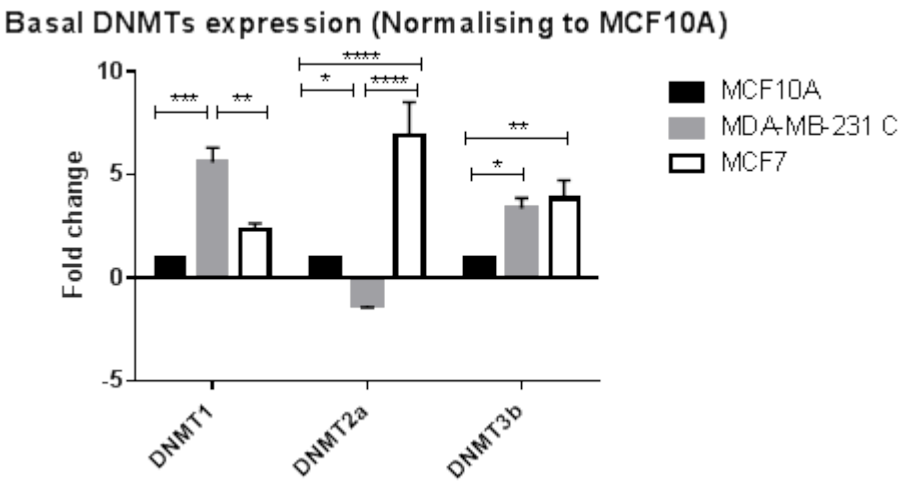
Data presented provides compelling evidence to draw a conclusion that psychological stress exerts an effect on the epigenetic landscape of breast cancer. Results produced in this project were sufficient to conclude the following novel contribution:

- 20 day exposure to cortisol induces aberrant DNA methylation patterns characterised by decrease in in *DNMT1* mRNA expression, specific promoter region hypermethylation of certain tumor suppressor genes, and global hypomethylation in TNBC cell lines.
- Cortisol treatment leads to the loss of methylation on the promoter region of *ESR1* gene in TNBC cell lines leading to the re-expression of functional ER.
- Stress causes loss of methylation of the promoter region of ESR1 gene in ER- breast cancer xenografts
- *DNMT*s change with ageing suggesting potential epigenetic changes through ageing in breast cancer tissue

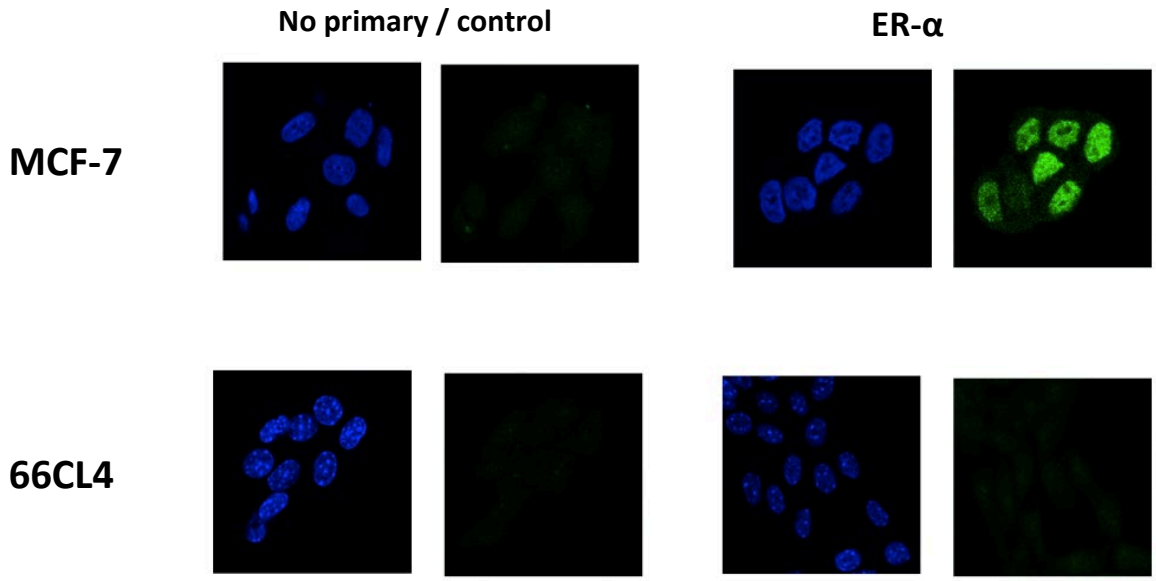
Appendices



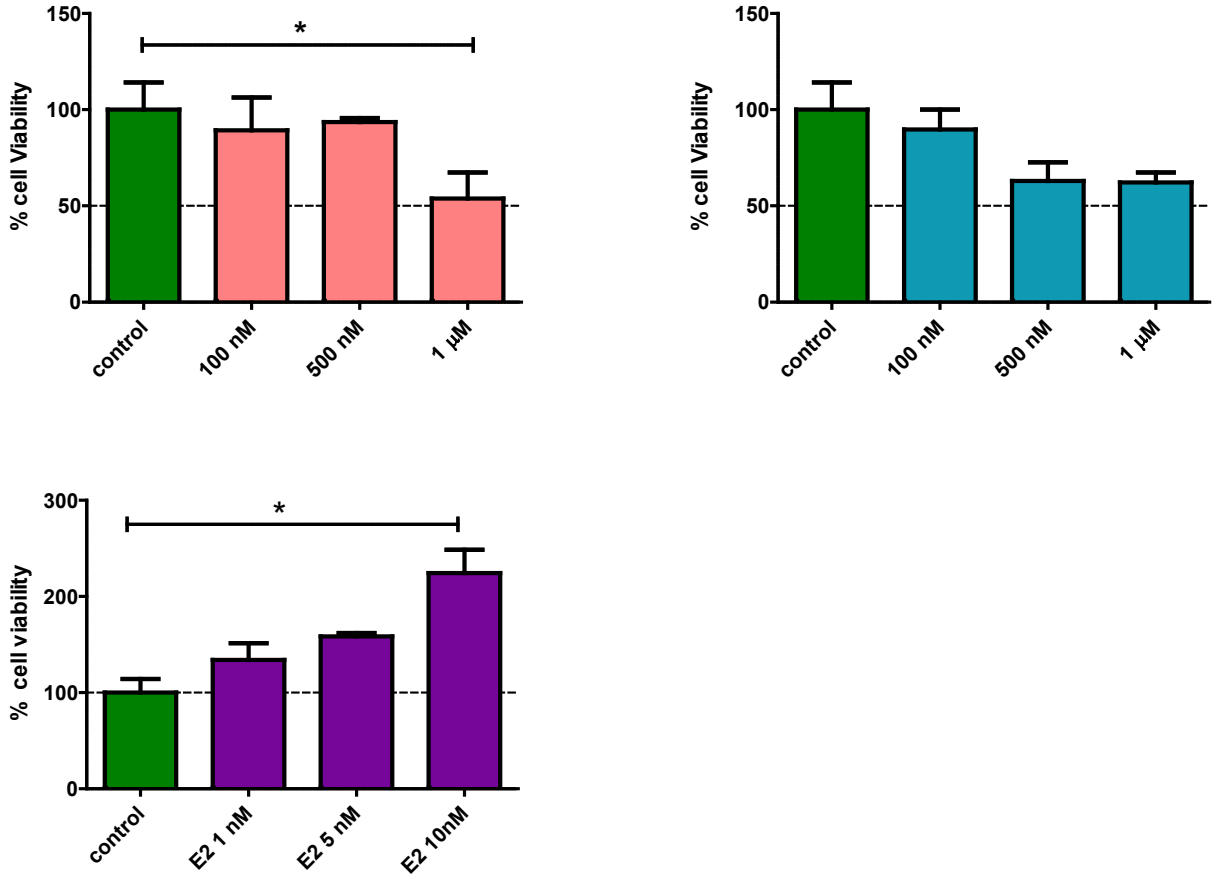
Appendix Figure 1*DNMT1* mRNA expressions in response to cortisol treatment MDA-MB-231cells were treated with 5µM of cortisol for 30 minutes, 24 hours, 7 days and 20 days. Total RNA was then extracted, and cDNA was synthesised to evaluate *DNMT*1 mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to control cells (untreated). Mean ± SEM expressed and one way ANOVA was used to compare the mean significance to control (untreated cells).* represents significant difference (* p<0.05, ** p<0.01, ***p<0.001)



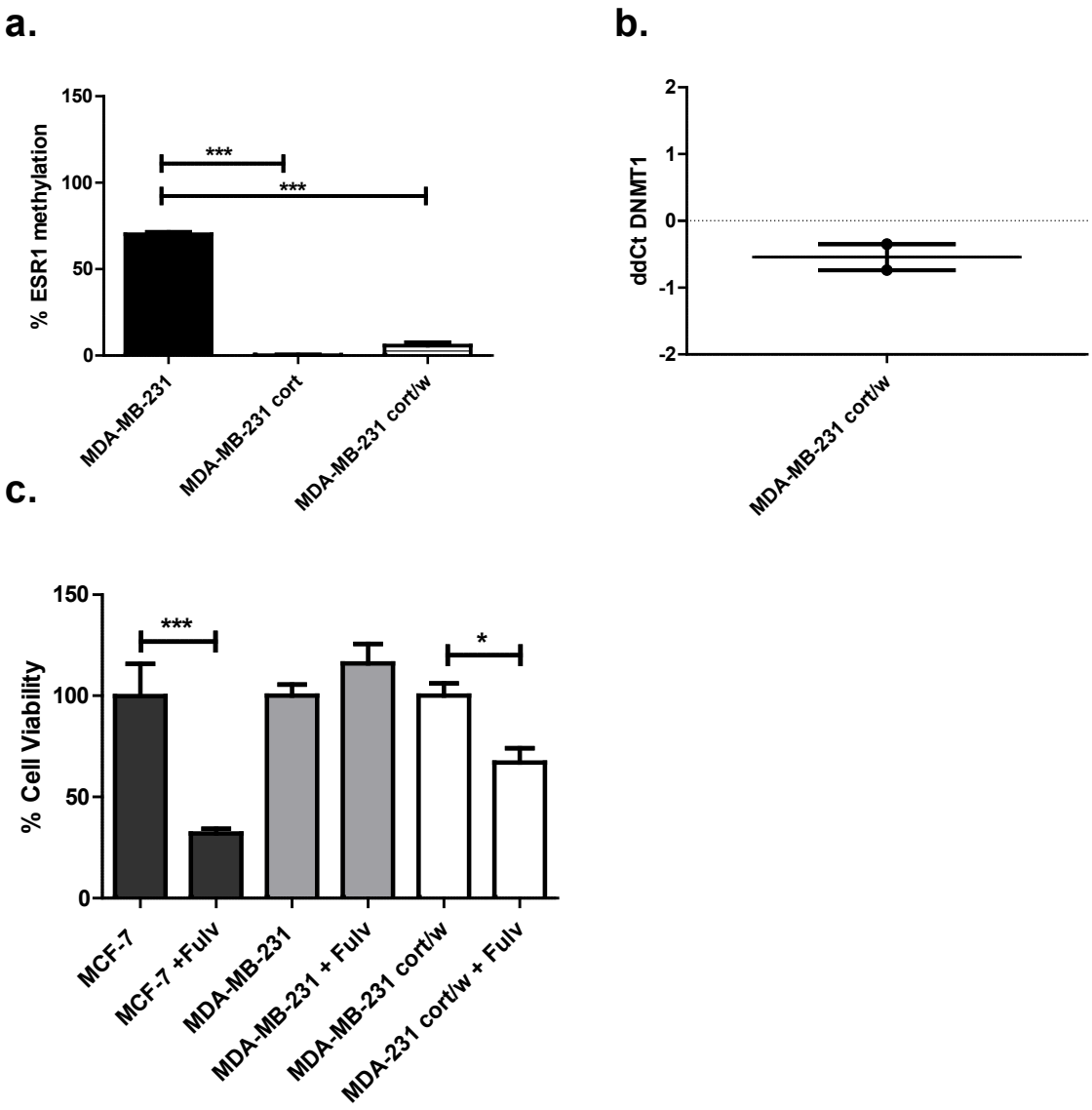
Appendix Figure 2*DNMTs* mRNA expressions in response to cortisol treatment.Total RNA was extracted from MDA-MB-231, MCF-7 and MCF10A cells., and cDNA was synthesised to evaluate *DNMT1, DNMT3a, and DNMT3b* mRNA expression using qRT-PCR. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the ΔΔCt method normalised to MCF10A cells. Mean ± SEM expressed and one way ANOVA was used to compare the mean significance to MCF10A cells.* represents significant difference (* p<0.05, ** p<0.01, ***p<0.001)



Appendix Figure 3Immunofluorescence results show that 66cl4 cell lines are ER negative breastcancer cell lines, MCF-7 cells were used as a positive control for ER expression. DAPI was used to stain the nucleus

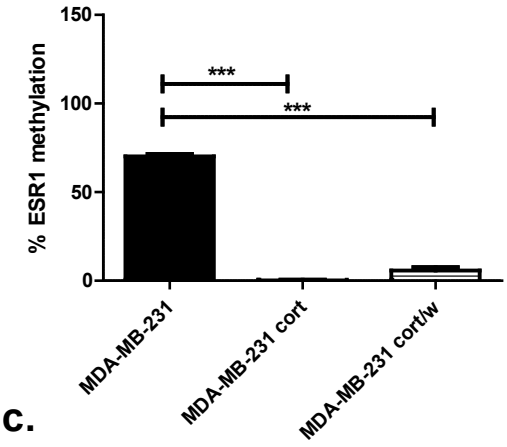


Appendix Figure 4 MTT results of MCF-7 cells treated with different doses of Fulvestrant, Tamoxifem, and Estradiol. MCF-7 cells were plated at a density of 3000/well in 200 µl of DMEM +10% FBS media. Cells were then treated with different doses of Fulvestrant and Tamoxifen (100 nM, 500nM, and 1µM), cells were also treated with different concentrations of Estradiol (E2) (1nM, 5nM, and 10nM). MCF-7 cells were incubated with treatment for 7 days, and an MTT was then preformed to determine cell viability. MTT analysis demonstrated a significant decrease in viability of MCF-7 when treated with 1µM of Fulvestrant, and around 50% decrease in viability when treated with 1µM of Tamoxifen. MTT results also demonstrated a significant increase in viability when treated with 10nM of E2. One-way ANOVA was used to compare between groups, with Bonferroni correction.* represents significant difference (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

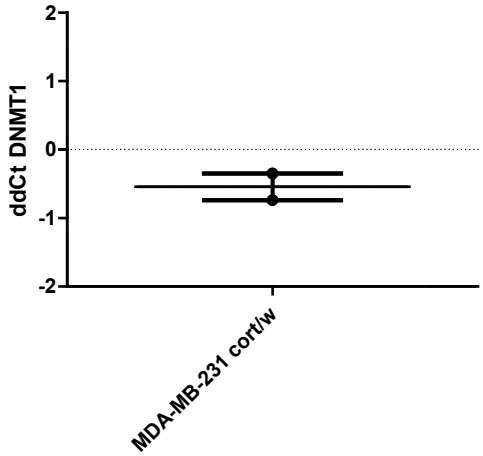


Appendix Figure 5 ESR1 remains un-methylated, and ER remains functional after two weeks of withdrawing cortisol. MDA-MB-231 cells were treated with cortisol for 20 days, after which cortisol was withdrawn (MDA-MB-231 cort/w) for two weeks. a. PCR demonstrates the loss of methylation on the promoter region of ESR1 gene in MDA-MB-231 cort/w cells. b. DNMT1 expression did not show any significant changes in MDA-MB-231 cort/w cells c. MCF-7 , MDA-MB-231 and MDA-MB-231 cort/w cells were incubated with 10-7 Fulvestrant (Fulv) for 7 days. Data are normalised to untreated cells. The ER+ cell line, MCF-7, significantly decreased in viability in response to Fulvestrant. MDA-MB-231 cells showed no change in cell viability in response to Fulvestrant. MDA-MB-231 cort/w cells significantly decreased in viability when treated with Fulvestrant, suggesting that the cells were still responsive to Fulvestrant, and the ER is still functional and activated. * $p<0.05$, ** $p<0.01$, *** $p< 0.001$

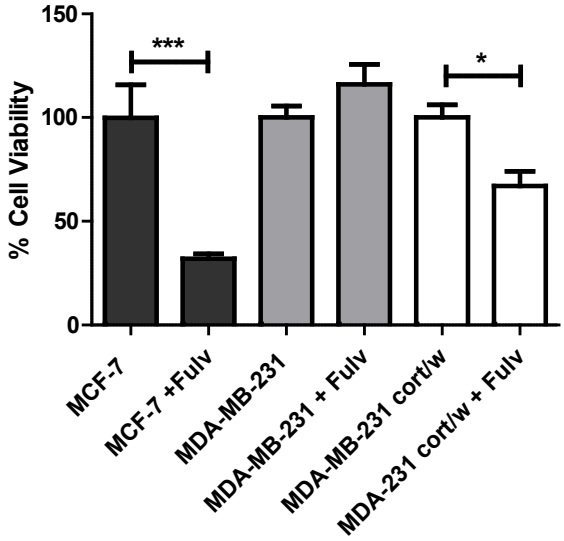
a.


% ESR1 methylation
150
100
50
0
***
***
MDA-MB-231
MDA-MB-231 cort
MDA-MB-231 cort/w

b.


ddCt DNMT1
2
1
0
-1
-2
MDA-MB-231 cort/w

c.


% Cell Viability
150
100
50
0
***
*
MCF-7
MCF-7 +Fulv
MDA-MB-231
MDA-MB-231 + Fulv
MDA-MB-231 cort/w
MDA-231 cort/w + Fulv

Appendix Table 1: List of genes from RT^2 Profiler ER Receptor Signalling array

Gene Symbol	Description	Gene Symbol	Description
AHR	Aryl hydrocarbon receptor	MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
AKAP1	A kinase (PRKA) anchor protein 1	MED1	Mediator complex subunit 1
APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
BCAR1	Breast cancer anti-estrogen resistance 1	MTA1	Metastasis associated 1
BCL2L1	BCL2-like 1	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
BDNF	Brain-derived neurotrophic factor	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)
BMP4	Bone morphogenetic protein 4	NCOA1	Nuclear receptor coactivator 1
BMP7	Bone morphogenetic protein 7	NCOA2	Nuclear receptor coactivator 2
BRCA1	Breast cancer 1, early onset	NCOA3	Nuclear receptor coactivator 3
C3	Complement component 3	NCOR1	Nuclear receptor corepressor 1
CAV1	Caveolin 1, caveolae protein, 22kDa	NCOR2	Nuclear receptor corepressor 2
CCL2	Chemokine (C-C motif) ligand 2	NOV	Nephroblastoma overexpressed gene
CCND1	Cyclin D1	NR0B1	Nuclear receptor subfamily 0, group B, member 1
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	NR0B2	Nuclear receptor subfamily 0, group B, member 2
CKB	Creatine kinase, brain	NR2F6	Nuclear receptor subfamily 2, group F, member 6
CTGF	Connective tissue growth factor	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
CTSD	Cathepsin D	NR5A2	Nuclear receptor subfamily 5, group A, member 2
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	NRIP1	Nuclear receptor interacting protein 1
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide	NRP1	Neuropilin 1

	1		
EBAG9	Estrogen receptor binding site associated, antigen, 9	PDZK1	PDZ domain containing 1
EFNA5	Ephrin-A5	PELP1	Proline, glutamate and leucine rich protein 1
EGR3	Early growth response 3	PGR	Progesterone receptor
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	PHB2	Prohibitin 2
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	PTCH1	Patched 1
ESR1	Estrogen receptor 1	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
ESR2	Estrogen receptor 2 (ER beta)	RALA	V-ral simian leukemia viral oncogene homolog A (ras related)
FOS	FBJ murine osteosarcoma viral oncogene homolog	RARA	Retinoic acid receptor, alpha
FOXA1	Forkhead box A1	S100A6	S100 calcium binding protein A6
FST	Follistatin	SAFB	Scaffold attachment factor B
G6PD	Glucose-6-phosphate dehydrogenase	SNAI1	Snail homolog 1 (Drosophila)
GPER1	G protein-coupled estrogen receptor 1	SOCS3	Suppressor of cytokine signaling 3
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	SPP1	Secreted phosphoprotein 1
IGF1	Insulin-like growth factor 1 (somatomedin C)	TFF1	Trefoil factor 1
IGFBP4	Insulin-like growth factor binding protein 4	TGFA	Transforming growth factor, alpha
IGFBP5	Insulin-like growth factor binding protein 5	TGFB3	Transforming growth factor, beta 3
IRS1	Insulin receptor substrate 1	THBS1	Thrombospondin 1
JUNB	Jun B proto-oncogene	VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
KLK3	Kallikrein-related peptidase 3	VEGFA	Vascular endothelial growth factor A
L1CAM	L1 cell adhesion molecule	WISP2	WNT1 inducible signaling pathway protein 2

LGALS1	Lectin, galactoside-binding, soluble, 1	WNT4	Wingless-type MMTV integration site family, member 4
LPL	Lipoprotein lipase	WNT5A	Wingless-type MMTV integration site family, member 5A
LTBP1	Latent transforming growth factor beta binding protein 1	XBP1	X-box binding protein 1

Appendix Table 2 List of genes from RT^2 Profiler Epigenetic Chromatin Modification Enzymez

Gene Symbol	Description	Gene Symbol	Description
ASH1L	Ash1 (absent, small, or homeotic)-like (Drosophila)	KMT2C	Myeloid/lymphoid or mixed-lineage leukemia 3
ATF2	Activating transcription factor 2	KMT2E	Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)
AURKA	Aurora kinase A	MYSM1	Myb-like, SWIRM and MPN domains 1
AURKB	Aurora kinase B	NCOA1	Nuclear receptor coactivator 1
AURKC	Aurora kinase C	NCOA3	Nuclear receptor coactivator 3
CARM1	Coactivator-associated arginine methyltransferase 1	NCOA6	Nuclear receptor coactivator 6
CDYL	Chromodomain protein, Y-like	NEK6	NIMA (never in mitosis gene a)-related kinase 6
CIITA	Class II, major histocompatibility complex, transactivator	NSD1	Nuclear receptor binding SET domain protein 1
CSRP2BP	CSRP2 binding protein	PAK1	P21 protein (Cdc42/Rac)-activated kinase 1
DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae)	PRMT1	Protein arginine methyltransferase 1
DZIP3	DAZ interacting protein 3, zinc finger	PRMT2	Protein arginine methyltransferase 2
EHMT2	Euchromatic histone-lysine N-methyltransferase 2	PRMT3	Protein arginine methyltransferase 3
ESCO1	Establishment of cohesion 1 homolog 1 (S. cerevisiae)	PRMT5	Protein arginine methyltransferase 5

ESCO2	Establishment of cohesion 1 homolog 2 (S. cerevisiae)	PRMT6	Protein arginine methyltransferase 6
HAT1	Histone acetyltransferase 1	PRMT7	Protein arginine methyltransferase 7
HDAC10	Histone deacetylase 10	PRMT8	Protein arginine methyltransferase 8
HDAC11	Histone deacetylase 11	RNF2	Ring finger protein 2
HDAC2	Histone deacetylase 2	RNF20	Ring finger protein 20
HDAC3	Histone deacetylase 3	RPS6KA3	Ribosomal protein S6 kinase, 90kDa, polypeptide 3
HDAC4	Histone deacetylase 4	RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5
HDAC5	Histone deacetylase 5	SETD1A	SET domain containing 1A
HDAC6	Histone deacetylase 6	SETD1B	SET domain containing 1B
HDAC7	Histone deacetylase 7	SETD2	SET domain containing 2
HDAC8	Histone deacetylase 8	SETD3	SET domain containing 3
HDAC9	Histone deacetylase 9	SETD4	SET domain containing 4
KAT2A	K(lysine) acetyltransferase 2A	SETD5	SET domain containing 5
KAT2B	K(lysine) acetyltransferase 2B	SETD6	SET domain containing 6
KAT5	K(lysine) acetyltransferase 5	SETD7	SET domain containing (lysine methyltransferase) 7
KAT6A	K(lysine) acetyltransferase 6A	SETD8	SET domain containing (lysine methyltransferase) 8
KAT6B	K(lysine) acetyltransferase 6B	SETDB1	SET domain, bifurcated 1
KAT7	K(lysine) acetyltransferase 7	SETDB2	SET domain, bifurcated 2
KAT8	K(lysine) acetyltransferase 8	SMYD3	SET and MYND domain containing 3
KDM1A	Lysine (K)-specific demethylase 1A	SUV39H1	Suppressor of variegation 3-9 homolog 1 (Drosophila)
KDM4A	Lysine (K)-specific demethylase 4A	SUV420H1	Suppressor of variegation 4-20 homolog 1 (Drosophila)

KDM4C	Lysine (K)-specific demethylase 4C	UBE2A	Ubiquitin-conjugating enzyme E2A
KDM5B	Lysine (K)-specific demethylase 5B	UBE2B	Ubiquitin-conjugating enzyme E2B
KDM5C	Lysine (K)-specific demethylase 5C	USP16	Ubiquitin specific peptidase 16
KDM6B	Lysine (K)-specific demethylase 6B	USP21	Ubiquitin specific peptidase 21
MBD2	Methyl-CpG binding domain protein 2	USP22	Ubiquitin specific peptidase 22
KMT2A	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	WHSC1	Wolf-Hirschhorn syndrome candidate 1

Appendix Table 3 List of genes of EpiTect Methyl Tumour Suppressor Genes array

Gene Symbol	Gene Description	Gene Symbol	Gene Description
ADAM23	ADAM metallopeptidase domain 23	HOXD11	Homeobox D11
BRCA1	Breast cancer 1	HS3ST2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2
CCNA1	Cyclin A1	HS3ST3B1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1
CCND2	Cyclin D2	HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4
CDH1	Cadherin 1, type 1, E-cadherin	ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
CDH13	Cadherin 13, H-cadherin (heart)	IGFBP7	Insulin-like growth factor binding protein 7
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57)	IGFBPL1	Insulin-like growth factor binding protein-like 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	JUP	Junction plakoglobin
ESR1	Estrogen receptor 1	KLK10	Kallikrein-related peptidase 10
GSTP1	Glutathione S-transferase pi 1	LOX	Lysyl oxidase
HIC1	Hypermethylated in cancer 1	MEN1	Multiple endocrine neoplasia I
MGMT	O-6-methylguanine-DNA methyltransferase	MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2
PRDM2	PR domain containing 2, with ZNF domain	MSX1	Msh homeobox 1
PTEN	Phosphatase and tensin homolog	MUC2	Mucin 2, oligomeric
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	MYOD1	Myogenic differentiation 1
PYCARD	PYD and CARD domain containing	PALB2	Partner and localizer of BRCA2

RASSF1	Ras association (RalGDS/AF-6) domain family member 1	PAX5	Paired box 5
SFN	Stratifin	PDLIM4	PDZ and LIM domain 4
SLIT2	Slit homolog 2 (Drosophila)	PER1	Period homolog 1
THBS1	Thrombospondin 1	PER2	Period homolog 2
TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c	PGR	Progesterone receptor
TP73	Tumor protein p73	PLAGL1	Pleiomorphic adenoma gene-like 1
APC	Adenomatous polyposis coli	PRKCDBP	Protein kinase C, delta binding protein
ATM	Ataxia telangiectasia mutated	PROX1	Prospero homeobox 1
BIRC5	Baculoviral IAP repeat containing 5	RARB	Retinoic acid receptor, beta
BMP6	Bone morphogenetic protein 6	RARRES1	Retinoic acid receptor responder (tazarotene induced) 1
BRCA2	Breast cancer 2, early onset	RB1	Retinoblastoma 1
CADM1	Cell adhesion molecule 1	RBP1	Retinol binding protein 1, cellular
CALCA	Calcitonin-related polypeptide alpha	RRAD	Ras-related associated with diabetes
CAV1	Caveolin 1, caveolae protein	RUNX3	Runt-related transcription factor 3
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27)	SFRP1	Secreted frizzled-related protein 1
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15)	SFRP2	Secreted frizzled-related protein 2
CDX2	Caudal type homeobox 2	SLC5A8	Solute carrier family 5 (iodide transporter), member 8
CHFR	Checkpoint with forkhead and ring finger domains	SLIT3	Slit homolog 3
CLSTN1	Calsyntenin 1	SYK	Spleen tyrosine kinase
CST6	Cystatin E/M	TERT	Telomerase reverse transcriptase
CTSZ	Cathepsin Z	TGFB2	Transforming growth factor, beta 2

CXCL12	Chemokine (C-X-C motif) ligand 12	TGFBI	Transforming growth factor, beta-induced
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	TGFBR1	Transforming growth factor, beta receptor 1
DAPK1	Death-associated protein kinase 1	TIMP3	TIMP metallopeptidase inhibitor 3
DSC3	Desmocollin 3	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d
EPB41L3	Erythrocyte membrane protein band 4.1-like 3	TWIST1	Twist homolog 1
EPCAM	Epithelial cell adhesion molecule	VHL	Von Hippel-Lindau tumor suppressor
FHIT	Fragile histidine triad gene	WIF1	WNT inhibitory factor 1
GADD45A	Growth arrest and DNA-damage-inducible, alpha	WT1	Wilms tumor 1
GPC3	Glypican 3	WWOX	WW domain containing oxidoreductase
HOXA5	Homeobox A5	ZMYND10	Zinc finger, MYND-type containing 10

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