Xenobiotic transporter expression in breast cancer patients treated with neoadjuvant systemic therapy: implications for therapy

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Three main xenobiotic transporters have been implicated in modulating breast cancer response to chemotherapy. These are Pgp (P-glycoprotein), MRP1 (multidrug resistance-associated protein 1), and BCRP (breast cancer resistance protein).

My first aim was to investigate expression of these proteins before and after neoadjuvant chemotherapy (NAC) for breast cancer to determine whether their levels define response to NAC or subsequent survival. Immunohistochemistry was performed for Pgp, MRP1, and BCRP on paraffin embedded tissue representing matched pairs of core biopsy (pre-NAC) and resection specimens (post-NAC) from 39 breast cancer patients. Pgp and MRP1 were found to be significantly up-regulated after chemotherapy but levels did not relate to response or survival. High post-NAC BCRP expression independently predicted for poorer disease free survival (hazard ratio of 4.04; p=0.013).

Evidence within the literature suggested that MRP1 up-regulation after chemotherapy may be driven by activated Notch1. My second aim was to determine whether activated Notch1 expression correlated with MRP1 expression in the same patient samples. Further immunohistochemistry to determine activated Notch1 expression revealed a significant correlation between post-NAC activated Notch1 and MRP1 expression (rho coefficient 0.6; p=0.0008). The hypothesis that inhibition of Notch signaling enhances killing of breast cancer cells by chemotherapeutics was developed. MTT assays were performed after treatment of breast cancer cells with combinations of doxorubicin and the Notch inhibitor DAPT. Minor additive inhibition of survival/proliferation was seen in the combination treatment, failing to provide strong support for the hypothesis.

The BCRP promoter has an oestrogen response element. My third aim was to investigate whether BCRP expression within breast tumours is regulated by oestrogen

and whether this impacts on cancer outcome. Immunohistochemistry was performed for BCRP in tumour samples from 51 patients receiving neoadjuvant endocrine therapy (NAET); matched core biopsy (pre-NAET) and resection specimens (post-NAET) were investigated. BCRP expression was significantly up-regulated after exposure to NAET ($p<0.0001$). High pre-NAET BCRP expression independently predicted for poorer DFS (hazard ratio of 17; $p=0.014$). Subsequent methylation analysis of cancer cell lines showed that the degree of methylation in the BCRP promoter region was potentially inversely correlated to the BCRP protein expression observed on immunoblotting. DNA was extracted from clinical sample and pyrosequencing analysis was performed. No such inverse correlation was observed in the clinical samples.

My work demonstrates that analysis of tumour samples pre- and post-neoadjuvant therapies provides a powerful way of investigating therapy-dependent changes in expression of molecules of interest, and may be critical for determining the prognostic or predictive value of some markers. Given the relatively small sample size of the cohort examined, future higher powered studies are required to determine the prognostic significance of BCRP expression.

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List of abbreviations

ANC: Axillary nodal clearance

5-aza-dC: 5-aza-2'-deoxycytidine

ABC: ATP-binding cassette

ADAM: A Disintegrin And Metalloproteinase

ATP: Adenosine triphosphate

BCRP: Breast cancer resistance protein

BCS: Breast conserving surgery

BK: Mr Baek Kim

BRCA1: Breast cancer 1, early onset

BSA: Bovine serum albumin

BW: Dr Bethany Williams (histopathologist; LTH Trust)

CpG: Cytosine phosphate guanine

CSL: CBF-1 (C-promoter binding factor 1), Suppressor of Hairless and Lag-1]

DAB: Diaminobenzidine

DAPT**:** N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DCIS: Ductal carcinoma in situ

DFS: Disease free survival

DMSO: Dimethyl sulfoxide

DNMT: DNA methyltransferases

DPBS: Dulbecco’s phosphate-buffered saline

E2: 17β-oestradiol

ER: Oestrogen receptor

ERE: Oestrogen response element

EV: Dr Eldo Verghese (breast histopathologist; University of Leeds / LTH Trust)

FFPE: Formalin-fixed paraffin-embedded

FTC: Fumitremorgin C

GSI: Gamma secretase inhibitors

H&E: Haematoxylin & Eosin

HER2: Human Epidermal Growth Factor Receptor 2

HRP: Horseradish peroxidase

IC: Inhibitory concentration

ICC: Inter-observer intraclass correlation coefficient

IDC: Invasive ductal carcinoma

ILC: Invasive lobular carcinoma

LB: Lysogeny Broth

LIMM: Leeds Institute of Molecular Medicine

LTHT: Leeds Teaching Hospital Trust

MAML: Mastermind-like

MAPK: Mitogen-activated protein kinase

MDR: Multidrug resistance

miRNA: MicroRNA

MMR: DNA-mismatch repair

MRI: Magnetic resonance imaging

MRP1: Multidrug resistance-associated protein 1

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC: Neoadjuvant chemotherapy

NAET: Neoadjuvant endocrine therapy

NBD: Nucleotide binding domain

NGAL: Neutrophil gelatinase-associated lipocalin

NICD: Notch intracellular domain

NSABP: National Surgical Adjuvant Breast and Bowel Project

NST: Neoadjuvant systemic therapy

OS: Overall survival

pCR: Pathological complete response

PCR: Polymerase chain reaction

PEPI: Pre-operative endocrine prognostic index

Pgp: P-glycoprotein

PPM: Patient pathway manager

PR: Progesterone receptor

PVDF: Polyvinylidene fluoride

RACE: Rapid amplification of cDNA ends

RECIST: Response evaluation criteria in solid tumours

ROC: Receiver operating characteristic

RTK: Receptor tyrosine kinase

SEER: Surveillance, Epidemiology, and End Results

siRNA: Small interfering RNA

SNB: Sentinel node biopsies

SNP: Single nucleotide polymorphisms

SRC: Steroid receptor co-activator

TACE: TNF-α converting enzyme

TBS: Tris-buffered saline

TBS-T: TBS-tween

TKIs: Tyrosine kinase inhibitors

TMA: Tissue microarrays

TMD: Transmembrane domain

TNM: Tumour node metastasis

TOPOIIα: Topoisomerase IIα

USS: Ultrasound scan

UTR: Untranslated region

List of presentations and publications

- **'Neoadjuvant chemotherapy induces expression levels of breast cancer resistance protein that predict disease-free survival in breast cancer'**

  B Kim, H Fatayer, AM Hanby, K Horgan, SL Perry, EMA Valleley, ET Verghese, BJ Williams, JL Thorne, TA Hughes. PLoS One 2013 May 2;8(5):e62766.

- **'Notch inhibition stops chemotherapy-induced activity of Multi-drug Resistance associated Protein-1 in breast cancer' B Kim,** SL Stephen, AM Hanby, K Horgan, SL Perry, J Richardson, EA Roundhill, EMA Valleley, ET Verghese, BJ Williams, TA Hughes, JL Thorne. *Under review for publication*

- **'Neoadjuvant chemotherapy induces changes in expression of breast cancer resistance protein that predict disease free survival in breast cancer'** Oral Presentation for the Patey Prize Session, Society of Academic and Research Surgery Meeting, London, Jan 2013

- **'Neoadjuvant chemotherapy induces changes in expression of breast cancer resistance protein that predict disease free survival in breast cancer'** Oral Presentation at the joint meeting of the Pathological Society of Great Britain & Ireland and the Dutch Pathological Society, Utrecht, Holland, Jan 2013

- **'Neoadjuvant endocrine therapy up-regulates expression of Breast Cancer Resistance Protein, but only pre-treatment levels predict survival'** Oral Presentation at the British Association of Surgical Oncology ~ The Association for Cancer Surgery 40th annual scientific conference, London, Nov 2013

- **'Notch-1 as a therapeutic target in neoadjuvant chemotherapy resistance acquired through MRP1 in breast cancer'** Poster Presentation at the Yorkshire Cancer Research Annual Scientific Meeting, Harrogate, June 2013

1.0 Introduction

1.1 Breast cancer incidence and survival

Breast cancer is the third commonest cause of death from cancer in United Kingdom with 11,762 deaths in 2011, which accounted for 7% of all deaths from cancer (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/mortality/uk-breast-cancer-mortality-statistics). After lung cancer, it is the second most deadly cancer in women with a mortality rate in the same year of 24 deaths per 100,000 women. Moreover, one in eight women will develop breast cancer during their lifetimes. The current 5-year survival estimate for patients diagnosed with breast cancer between 2005 and 2009 is 85% (Office for National Statistics: http://www.ons.gov.uk/ons/rel/cancer-unit/breast-cancer-in-england/2010/sum-1.html), which is relatively high as compared to the other common solid cancers, such as colon cancer with a 5-year survival rate of 55%. However, despite this relatively successful treatment of breast cancer overall, some subtypes of breast cancer continue to give poorer outcomes. For example, triple negative breast cancer (see section 1.2) has an increased likelihood of distant recurrence and death within 5 years of diagnosis when compared to non-triple negative breast cancers (hazard ratio of 2.6 and 3.2; $p<0.0001$ and $p<0.001$ respectively) (Dent *et al*., 2007).

1.2 Breast cancer presentation and diagnosis

Primary breast cancers typically either present as palpable lumps, usually initially identified by the patient themselves or by clinicians, or as impalpable lesions that were detected by mammographic screening. The diagnosis of breast cancer is made by microscopic examination of core biopsies taken from the breast lesion. Pathological assessment includes examination of the epithelial cell morphology in breast tissue. In normal breast tissue, terminal duct lobular units are seen surrounded by stroma. In

breast cancer, epithelial cells within ducts or lobules have a disorganised appearance and expand in a non-uniform manner. When cancer cells do not invade across the basement membrane and hence are confined within the ducts and lobules, they are termed ductal or lobular carcinoma in situ. When there is invasion of the surrounding tissue, they are termed invasive ductal or invasive lobular carcinoma. These diagnostic cores of are particular relevance to my work since I have used the material that was not required for diagnostic purposes extensively in this thesis. Having diagnosed cancer, histopathologists also use these diagnostic cores to classify the disease using various systems that guide subsequent treatment.

Breast cancer classifications

Breast cancers are classified according to histopathological subtypes, tumour grade, the international Tumour Node Metastasis (TNM) staging, and molecular subtype. Classification of breast cancer has particular importance as it is used to help define therapies and determine prognosis.

The two commonest histopathological subtypes are invasive ductal and invasive lobular carcinomas. Li *et al* analysed the Surveillance, Epidemiology, and End Results (SEER) data from the National Cancer Institute from the United States. They determined that of 190,458 invasive breast cancer cases analysed, 72.8% were invasive ductal carcinomas, 7.6% were invasive lobular carcinomas, and 4.7% were invasive mixed ductal-lobular carcinomas (Li *et al*., 2003). The remaining 14.9% consists of numerous other rare histological breast cancer subtypes such as invasive tubular carcinoma, invasive medullary carcinoma, invasive papillary carcinoma, and invasive mucinous carcinoma. Depending on the morphology of breast cancer cells, they are assigned a grade of 1 to 3 according to the modified Bloom-Richardson

grading system, with higher grade tumours having worse prognoses. Higher grade tumours have more aggressive features with a lower degree of tubule formation, and higher degrees of mitotic activity and nuclear pleomorphism (Elston and Ellis, 1991).

The extent of breast cancer in patients is staged according to the Tumour Node Metastasis (TNM) classification of the American Joint Committee on Cancer (http://www.cancerstaging.org/staging/)(Allen et al., 2002). Table 1.1 highlights the key components of the staging system which is confirmed post-operatively when pathological specimens are examined.

T stages (tumour)	T1	Tumour size ≤2cm
	T2	Tumour size >2cm but ≤5cm
	T3	Tumour size >5cm
	T4	Tumour spread to the chest wall or breast skin envelope
N stages (nodes)	N0	No cancer cells in the axillary lymph nodes
	N1	Metastases in 1 to 3 axillary lymph nodes
	N2	Metastases in 4 to 9 axillary lymph nodes
	N3	Metastases in ≥10 axillary lymph nodes or metastases in the ipsilateral supraclavicular lymph nodes
M stages (metastases)	M0	No distant metastases
	M1	Presence of distant metastases

Table 1.1: TNM staging for breast cancer

Breast cancers are also classified into molecular subtypes (Table 1.2) depending on the expression of oestrogen and progesterone receptor in the cell nucleus, as well as HER2 (human epidermal growth factor receptor 2) protein expression on the cell surface as well as gene amplification.

Molecular subtype	Receptor status
Luminal A	ER+ and/or PR+, HER2 -
Luminal B	ER+ and/or PR+, HER2+
Triple negative/basal-like	ER-, PR-, and HER2-
HER2 type	ER-, PR-, and HER2+

Table 1.2: Breast cancer molecular subtypes

Breast cancer classifications described so far are important in identifying subgroups of patients who have poorer prognosis. Patients with higher grade breast cancers, advanced TNM staging, and basal-like phenotypes have the worst prognosis. These patients have higher likelihood of tumour recurrence in the breast or the axilla, as well as other distant sites, which usually subsequently leads to death.

1.3 The importance of systemic therapy

Treatment of breast cancer is multi-modal with patients receiving combinations of surgery, radiotherapy, chemotherapy, endocrine therapy, and/or the targeted biological therapy Herceptin. The first two modalities are loco-regional therapies that treat the breast and the axillary lymph nodes. The latter modalities are systemic therapies which

in early breast cancer are administered to treat occult metastatic disease, with an aim of reducing the risks of tumour recurrences and prolong survival.

Loco-regional therapies

Surgery is indicated for patients without obvious evidence of distant metastasis, which applies to the majority of patients seen in the clinic, and is known as early operable breast cancer. Surgery consists of breast conserving surgery (BCS) or mastectomy of the breast, and sentinel node biopsies (SNB) or axillary nodal clearance (ANC) in the axilla. The aim of surgery is to resect the cancer with clear margins in order to minimise the likelihood of local tumour recurrence. In general, patients are offered BCS if the tumour size to breast volume ratio is small enough to achieve clear margins whilst minimising cosmetic distortion to the breast. BCS is applicable to the majority of patients who present with T1 tumours. The feasibility of BCS for T2 tumours depends on the patient's breast volume, while the majority of patients with ≥T3 tumours will receive mastectomies. The SNB is performed to stage the axilla (i.e. to detect the presence or absence of tumour metastasis in the first draining axillary lymph nodes). The majority of patients who have evidence of metastatic cancer cells in the sentinel nodes subsequently undergo a second operation, ANC, to remove the remaining axillary lymph nodes with an aim of resecting further potentially involved lymph nodes.

Radiotherapy is administered to patients after BCS to reduce survival of any potential residual cancer cells. It has been shown to reduce the rate of loco-regional tumour recurrence in the BCS setting, meaning that patients having BCS have a very similar disease free survival (DFS) as those having mastectomies. Poggi *et al* showed the estimated 20-year DFS rate for patients treated with mastectomies to be 67%, versus 63% in patients treated with BCS and radiotherapy (p=0.64), at a median follow-up of

18.4 years (Poggi *et al*., 2003). Post-mastectomy radiotherapy is not always used, and is reserved for patients who present with large (T3 or 4) and/or substantially node positive (N2 or 3) disease (Dragun *et al*., 2012).

Systemic therapies

Chemotherapy is administered intravenously to patients who are at increased risk of having occult distant metastases and this is predicted by positive axillary lymph nodes (Rossi *et al*., 1981), grade 3 or T3 tumours, or those with triple negative breast cancer. This group of patients have the worst prognostic features, therefore aggressive treatment is indicated, and chemotherapy provides significant survival advantages (Goldhirsch *et al*., 2009). Chemotherapy agents limit proliferation of cancer cells and induce their apoptosis, but also have harmful effects on normal cells. The aim is to kill cancer cells, while minimising damage to normal tissues. Common chemotherapy agents that are in current clinical use include anthracyclines such as epirubicin (often given in combination with the alkylating agent cyclophosphamide), and taxanes such as docetaxel. Epirubicin intercalates with DNA, causing DNA damage and subsequent interference with DNA and RNA synthesis (Cersosimo and Hong, 1986). Cyclophosphamide is an alkylating agent which binds to DNA and results in the disruption of cell division (Awad and Stuve, 2009). By contrast, taxanes do not target the DNA directly. Microtubules are important in cell division and growth, and taxanes interact with tubulin units, resulting in mitotic problems and apoptosis (Cortes and Baselga, 2007). A significant survival gains from chemotherapy administration were demonstrated by the National Surgical Adjuvant Breast and Bowel Project (NSABP), which showed that chemotherapy achieved a 58% reduction in recurrence rate and a 40% reduction in mortality rate when compared to surgery alone at 8 years of follow-up (Fisher *et al*., 2004a). However, chemotherapy can cause substantial side-effects such as febrile neutropenia, pulmonary embolism, congestive heart failure, and neuro-motor

and sensory toxicity (Smith *et al*., 2004). It would be advantageous if it was possible to improve the clinical utility of chemotherapy agents by enhancing the killing of cancer cells whilst reducing the drug side-effects.

Endocrine therapy is offered to patients on the basis of positive expression of the oestrogen receptor (ER) within the nuclei of the tumour cells. Active ER signalling is a key driver of proliferation of breast epithelial cells (Tyson *et al*., 2011). ER can be activated by at least 2 defined pathways: the nuclear and non-nuclear pathways. In the former, oestrogen-bound ER activates transcription by binding to the oestrogen response element (ERE) of the target genes, including c-Myc and Survivin, leading to enhanced tumour cell proliferation (Welboren *et al*., 2007). In the latter, ER interacts with signalling molecules such as steroid receptor co-activator (SRC), and receptor tyrosine kinases (RTK) in the cytoplasm. This, in turn, activates downstream kinases that phosphorylate ER and other transcription factors to activate transcription (Giuliano *et al*., 2011), and promote tumour growth/survival. This mechanism of ER activation is independent of oestrogen but is dependent on EREs. ER protein expression is quantified by immunohistochemistry using the Allred scoring system (Allred *et al*., 2012). The total score in whole numbers ranges from 0 to 8 (note: a score of 1 is not possible), with an intensity score ranging from 0 to 3 added to a score quantifying the proportion of tumours cells staining positively ranging from 0 to 5. A score of ≥3 out of 8 is defined as being positive for ER expression from a clinical utility viewpoint.

The aim of endocrine therapies is to interfere with the up-regulated ER signalling in ER positive tumours, to inhibit ER-dependent growth and survival. To achieve this, pre-menopausal breast cancer patients receive tamoxifen, whereas post-menopausal breast cancer patients receive aromatase inhibitors such as anastrozole, letrozole, or exemestane. Tamoxifen binds to the oestrogen receptor and antagonises the action of

oestrogen by causing conformational changes to the receptor, which in turn inhibit the activation of transcription at EREs. A large trial in node negative, ER positive breast cancer patients demonstrated that tamoxifen treatment of at least 5 years resulted in significant improvement in outcome as compared to placebo in terms of DFS (hazard ratio, HR, 0.58) and overall survival, OS (HR of 0.8) at 15 years (Fisher *et al*., 2004b). Aromatase inhibitors target the enzyme aromatase, which is involved in the conversion of androgen to oestrogen, and hence inhibition of this enzyme results in the reduction of oestrogen level. Aromatase is not only specific to breast cancer cells, and is also present in ovary, liver, and muscle amongst other tissues. In post-menopausal women, ovarian oestrogen production decreases, and the aromatase activity in breast and adrenal tissues contributes significantly to oestrogen synthesis. Aromatase inhibitors cause a systemic reduction in oestrogen levels to exert the anti-tumourigenic effect (Bhatnagar, 2007). Aromatase inhibitors are superior compared to tamoxifen in terms of DFS in post-menopausal women with ER positive breast cancer (Cuzick *et al*., 2010).

HER2 overexpression stimulates tumour cell proliferation. Therefore, patients who have HER2 overexpression are treated with the monoclonal antibody Herceptin (trastuzumab) that reduces tumour cell proliferation. On binding of growth factor ligands, HER receptors (HER1 to HER4) form dimers with the same type of HER receptor (homodimers) or with other HER receptors (heterodimers). No known ligand exists for HER2 receptor on its own, but dimerisation of HER2 receptor with other HER receptors (HER1, HER3, or HER4) results in activation of intracellular signalling pathways involving MAPK, which stimulate tumour proliferation, and PI3K-Akt, which promotes tumour cell survival (Rubin and Yarden, 2001). Herceptin binds to the HER2 receptor and prevents dimerisation and subsequent activation of the intracellular signalling pathways described (Roukos, 2011). A randomised clinical trial by Slamon *et*

al in patients with HER2 overexpression showed that the addition of Herceptin to chemotherapy resulted in a 20% reduction in relative risk of death at median follow-up of 30 months when compared to chemotherapy alone (Slamon *et al*., 2001). Herceptin is a relatively modern systemic therapy when compared to chemotherapy and endocrine therapy.

1.4 The emergence of neoadjuvant systemic therapy

Traditionally systemic therapies have been administered in the adjuvant setting (i.e. after surgery). Chemotherapy is administered as a combination of chemotherapy agents or as a monotherapy that consists of typically 4 to 8 cycles. Each cycle consists of chemotherapy administration for 5 consecutive days, followed by a rest period lasting 3 to 4 weeks, which allows patients to recover from the side-effects mentioned in the previous section. In contrast, patients usually receive endocrine therapy as oral medication for a total of 5 years. For patients who require both treatment modalities, chemotherapy is administered initially and endocrine therapy is commenced once the chemotherapy treatment is completed (Mariotto *et al.*, 2002).

Alternatively, patients who require chemotherapy or endocrine therapy can receive either treatment prior to surgery, as a neoadjuvant treatment. This has a number of potential benefits. One aim is to reduce the tumour size (i.e. down-stage) prior to surgery, which may enable BCS instead of mastectomy in patients who otherwise would have been obligate candidates for mastectomy. This benefits patients as it may allow less distressing surgery with consequent benefits to their psychological well-being (Parker *et al*., 2007). There is no reduction in survival by a neoadjuvant approach (Mieog *et al*., 2007). Patients who would already be candidates for BCS, such as patients with T1 tumours, are not offered neoadjuvant systemic therapy (NST) since

there are no obvious surgical advantages. A further potential advantage of NST is that therapy regimens can be modified based on the response of primary tumours with an ultimate aim of improving response (Rigter *et al*., 2013). In the adjuvant setting this is of course not possible since the primary tumour has been resected, and any occult metastases (the actual targets of systemic therapies) cannot be monitored. It is hoped, although firm data are lacking, that such fine-tuning of therapy regimens would improve outcomes. Patients are monitored by using magnetic resonance imaging (MRI) or ultrasound scan (USS) in order to assess tumour responses to NST. Poor response observed leads to abandoning the neoadjuvant therapy, and proceeding with surgery.

Substantial proportions of breast cancer patients receive neoadjuvant chemotherapy (NAC) compared to adjuvant chemotherapy, with a reported NAC administration of up to 31 to 39% in a range of institutions (Fisher *et al.*, 2012, Kennedy *et al.*, 2010). Patients treated with NAC have equivalent benefit in terms of overall survival and avoidance of loco-regional recurrence compared to those treated with adjuvant chemotherapy (Mieog *et al*., 2007). Patients undergo an initial breast core biopsy that establishes the diagnosis of breast cancer (see section 1.2 above). Those selected for NAC are then subjected to cycles of a chemotherapy regime that typically consists of epirubicin and cyclophosphamide at Leeds Teaching Hospital Trust (LTHT). However, NAC regimes are not standardised nationally. The tumour response to NAC is monitored using MRI during treatment and the regime can be switched, usually to a taxane-based therapy (Antolin *et al*., 2011, Walker *et al*., 2011, Rastogi *et al*., 2008), if the tumour fails to respond (i.e. there is no reduction in tumour size). Patients undergo a baseline MRI scan prior to commencing NAC, followed by an interval MRI scan to assess response after the second cycle of NAC. This is subsequently followed by a final MRI scan at completion of final NAC cycle. The decision to switch chemotherapy regimen occurs after the second cycle of NAC, and all patients receive at least six

cycles of NAC in total unless there is tumour progression. Based on the MRI assessment of tumour response (Table 1.3) (Therasse *et al*., 2000), patients subsequently undergo BCS or mastectomy.

Classification of MRI response to NAC	Definition
Complete response	Disappearance of all tumour lesions
Partial response	≥30% reduction in tumour size
Stable disease	<30% reduction or <20% increase in tumour size
Progressive disease	≥20% increase in tumour size

Table 1.3: Response Evaluation Criteria in Solid Tumours (RECIST) classification of tumour response to NAC assessed by MRI

Response to NAC can also be assessed post-operatively by measuring the residual pathological tumour size after surgery. A small proportion of patients achieve pathological complete response (pCR), where no tumour cells are identified after NAC on post-operative histopathology. These patients still require surgery, however, since an MRI scan alone is not sensitive enough to reliably predict this response, which is only evident after surgery by pathological examination. A recent meta-analysis showed that pCR was seen in 17.1% (Kong *et al*., 2011) of patients, and those who achieved pCR have a 5-year DFS rate of above 90%, as opposed to 50 to 70% in the non-pCR group (Tanei *et al*., 2011). However, definitions of pCR can be variable (Mailliez *et al*., 2010) according to the classification systems used; the NSABP classification defines pCR as the absence of invasive tumour cells, whereas the Honkoop's classification demands that there is also an absence of in situ tumour cells. Furthermore, there are no standardised laboratory protocols for processing post-NAC breast tissue samples,

such that there are substantial variations in the histopathological processing and examination of excised breast tissue at different centres.

Similarly, neoadjuvant endocrine therapy (NAET) is used to attempt tumour down-staging prior to surgery. Patients will typically receive NAET for 3 to 4 months prior to undergoing surgery (Colleoni and Montagna, 2012). However, NAET duration of up to 12 months has been reported (Macaskill and Dixon, 2007). The endocrine therapy is also continued after surgery for a total duration of 5 years. The common side-effects of anti-oestrogenic therapy include hot flushes, arthralgia, and reduction in bone density. These side-effect profiles are potentially less severe than with NAC. The majority of patients who receive NAET are post-menopausal. This is in contrast to NAC where most patients are pre-menopausal, and are able to tolerate better the more severe side-effects of chemotherapy. Studies have shown no difference in tumour response rates between the aromatase inhibitors anastrozole, letrozole, and exemestane in the neoadjuvant setting (Ellis *et al*., 2011). Therefore, no particular aromatase inhibitor is preferentially used for NAET at LTHT. The monitoring of response to NAET treatment is assessed using USS as opposed to MRI, since there is no proven benefit in the use of MRI to monitor response in patients treated with NAET (Kaufmann *et al*., 2012). However, no consensus exists regarding the timing of USS during NAET, and response to NAET is more often assessed by a combination of clinical examination and USS (Smith *et al.*, 2005). In the PROACT (Pre-Operative Arimidex Compared to Tamoxifen) trial, 262 ER positive breast cancer patients who were deemed to have inoperable breast cancer or scheduled for mastectomy were treated with 3 months of NAET. As a result, 15 patients (5.7%) were able to receive mastectomies that were originally deemed to have inoperable breast cancer. Furthermore, 98 patients (37.4%) who were originally scheduled for mastectomy achieved sufficient down-staging to enable breast conserving surgery (Cataliotti *et al*., 2006). Moreover, the IMPACT

(Immediate Preoperative Anastrozole, Tamoxifen, or Combined With Tamoxifen) trial showed that of 124 patients who required mastectomy at baseline, a BCS rate of 44% was achieved with 3 months of neoadjuvant anastrozole (Smith *et al*., 2005). Compared to NAC, pCR is rarely seen with NAET (Chia *et al*., 2010), which probably relates to the anti-proliferative effect of the latter compared to the cytotoxic effect of the former.

1.5 Predicting response to neoadjuvant systemic therapy

The majority of patients treated with neoadjuvant systemic therapy do not achieve pCR and achieve partial responses. If this is insufficient to achieve tumour down-staging, patients are likely to require mastectomies and therefore the primary goal of the neoadjuvant systemic therapy is not achieved. These patients end up having delayed surgery with no benefit, and there are cost implications regarding MRI and USS monitoring of tumour response, which would have been unnecessary if these patients were treated with mastectomy followed by adjuvant systemic therapy. It would be of considerable benefit to be able to select accurately the patients who are likely to respond to neoadjuvant systemic therapies, and treat the remaining patients with surgery followed by adjuvant therapies. Clinico-pathological factors that are associated with improved response to NAC include invasive ductal carcinomas, ER negativity, high tumour grade, and increased Ki-67 expression (Kaufmann *et al*., 2012). Clinico-pathological parameters that predict favourable response to NAET include positive progesterone receptor (PR) expression, lack of HER2 overexpression, and high ER expression (Macaskill and Dixon, 2007). However, relying on clinico-pathological parameters alone is inadequate in predicting response to NST in individual patients. For example, not all ER negative breast cancer patients respond to NAC in the same manner. Therefore, further research is required to identify more accurate predictive molecular markers.

There are studies emerging in which potential molecular predictive markers have been studied in the context of NAC. Using core biopsy tissues, one such study showed a positive correlation between thymosin beta 15 mRNA levels and pCR rates in patients with triple negative breast cancers, but not in luminal breast cancers (Darb-Esfahani *et al*., 2012). Wenners *et al* performed immunohistochemistry on tissue microarrays (TMA) constructed from core biopsy tissues, and showed that neutrophil gelatinase-associated lipocalin (NGAL) expression was associated with improved pCR rate in a low-risk subset of patients with ER positive node-negative breast cancer (Wenners *et al*., 2012). Chen *et al* examined the expression of eleven candidate molecular markers using immunohistochemistry on core biopsy tissues. Multivariate analysis showed that the lack of β-tubulin III, bcl-2, and ERCC1 expression all independently predicted pCR in patients receiving taxane-based NAC (Chen *et al*., 2012b). Wang *et al* also performed immunohistochemistry on core biopsy tissues and showed that the lack of tau expression resulted in an improved response to taxane-based NAC (Wang *et al*., 2013a). These studies show that significant correlations are only present in selected tumour subtypes, suggesting that tumour heterogeneity plays an important role.

In the context of NAET, there are far fewer studies into predictive markers, with only Ki-67 as a candidate. Paired biopsy samples in 158 patients were examined for a change in Ki-67 expression pre-NAET and after 2 weeks of NAET. At median follow-up of 37 months, patients with high Ki-67 expression after two weeks of NAET had a lower DFS than those with lower Ki-67 (Hazard ratio of 1.95; p=0.004) (Dowsett *et al*., 2007). However, the baseline Ki-67 expression levels prior to NAET did not predict DFS. This study suggests that Ki-67 may be a marker of response to NAET.

Molecular markers	Breast cancer molecular subtype	Sample size	mRNA or protein expression	Positive/negative correlation with pCR
Thymosin beta 15	Basal	61	mRNA	Positive
NGAL	Luminal	487	Protein	Positive
β-tubulin III, bcl-2, and ERCC1	All	91	Protein	Negative
Tau	All	113	Protein	Negative

Table 1.4: Summary of studies investigating predictive molecular markers in breast cancer patients treated with NAC (Darb-Esfahani *et al*., 2012, Wenners *et al*., 2012, Chen *et al*., 2012b, Wang *et al*., 2013a).

1.6 Mechanism of resistance to chemotherapy

Studying predictive molecular markers can have importance in gaining further insight into the underlying mechanisms that are responsible for chemoresistance in breast cancer patients. The studied markers in Table 1.4 have been shown to have expression levels that correlated with tumour response to NAC in terms of pCR rate, and hence subsequent survival since patients who achieve pCR have improved survival. Therefore, their expression levels have clinical utility in predicting which patients respond more favourably to NAC and subsequent survival. Furthermore, examining the underlying mechanisms responsible for defining their expression levels may ultimately lead to therapies designed to improve sensitivity to chemotherapy, and result in improved response and survival.

The mechanisms of resistance to chemotherapy are highly complex and large numbers of molecules are involved. Different pathways involved in chemoresistance can be broadly classified into the alteration in protein expression affecting drug transport into the intracellular compartment (ABC transporters, LRP), modification in the expression and function of the molecules targeted by chemotherapeutics (DHFR, β-Tubulin, tau, MAP4), alterations in the DNA repair mechanism (Topoisomerase II, mismatch repair proteins), alterations in the enzyme affecting drug metabolism (cytochrome P450, GST, ALDH), and alteration in molecules responsible for the regulation of apoptosis (caspase-3, p27, p53, bcl-2, PTEN, and p27) (Rivera, 2010).

The following molecules have been highlighted due to their importance in breast cancer chemotherapy resistance, although there is a wide literature and common themes are difficult to identify. One of the key mechanisms of chemoresistance is driven by ATP-dependent drug efflux pumps, collectively termed ATP-binding cassette (ABC) transporters. Their substrates include the majority of chemotherapeutic agents currently in use, thereby the molecules potentially confer multidrug resistance (MDR). ABC transporters can reduce the intracellular concentration of chemotherapeutics, and therefore reduce their efficacy. These molecules are discussed in more detail in section 1.8. p53 has an important role in regulation of apoptosis, cell cycle progression, and DNA repair. When compared to the wild-type p53, cells with mutant p53 show greater resistant to chemotherapeutics (Lai *et al*., 2012). This is attributed to the failure of cells to undergo apoptosis following treatment with chemotherapeutics. Breast cancer 1, early onset (BRCA1) is a tumour suppressor gene which is involved in regulation of transcription and DNA repair. Its overexpression can result in resistance to platinum-based chemotherapeutics (Husain *et al*., 1998). DNA Topoisomerase II is an enzyme involved in DNA replication and repair. Anthracyclines exert their action by interfering with topoisomerase IIα (TOPOIIα). Studies have shown that TOPOIIα gene

amplification resulted in enhanced benefit from anthracyclines for breast cancer patients who were HER2-positive (Barrett-Lee, 2005). Therefore, reduction in topoisomerase II function results in resistance to anthracyclines. Taxanes target β-tubulin which is involved in the formation of microtubules. Overexpression or mutations of β-tubulin has been shown to result in resistance to taxanes (Kamath *et al*., 2005).

1.7 Mechanism of resistance to endocrine therapy

Similarly, large numbers of molecules are involved in endocrine therapy resistance, and the mechanism of resistance can be broadly classified into the alteration of ER expression and its activity (ERα36, microRNAs, EGFR, HER2), alterations in the expression of ER co-repressors (NCoR) and co-activators (SRC3), change in the activity of transcriptional factors involved in mediating ER signalling in the non-nuclear pathway (AP-1, SP-1, NFκβ), alterations in receptor tyrosine kinase (RTK) signalling due to its cross-talk with ER (EGFR, HER2, PI3K, MAPK), and alterations in the expression of cell-cycle regulators (MYC, Cyclin E1, p21) (Giuliano *et al*., 2011). Some of the mechanisms involved in endocrine resistance are further highlighted below.

Loss of ER expression can result in endocrine therapy resistance, and is in part controlled by post-transcriptional mechanisms. For example, ER mRNA stability can be affected by miR-206, and can result in the loss of ER expression (Adams *et al*., 2007). ER exerts its effect on gene expression by binding to a group of regulatory proteins to form the transcription initiation complex. High expression of such regulatory proteins, such as SRC3, is associated with tamoxifen resistance (Osborne *et al*., 2003). Increased activity of transcriptional factors, such as NFκB (Zhou *et al*., 2007), is also associated with resistance to endocrine therapy. Cross-talk between ER and RTK signalling can also result in endocrine therapy resistance. Specifically, ER causes up-

regulation of RTK signalling, such as MAPK and PI3K/Akt, which in turn causes phosphorylation of ER and its co-regulatory proteins, resulting in the activation of ER in the presence of anti-oestrogens (Giuliano *et al*., 2011).

1.8 The importance of ABC transporters

1.8.1 Classification of ABC transporters and the distribution of their expression

49 human ABC genes have been identified (Leonard *et al*., 2003), and there are 7 subfamilies termed *ABCA* to *ABCG*. The following table adapted from Gottesman *et al* outlines the list of known ABC proteins according to each subfamily (Table 1.5).

Subfamily	Nomenclature	Protein name	Known tissue or cellular localization
ABCA	ABCA1	ABC1	Placenta, liver, lung, adrenal glands
	ABCA2	ABC2	Brain, monocytes
	ABCA3	ABC3	Apoptotic cells
	ABCA4	ABCR	Retina
	ABCA7	KIAA0822	Brain
ABCB	ABCB1	Pgp	Intestine, liver, kidney, placenta, blood-brain barrier
	ABCB2 and 3	TAP1 and 2	Endoplasmic reticulum membrane
	ABCB4	Pgp3	Liver
	ABCB7 and 8	ABC7 and M-ABC1	Mitochondrial membrane
	ABCB11	BSEP	Liver
ABCC	ABCC1	MRP1	All tissues
	ABCC2	MRP2	Liver, kidney, intestine
	ABCC3	MRP3	Pancreas, kidney, intestine, liver, adrenal glands

	ABCC4	MRP4	Prostate, testis, ovary, intestine, pancreas, lung
	ABCC5	MRP5	Most tissues
	ABCC6	MRP6	Liver, kidney
	ABCC7	CFTR	Liver, pancreas, intestine, bronchial and sweat glands
	ABCC8 and 9	SUR1 and 2	Pancreas
ABCD	ABCD1 and 2	ALD/ALDL1	Peroxisomal membrane
	ABCD3 and 4	PXMP1 and PXMP1L	Peroxisomal membrane
ABCE/F	ABCE1/ABCF1	OABP/ABC50	Unknown
ABCG	ABCG1	ABC8	Central nervous system
	ABCG2	BCRP	Placenta, intestine, breast, liver

Table 1.5: List of ABC transporters and their distributions of expression (Gottesman *et al*., 2002)

1.8.2 Roles in normal physiology and multidrug resistance

ABC transporters have normal physiological roles as well as roles in the multidrug resistance (MDR) phenotype. Table 1.5 shows that the majority of ABC transporters are expressed in vital organs responsible for excreting waste products of metabolism and potentially harmful chemicals. Their physiological function is to excrete xenobiotics and their metabolites, including phospholipids, ions, steroids, and amino acids. Therefore, the transporters have important roles in tissue defence, and prevent accumulation of potentially harmful compounds. They are present at the blood-brain barrier, luminal membranes of kidney, and the brush border membranes of intestinal cells amongst other locations (Klein *et al*., 1999b). For example, Pgp, MRP1/2/4, and BCRP provide protection to the brain at the blood-brain barrier. In the liver, Pgp, BSEP,

and MRP1-3 excrete xenobiotics into bile and blood stream. In the kidney, Pgp, MRP2/4, and BCRP excrete xenobiotics into urine. In the mammary gland of lactating animals, BCRP is involved in secretion of nutrients into milk (Huls *et al*., 2009).

ABC transporter expression is also associated with MDR phenotype. This has been demonstrated experimentally repeatedly by growth of cell lines in medium containing a single specific chemotherapeutic agent thereby selecting for resistance; the resultant resistant cell lines typically show a MDR phenotype against multiple unrelated drugs, with deregulation of ABC transporters identified as being responsible. The wide range of relevant substrates of the most well studied ABC transporters are highlighted in Table 1.6 (Leonessa and Clarke, 2003, Chang, 2010, Doyle and Ross, 2003). This demonstrates that ABC transporters efflux most chemotherapeutics that are in current clinical use, and hence their basal expression in tumours or their change in expression on exposure to chemotherapy agents potentially has high clinical relevance.

ABC transporter	Examples of known substrates
Pgp	Anthracyclines, colchicine, cortisol, dexamethasone, diltiazem, methotrexate, mitoxantrone, nicardipine, taxanes, verapamil, vincristine.
MRP1	Anthracyclines, cyclophosphamide, folic acid, methotrexate, vincristine.
BCRP	Anthracyclines, folic acid, methotrexate, mitoxantrone, sulphasalazine, topotecan.

Table 1.6: Example of common substrates for ABC transporters

1.8.3 Molecular structure of ABC transporters

ABC transporters are active pumps that efflux substrates against their concentration gradient. Their molecular structure consists of a nucleotide binding domain (NBD) in the cytoplasm and a transmembrane domain (TMD), composed of six transmembrane helices. Two or more TMDs are required to form an active ABC transporter. Two highly conserved peptide motifs within the NBD, Walker A and B, characterises the ABC transporters (Klein *et al*., 1999a), and are involved in the binding of ATP molecules. When a substrate binds to the transporters, ATP hydrolysis occurs, causing a conformational change at the cytosolic point of entry to the trans-membrane channel. This structural change moves the substrate through the protein channel and the plasma membrane, causing its export to the extracellular space. A further ATP hydrolysis at its binding site restores the protein to its original conformation, enabling it to bind to its substrate again (Sauna and Ambudkar, 2001).

The vast majority of studies into the roles of these genes in MDR so far have focused on the 170 kDa ABC transporter P-glycoprotein (Pgp), the product of the *ABCB1 (MDR1)* gene. It was discovered by selecting Chinese hamster ovarian cell lines with colchicine (Juliano and Ling, 1976), which then showed an MDR phenotype to a wide range of drugs. The gene encoding for Pgp, *MDR1*, was identified and cloned from the human KB carcinoma cell line (Ueda *et al*., 1986). The structure of Pgp consists of 12 transmembrane helices, split into two TMDs that are linked by an intracellular ATP-binding domain. Substrates of Pgp are highlighted in Table 1.6.

Another well-studied ABC transporter is the 190 kDa Multidrug resistance-associated protein 1 (MRP1), the product of the *ABCC1* gene. It was discovered by doxorubicin-selection of drug resistant H69AR cells from the initially drug sensitive small cell lung

cancer cell line H69, and by subsequent cloning of the MRP1 cDNA (Cole *et al*., 1992). The selected cell line did not express Pgp, and both the mRNA and protein expressions of MRP1 was increased compared to the unselected parental cell line. MRP1, which has 17 transmembrane helices, shares less than 15% of amino acid identity compared to Pgp. Compared to Pgp, MRP1 has an additional TMD of 5 transmembrane helices. It has similar substrate specificity to Pgp, including anthracyclines and methotrexates. It is worth noting that MRP1 does not efflux paclitaxel (Leonard *et al*., 2003).

The 72 KDa BCRP is a less well-studied ABC transporter, especially in breast cancer. It is a product of the *ABCG2* gene. BCRP was first identified in a human breast cancer cell line MCF7/AdrVp (Ni *et al*., 2010), a multidrug resistant cell line that does not express Pgp or MRP1. The cell line was developed by co-selecting the parental cell line in doxorubicin and verapamil, the latter being a Pgp inhibitor and therefore avoiding Pgp-mediated resistance (Leslie *et al*., 2005). Substrates of BCRP include doxorubicin, methotrexate, and mitoxantrone. Similar to MRP1, paclitaxel is not a substrate of BCRP (Leslie *et al*., 2005). In contrast to Pgp and MRP1, structurally BCRP consists of six transmembrane helices only, and an ATP-binding domain. To function as an active transporter, BCRP has to homodimerise.

1.8.4 Regulation of Pgp, MRP1, and BCRP

Patients with high ABC transporter expression might be expected to have poor responses to chemotherapy, and have lower survival rates. Therefore, manipulation of molecular mechanisms involved in the regulation of ABC transporter expression in cancer cells could potentially lead to improved efficacy of chemotherapy agents. Common regulation and cancer-specific deregulation mechanisms for ABC transporter

expression can be broadly classified into changes in genomic copy number, transcriptional regulation, and post-transcriptional regulation. Below I give specific examples in each of these categories for Pgp, MRP1 and BCRP (Nakanishi and Ross, 2012, Kuo, 2009).

Teeter *et al* selected Chinese hamster ovarian cells with increasing doses of vincristine to develop MDR. Cytogenetic analyses of the cell lines revealed *MDR1* gene amplification as an underlying mechanism of chemoresistance (Teeter *et al*., 1986). Transcriptional regulation is defined by transcriptional factors. However, their binding to the target gene can be altered in cancer due to changes in DNA methylation and chromatin structure. Tada *et al* showed that in bladder cancer, *MDR1* mRNA expression was up-regulated after exposure to chemotherapy. In this study, the frequency of patients with hyper-methylated *MDR1* promoter region was reduced from 50% to 17% after exposure to chemotherapy (Tada *et al*., 2000). Therefore, an inverse correlation was detected between the degree of methylation at the CpG (cytosine phosphate guanine) sites of the *MDR1* promoter region and the mRNA levels. An example of post-transcriptional regulation is demonstrated by Randle *et al*. The 5'-untranslated region (UTR) of the *MDR1* mRNA contains considerable secondary structure, with double-stranded hair-pin loops. This sequence influenced the translational efficiency of transcripts and allowed regulation under cytotoxic stress (Randle *et al*., 2007).

Yasui *et al* conducted cytogenetic analyses of parental cancer cell lines versus chemotherapy resistance cancer cell lines and determined that *ABCC1* genomic copy number was amplified in the resistant cell lines compared to the parental cell lines (Yasui *et al*., 2004). Wild-type p53 is involved in the repression of transcription at the *ABCC1* promoter sites, and the loss of p53 expression in colorectal cancer has been

shown to correlate with increased MRP1 expression (Fukushima *et al*., 1999). Borel *et al* examined *ABCC1* mRNA expression amongst other ABC transporters in hepatocellular carcinoma compared to adjacent healthy liver. Higher mRNA levels were observed in the tumour tissue compared to healthy liver tissue. Furthermore, an inverse relationship was detected between microRNA expression (miR-199a/b and miR-296) and *ABCC1* mRNA expression (Borel *et al*., 2012).

Similarly to *ABCB1* and *ABCC1*, comparative genomic hybridisation studies have shown *ABCG2* genomic copy number amplifications in cancer cell lines treated with mitoxantrone and doxorubicin (Nakanishi and Ross, 2012). Oestrogen and its derivatives can act as either substrates or inhibitors (Staud and Pavek, 2005) for BCRP, which contains an oestrogen response element (ERE) in its promoter site. Ee *et al* showed that oestrogen enhanced BCRP mRNA expression in T47D cell lines (Ee *et al*., 2004), demonstrating transcriptional regulation of BCRP expression. In contrast, Imai *et al* showed that BCRP expression on western blot, but not at the mRNA level, was reduced with increasing levels of 17β-oestradiol in MCF7 cell lines, and the authors inferred that BCRP expression was regulated post-transcriptionally (Imai *et al*., 2005).

1.8.5 ABC transporter directed therapies

The efflux activity of xenobiotic transporters can be inhibited by various targeted drugs (e.g. valspodar, biricodar, and tariquidar). However, they are not currently in routine clinical use, and have mostly been evaluated in phase II and III trials (Modok *et al*., 2006). Their lack of advance in the clinical setting may be due to the fact that the transporters have a wide range of physiological roles and hence the inhibitors may cause non-specific toxicity. Verapamil, a Pgp inhibitor, was first used in clinical trials in

1980s, but had unacceptable levels of side-effects, including cardiotoxicity and myelotoxicity (Ozols et al., 1987, Mross *et al*., 1993). This was followed by valspodar, a second generation Pgp inhibitor, which had a reduced side-effect profile. However, phase III clinical trials using valspodar in ovarian or peritoneal cancer have not been encouraging. 762 patients with advanced ovarian or primary peritoneal cancer were treated with carboplatin and paclitaxel with or without valspodar in a phase III clinical trial. The result showed no significant improvement in time to disease progression or overall survival from the addition of valspodar. Furthermore, central and peripheral nervous system and gastrointestinal toxicities were more frequently seen in patients treated with valspodar (Lhomme *et al*., 2008). An MRP1 inhibitor, Sulindac, has been evaluated in phase I clinical trial in combination with epirubicin (O'Connor *et al*., 2007). However, as yet no further phase II or III trials have been reported. As shown in Table 1.6, there is a considerable overlap in substrate specificities between the ABC transporters. Therefore, a limitation of these approaches may be that inhibition of a single transporter may not result in effective clinical response as it is possible, or even probable, that inhibition of a single transporter may lead to increased compensatory substrate efflux by the other transporters. However, further research is required to study the potential interaction and molecular cross-talk between the transporters. Apart from Pgp, MRP1, and BCRP, there are numerous other less well-studied ABC transporters (Table 1.5). It is possible that these other transporters also have key roles in chemoresistance.

1.8.6 ABC transporter expression in cancer

Pgp and MRP1 expression has been detected using immunohistochemistry, qPCR, and flow cytometry in a range of tumours including breast cancer, myeloma, lung cancer, ovarian cancer, and leukaemia (Leonard *et al*., 2003). Of note, in acute myeloid leukaemia Pgp expression is up-regulated in patients who suffer from disease relapse

(Han *et al*., 2000). Other studies have shown that the intrinsic expression levels of ABC transporters correlate with clinico-pathological parameters that are associated with poor patient outcome. For example, MRP1 expression in untreated hepatocellular carcinoma correlates with tumour grade, size, and the degree of microvascular invasion (Fletcher *et al*., 2010). Similarly a study by Weinstein *et al* found that Pgp expression in colon cancer correlated with vessel wall invasion and lymph node metastasis (Weinstein *et al*., 1991).

In breast cancer, the majority of published studies have focused on the expression of Pgp since it was the first of the three to be discovered (Leonessa and Clarke, 2003). Many previous studies have focused on measuring expression levels prior to adjuvant (post-surgery) chemotherapy. A meta-analysis (Trock *et al*., 1997), including 21 immunohistochemical studies, showed that Pgp expression was seen in 48.5% of breast tumours. However, the incidence ranges widely from 0 to 80% (Leonard *et al*., 2003). This may reflect the differences in quantification of expression, heterogeneity between the patient cohorts, or the differences in the specificity of antibodies used. Indeed, antibodies JSB-1, C494, and C219, which have been used for “Pgp” detection, have been reported to have cross-reactivity with proteins other than Pgp (Trock *et al*., 1997).

Studies examining Pgp expression in normal breast tissues have shown variable results. Van der Valk *et al* used three antibodies (JSB-1, C219, and MRK16) for immunohistochemistry and showed that Pgp expression was weak and confined to the breast epithelial cells (van der Valk *et al*., 1990). Ro *et al* showed that Pgp expression was present in the adjacent normal or hyperplastic tissue in 67% of the 40 breast cancer specimens examined by immunohistochemistry using C219 antibody (Ro *et al*., 1990). However, Pgp expression was undetectable in the normal breast tissue samples

with immunoblots using the same antibody, compared to the breast cancer tissue samples (Sanfilippo *et al*., 1991) where Pgp expression was detected in 10 out of 34 cases. Such studies examining MRP1 and BCRP expression in normal breast tissues are limited. Linn *et al* (Linn *et al*., 1997) showed that MRP1 expression was observed in the normal breast epithelium as well as the malignant cells using immunohistochemistry.

Regarding comparisons to clinico-pathological parameters, there are no studies that show clear correlations between Pgp expression and tumour grade or stage. Pooled analysis from 4 studies suggests a correlation between Pgp expression and tumour stage (Leonessa and Clarke, 2003), with Pgp expression observed in 23% of T1 tumours, compared to 42% of T4 tumours. This correlation needs to be interpreted with caution due to the heterogeneity in study design and patient cohort in each study. Data from pooled studies (n=5) showed that MRP1 expression was observed in 49% of the cases using immunohistochemistry in untreated breast cancer patients. Similarly to Pgp, studies examining MRP1 expression did not detect correlations with tumour stage, grade, or ER status (Leonessa and Clarke, 2003).

In terms of predicting survival, studies by Vargas-Roig *et al* (Vargas-Roig *et al*., 1999) and Honkoop *et al* (Honkoop *et al*., 1998) both show that Pgp expression detected by immunohistochemistry did not predict disease free survival (DFS) or overall survival (OS) in breast cancer, where patients in both cohorts received doxorubicin. The Austrian Breast and Colorectal Cancer Study Group carried out a large study (n=516) investigating the role of MRP1 in breast cancer (Filipits *et al*., 2005). Using immunohistochemistry, they determined that MRP1 expression independently predicted for poor DFS. BCRP expression has been studied using immunohistochemistry in lung (Kim *et al*., 2009) and pancreatic cancer (Lee *et al*.,

2012). These studies have shown BCRP expression to be associated with poor DFS. A limited number of studies have used tissues from patients treated with NAC to study xenobiotic transporter expression in breast cancer. It is of clear interest to examine their expression in the context of NAC, which has become a key treatment modality in breast cancer. In patients treated with adjuvant chemotherapy, only the intrinsic initial expression of the xenobiotic transporters can be determined since the tumour has been resected, and no further tumour tissue is available post-adjuvant chemotherapy unless patients develop tumour recurrences. NAC treatment protocols enable examination of the effect of chemotherapy in modulating xenobiotic transporter expression, and whether these expression levels correlate with patient outcome.

1.8.7 Introduction summary

Breast cancer is a relatively common disease, and NST is an important treatment modality. ABC molecules potentially impact on response to NST, and further investigation of how they impact on response, and how NST impacts on their expression is warranted.

2.0 Hypothesis and Aims

My hypothesis is that expression of xenobiotic transporters impacts on neoadjuvant systemic therapy response in breast cancer.

1. The primary aim of the project was to investigate whether expression of Pgp, MRP1, and BCRP either pre- or post-NAC correlates with treatment outcome.

2. The secondary aim was to examine whether Notch1 activity impacts on MRP1 expression, and thereby on response to chemotherapy.

3. The tertiary aim was to examine whether NAET modulates BCRP expression, and whether epigenetic mechanisms are involved.

3.0 Materials and methods

3.1 Cohort selection

Ethical approval was obtained from Leeds (East) Research Ethics Committee (reference 06/Q1206/180).

Cohort 1 – Neoadjuvant chemotherapy

136 breast cancer patients treated with NAC at Leeds Teaching Hospitals NHS Trust (LTHT) from 2005 to 2009 were identified using the LTHT computer database, Patient Pathway Manager (PPM). Patients to be studied further were subject to careful cohort selection in order to limit tumour heterogeneity. Relevant data on patient and tumour characteristics were collected. Strict inclusion criteria for the study included patients who had a minimum of 3 year clinical follow-up after NAC, post-operative radiotherapy, grade 2 or 3 invasive ductal carcinoma (IDC) on core biopsy specimen, and NAC regimen consisting of anthracyclines +/- taxanes. Exclusion criteria included inflammatory breast carcinoma, invasive lobular carcinoma (ILC), and those who received adjuvant chemotherapy as well as NAC. This identified a cohort of 45 patients. Relevant clinical data are outlined in table 3.1. Disease free survival (DFS) was defined as survival free of local or distant disease post-surgery. 21 patients were diagnosed with axillary metastasis and nodal tissues were available in 15 cases. In 2/15 cases the final pathology diagnosis was micrometastasis, where tumour size was less than 2mm. This was deemed insufficient for immunohistochemistry, and hence 13 matching patient lymph node blocks were available for immunohistochemical analysis of tumour cells.

Characteristic	Categories	No. of patients (%) n=45
Age	<45	21 (47)
	>45	24 (53)
Grade (pre-NAC)	2	15 (33.3)
	3	30 (66.7)
Stage (pre-NAC)	T2	26 (57.8)
	T3	16 (35.6)
	T4	3 (6.6)
Stage (post-NAC)	T0	6 (13.3)
	T1	14 (31.1)
	T2	17 (37.8)
	T3	6 (13.3)
	T4	2 (4.5)
Tumour size change	Increase	8 (17.8)
	Decrease	31 (68.9)
	pCR	6 (13.3)
MRI response	Minimal	9 (20)
	Partial	30 (66.7)
	Complete	6 (13.3)
NAC regimen	epirubicin + cyclophosphamide (EC)	13 (28.9)
	EC + taxanes	32 (71.1)
Lymphovascular invasion	Positive	17 (37.8)
Axillary metastasis	Positive	21 (46.7)
Estrogen receptor	Positive	26 (57.8)
Her2	Positive	9 (20)
Surgery	breast conserving	17 (37.8)
	Mastectomy	28 (62.2)
Follow up	median: 4.5 years (range 3-8.8 years)	
Recurrence		17 (37.8)
Death		10 (22.2)

Table 3.1: Clinico-pathological characteristics of the NAC cohort (note: a relatively high number of patients suffered from recurrences, reflecting the aggressive nature of disease presentation in this cohort)

Cohort 2 – Neoadjuvant endocrine therapy

144 breast cancer patients treated with NAET at LTHT from 2005 to 2013 were identified using the LTHT computer database, Patient Pathway Manager (PPM). Patients who were part of the POETIC or NEO-EXCEL clinical trials were excluded from further consideration. In the former trial the length of duration of NAET was only 2

weeks (Dowsett *et al.*, 2011), and in the latter trial NAET was combined with celecoxib. Therefore, 86 patients were available for further cohort selection. Inclusion criteria for the study included NAET duration of 1 month to 1 year, IDC or ILC, Allred score for ER expression of 7 or 8, no change in NAET regime, and lack of HER2 overexpression. This identified a cohort of 51 patients. Relevant clinical data are outlined in Table 3.2. Patients were mostly post-menopausal and this is reflected by administration of aromatase inhibitors in the majority. Most patients presented with lower grade and stage tumours, reflected by the lower recurrence rates.

Characteristic	**Categories**	**No. of patients (%) n=51**
Age	Median: 67 years old (range 40-95 years old)	
Histological type	IDC	38 (74.5)
	ILC	7 (13.7)
	IDC/ILC	7 (13.7)
Grade	1	15 (29.4)
	2	30 (58.8)
	3	6 (11.8)
Stage (pre-NAET)	T1	17 (33.3)
	T2	26 (51)
	T3	5 (9.8)
	T4	3 (5.9)
Stage (post-NAET)	T1	19 (37.3)
	T2	25 (49)
	T3	7 (13.7)
Tumour size change	Increase or no change	22 (43.1)
	Decrease	29 (56.9)
Lymphovascular invasion	Positive	11 (21.6)
Receptor status (4/8 as cut-off for positive expression)	ER	51 (100)
	PR	37 (78.7)
NAET duration	Median: 90 days (range 30-362 days)	
NAET regimen	Anastrozole	24 (47.1)
	Letrozole	20 (39.2)
	Tamoxifen	7 (13.7)
Axillary metastasis	Positive	21 (41.2)
Surgery	breast conserving	22 (43.1)
	Mastectomy	29 (56.9)
Adjuvant chemotherapy		6 (11.8)
Recurrence	Median follow-up	7 (13.7)

Table 3.2: Clinico-pathological characteristics of the NAET cohort

3.2 Haematoxylin & Eosin staining and step sectioning

Archival formalin-fixed paraffin-embedded (FFPE) matching breast core biopsy and resection blocks were obtained for the selected patients. Single core biopsy (pre-NAC/NAET) blocks were available, compared to the numerous resection blocks (post-NAC/NAET). A review of histopathology reports was required to identify appropriate resection blocks that contained tumour. To ensure the resection block contained tumour cells, haematoxylin & Eosin (H&E) staining was performed on the selected blocks.

Blocks were sectioned at depths of 5µm with a microtome, and sections were placed onto single SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Slides were then air-dried, and incubated at 37^{o}C. Sections were dewaxed in xylene, and rehydrated with graded ethanol. They were then stained in Mayer's haematoxylin, Scotts substitute, and eosin. Following this, sections were dehydrated in ethanol and xylene, and mounted in DPX (Fluka, Gillingham, UK). The stained slides were then examined under a microscope to detect the presence of tumour cells. The slides were also examined by a pathologist (Dr Eldo Verghese, EV, Leeds Institutes of Molecular Medicine, University of Leeds and Department of Histopathology, LTHT) to confirm the findings.

In addition, 8 patients receiving NAC were reported to have achieved pCR in the pathology reports. In order to confirm the absence of tumour tissue in these cases, a single resection block was selected per patient. These blocks had evidence of fibrosis, or the presence of a guide wire tip, and hence corresponded to the original tumour location prior to NAC. Step-sectioning was performed at intervals of 100µm and four consecutive sections of 5µm thickness were placed onto the SuperFrost Plus slides,

and immunohistochemistry (Section 3.4) was performed at each level (Table 3.3) with the monoclonal mouse anti-human cytokeratin AE1/3 antibody (M 3515; Dako, Glostrup, Denmark). This revealed residual tumour cells in 2 out of 8 cases (Appendix 3, Fig S1). Therefore, the final cohort of patients for whom pre- and post-NAC tumour tissue was available was increased to 39/45 cases.

3.3 Antibody selection

Many monoclonal antibodies are available for Pgp, including; C219, JSB1, MRK16, UIC2, and 4E3 (Beck *et al*., 1996). Fewer monoclonal antibodies are available for MRP1 (QCRL1, QCRL3, and MRPr1), and BCRP (BXP21 and BXP34). UIC2 was chosen to detect Pgp expression as it has been widely used for immunohistochemistry on breast tissue (Mechetner *et al*., 1998, Hegewisch-Becker *et al*., 1998, Tsukamoto *et al*., 1997), and has also been validated by the presence of a single band at 170kDa (Mechetner and Roninson, 1992) in western blot experiments. Similarly, the QCRL1 monoclonal antibody was chosen for MRP1 as it had been used on breast cancer tissues (Filipits *et al*., 1996), and was validated by a specific western blot at 190kDa on breast cancer cell lines (Morrow *et al.*, 2006). In the aforementioned study, MRP1 staining was mostly observed in tumour cells, with occasional weak staining in some stromal cells. Both membranous and cytoplasmic staining patterns were observed. For BCRP, BXP21 showed a range of positive staining in a variety of human tumours including FFPE breast tissue (Diestra *et al*., 2002). Both homogeneous and heterogeneous staining intensity were observed that was mostly tumour-specific, but some staining was observed in the endothelial cells. The staining patterns were mixed membranous and cytoplasmic. Western blot experiments using BXP21 on breast cancer cell lines showed a presence of a specific band at 72kDa (Morrow *et al*., 2006). Therefore, BXP21 monoclonal antibody was chosen (Table 3.3). Anti-activated Notch1 antibody detects only the active, cleaved intracellular domain of Notch1 (Notch intra-

cellular domain, “NICD”). It has been used on breast tissue for immunohistochemistry (Efstratiadis *et al*., 2007), and on western blots with breast cancer cell lines (Hao *et al*., 2010). Strong nuclear staining was observed in breast cancer cells with this antibody, but normal ductal epithelial cells stained negative or weakly positive.

Antigen	Antibody	Type	Code and manufacturer
Pgp	UIC2	Mouse monoclonal IgG_{2a} anti-human	sc-73354, Santa Cruz Biotech., Santa Cruz, USA
MRP1	QCRL1	Mouse monoclonal IgG_1 anti-human	sc-18835, Santa Cruz Biotech., Santa Cruz, USA
BCRP	BXP21	Mouse monoclonal IgG_{2a} anti-human	ab3380, Abcam, Cambridge, UK
Notch1 NICD	Anti-activated Notch1 antibody	Rabbit polyclonal IgG anti-human	ab8925, Abcam, Cambridge, UK
Cytokeratin	AE1/3	Mouse monoclonal IgG_1 anti-human	M3515, Dako, Glostrup, Denmark

Table 3.3: Selected antibodies for immunohistochemistry

3.4 Immunohistochemistry

The protocol below describes my generic immunohistochemistry method; modifications made for use of specific individual antibodies are noted in section 3.5, and Table 3.4. FFPE breast tissue blocks were sectioned at depths of 5µm with a microtome, and the sections were placed onto SuperFrost Plus slides (Menzel-Glaser, Braunschweig,

Germany). Slides were then air-dried, and incubated at 37^{o}C for 16 hours. Sections were dewaxed with xylene and rehydrated with graded ethanol. This was followed by a 5 minute wash with water. The sections were then immersed in 10mM citric acid buffer (pH 6.0), and epitope retrieval was performed by heat using a 900W microwave or pressure cooker (10 min). Sections were then immersed in 0.3% H_2O_2 (10 min) to block endogenous peroxidase activity. Slides were rinsed in Tris-buffered saline (TBS), and mounted on Sequenza racks (Thermo Scientific, USA). Non-specific binding activity for primary antibodies was blocked, when required, using 100µl of casein solution (SP5020; Vector Labs, Burlingame, USA) diluted 10-fold (20 min) in TBS or antibody diluent reagent solution (Invitrogen, USA). Otherwise 100µl of antibody diluent reagent solution was added, and then 100µl of the primary antibody diluted in the same diluent was added for either 1 hour at room temperature or 16 hours at 4^{o}C. Slides were then rinsed twice with TBS-tween (TBS-T), followed by a single wash with TBS. 1.25ml of 10% v/v Tween-20 was added to 1 litre of TBS solution to form TBS-T (Appendix Section 1). Each wash lasted 5 minutes. Slides were incubated in a horseradish peroxidase (HRP) conjugated polymer secondary antibody (Dako, Glostrup, Denmark) for 30 minutes. The slides were then re-washed with TBS-T and TBS as before, and then 100µl of diaminobenzidine (DAB) was added to each slide for exactly 10 minutes, followed by a wash in water for 5 minutes. The slides were stained with haematoxylin for one minute, followed by a one minute wash in water. This was followed by a wash in Scott's tap water (refer to Appendix Section 1 for recipe) for one minute, and a subsequent one minute wash in water. The slides were dehydrated with graded ethanol and xylene, and mounted in DPX (Fluka, Gillingham, UK). The recipe list for all the reagents used for immunohistochemistry and other experiments are listed in the appendix section 1.

3.5 Antibody optimisation and cohort staining

Conditions for use of the antibodies in immunohistochemistry with breast cancer tissue were optimised using a tissue microarray (TMA) of 30 cores of randomly selected and fully anonymised breast carcinomas, normal breast tissue, and placenta tissues. The presence of placenta tissue was particularly important since this tissue is known to express the xenobiotic transporters highly (as corroborated by reviewing immunohistochemistry results compiled on the human protein atlas project http://www.proteinatlas.org) and therefore this tissue acted as a positive control. Negative controls were performed by omitting the primary antibody, which produced no staining in the TMA cores. Optimised staining of TMA tissues for each antibody is shown in Appendix section 3, Figure S2. Variables were method of antigen retrieval, antibody concentration, length of incubation and method of blocking (Table 3.4). Conditions were chosen for xenobiotic transporters that allowed a range of tumour-specific cytoplasmic and membranous staining intensities in different cores with minimal staining in fibroblast or normal breast cells. For Notch1 NICD, conditions were chosen that allowed a nuclear-specific staining in tumour cells. Optimisation slides were reviewed by breast histopathologist EV.

Antigen	Antibody	Optimised primary antibody concentration	Optimised antigen retrieval methods	Additional blocking steps	Antibody incubation period
Pgp	UIC2	1/2000	No retrieval	10% casein	1 hour
MRP1	QCRL1	1/50	Microwave	None	1 hour
BCRP	BXP21	1/50	No retrieval	None	16 hours
Notch1 NICD	Anti-activated Notch1 antibody	1/100	Microwave	10% casein	1 hour
Cytokeratin	AE1/3	1/50	Pressure cooker	None	1 hour

Table 3.4: Conditions for use of antibodies for immunohistochemistry

Core (pre-NAC/NAET) and resection (post-NAC/NAET) tissues for the whole cohort were sectioned at depths of 5µm. To enable direct comparison of the staining pattern in these matched tissues, the core and resection tissues were placed on the same slide (Fig 3.1). This methodology is unique in the literature for the proteins of interest, and potentially reduces experimental variation.

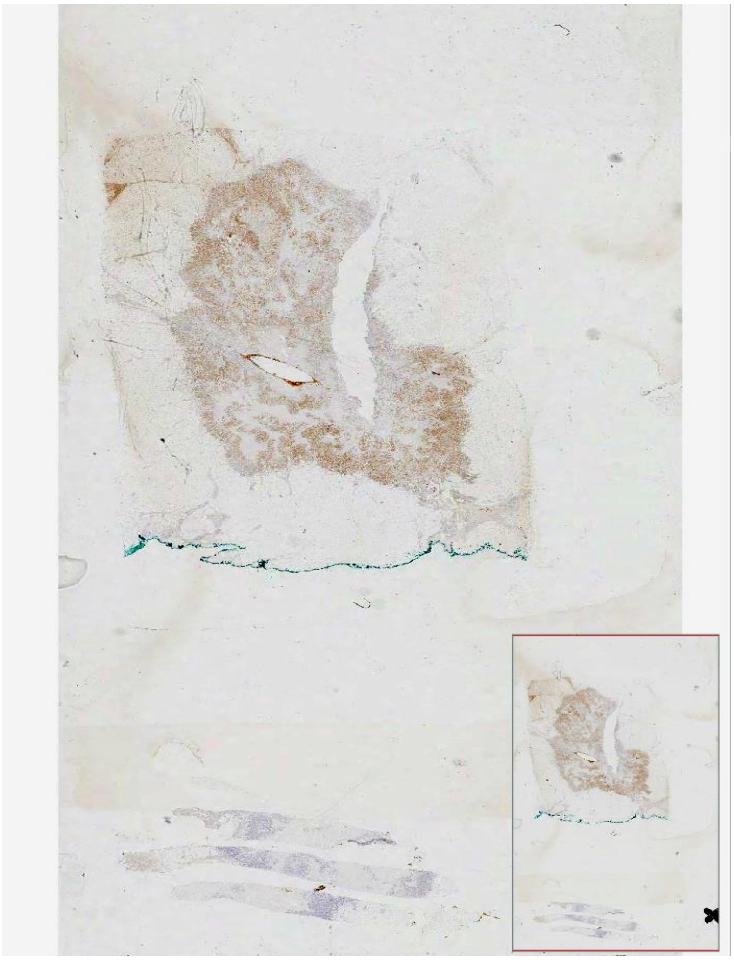


Figure 3.1: Image of core (bottom) and resection (top) tissues placed on the same slide

3.6 Scoring protocol for xenobiotic transporters

Stained sections were digitally scanned using Scanscope XT (Aperio®) at 20x magnification and were observed using ImageScope (Aperio®). Staining was scored initially manually by two independent scorers (BK and Dr Bethany Williams (BW), a histopathologist; Department of Histopathology, LTHT) in order to demonstrate that staining could be reproducibly assessed. Scoring was then performed using a semi-automated procedure, which was validated against the initial manual scores. First, weighted histoscores (van Nes *et al*., 2012) [0 to 300 = (1 x % of tumour cells weakly stained) + (2 x % moderately stained) + (3 x % strongly stained)] were determined independently by BK and BW on a randomly selected tumour field containing at least 100 tumour cells on each of a total of 54 slides with an equal distribution between the core and resection tissues, and between the three antibodies (Appendix Section 2, Table S1). Inter-observer intraclass correlation coefficient (ICC) was then calculated, resulting in an ICC of 0.87 between the two independent observers. Imagescope (Aperio®) software was then used to mark the identical tumour regions digitally (Fig 3.2). The positive brown pixel count algorithm (http://tmalab.jhmi.edu/aperiou/userguides/Positive_Pixel.pdf) was then applied to these regions. Three intensity ranges for each pixel were defined (counts of <100 defined as weakly positive, 100 to <175 as moderate, and >=175 as strong). Pixels not counted as brown were defined as negative. These values were used to generate automated histoscores: (1 x % weakly positive pixels within epithelial region) + (2 x % moderate) + (3 x % strong). ICCs were calculated for BK versus Imagescope (0.83), and for BW versus Imagescope (0.82). These values were deemed to be satisfactory to validate the use of Imagescope to perform semi-automated scoring protocol for the entire cohort. Then, BK manually marked all epithelial tumour cell regions of the cores and resections and Imagescope was used to generate semi-automated histoscores.

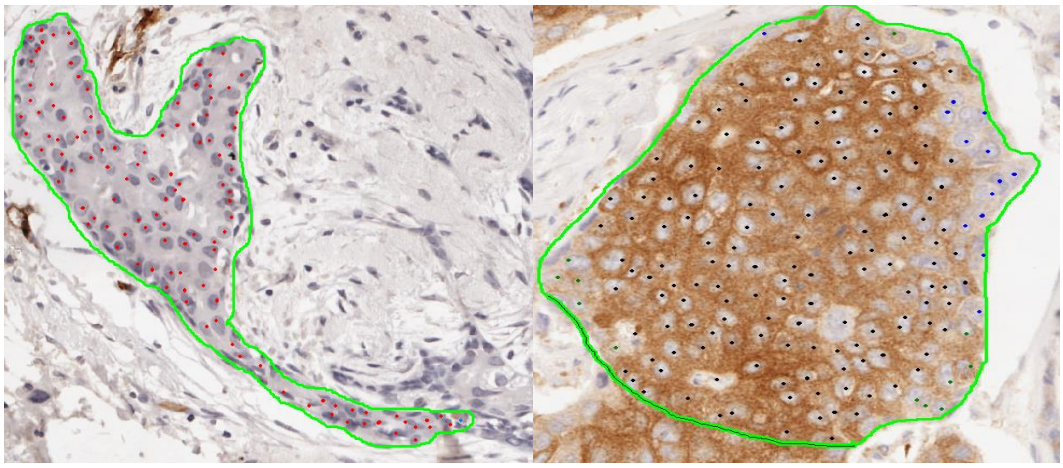


Figure 3.2: An example of manual versus automated scoring. Imagescope was used to mark out the tumour region (inside green boundary) and to generate an automated score within this region. For manual scoring of the same region: individual dots within the marked regions show examples of manual cell counting with different colours representing the different bands of staining intensity: on the left the staining was negative and hence the dots are marked in red colour predominantly, whereas on the right the staining was strong and dots are marked in black colour predominantly. The blue and green dots represent weaker staining observed within the marked region.

3.7 Scoring protocol for Notch1 NICD

Notch1 NICD nuclear staining was scored using the Allred method (Mohammed *et al*., 2012, Allred et al., 2012). This generates a total score in whole numbers ranging from 0 to 8 (note: a score of 1 is not possible), with an intensity score ranging from 0 to 3 added to a score quantifying the proportion of tumours cells staining positively ranging from 0 to 5. Two independent observers (BK and BW) performed the scoring manually (Appendix Section 2, Table S2). Weighted Kappa co-efficients (*k*) were determined to compare the degree of agreement between the two observers. This resulted in a

k=0.78 for the core specimens, and a *k*=0.9 for the resection specimens. This resulted in an overall *k*=0.89 (n=58). The average of the two scores was used as the final score to indicate the degree of Notch1 NICD expression.

3.8 Cell culture

MCF7, HB2, and T47D cells were obtained from Dr Thomas Hughes (Leeds Institutes of Molecular Medicine, University of Leeds), and were routinely maintained in D-MEM, GlutaMAX^{TM} I, 4500mg/L D-Glucose, 110mg/L Sodium Pyruvate (31966; Invitrogen) with 10% fetal calf serum (Sigma-Aldrich, USA). Cell line identities were confirmed by Short Tandem Repeat DNA profiling (Leeds Genomic Service). Cell lines were tested for mycoplasma at the start of the project and at every 6 months intervals (MycoAlert Mycoplasma detection assay, Lonza, Basal, Switzerland), which showed that the cell lines were consistently negative for mycoplasma. Cells were grown routinely in 75 cm^2 tissue culture flasks (430641; Corning life sciences) at 37^{0}C in humidified 5% carbon dioxide/air. No antibiotics were used routinely. The cells were passaged approximately every 72 hours when almost fully confluent. The media was removed from the flask, and the cells were washed with 10ml of Dulbecco's phosphate-buffered saline (DPBS) (14190-094; Invitrogen) prior to dissociation with 2.5ml of trypsin (0.05% v/v) for 5 minutes at 37^{o}C. The detached cells were suspended by adding 7.5ml of fresh culture medium to inactivate the trypsin, and centrifuged at 400g for 5 minutes. The resulting pellet was re-suspended in 10ml of fresh culture medium, and 1ml of the resulting solution was added to 14ml of fresh culture medium in a new flask.

H929 and HL60 cell lines were obtained from Dr Elizabeth Valleley (Leeds Institutes of Molecular Medicine, University of Leeds), and were routinely maintained in RPMI 1640, GlutaMAX^{TM} I (61870-010; Invitrogen) with 10% fetal calf serum. Cells were grown routinely in 75 cm^2 tissue culture flasks at 37^{0}C in humidified 5% carbon dioxide/air.

Since these cells are maintained in suspension culture, passaging was performed approximately every 5 days by adding 1ml of suspended cells to 14ml of fresh culture medium.

3.9 Growth/proliferation assays for response to drugs

MTT assays were used to assess proliferation/survival of cultures after treatment with various drug combinations. Assays were performed using 48 well cell culture multiwell plates (677180; Greiner Bio-one). Each well was seeded with the appropriate number of MCF7 or T47D cells diluted in 250µl of culture medium. Appropriate numbers were determined to enable log phase growth with the culture nearing confluence at either 3 or 5 days (10,000 and 7,500 seeding cell numbers respectively for MCF7 cells) (Appendix Section 3, Fig S3). For the 5 day experiment, 10,000 cells were seeded for T47D cells (Appendix Section 3, Fig S4). A haemocytometer was used to count viable cells that appeared refractile, as opposed to dead cells which that stained blue and non-refractile; the chambers were loaded by mixing 5µl of cells suspended in medium with 5µl of 0.1% trypan blue (T8154; Sigma-Aldrich). Viable cell concentrations were determined using the formula below, taking account of the 50% dilution.

Concentration (number of cells/ml) = {(number of cells counted in 1mm x 1mm grid) x 10,000} x 2

Cell counting was performed in four 1mm x 1mm haemocytometer squares on each occasion and the final concentration was taken from the mean. Cells were incubated in the same conditions as described in the previous section to allow them to adhere and after 24 hours dose-response experiments were performed with up to 1µM concentration of doxorubicin hydrochloride (BPE2516-1; Fisher scientific) alone and up

to 10µM concentration of DAPT (sc-201315; Santa cruz) alone. Each experimental condition was performed for three wells to generate triplicate values. Negative controls included wells without doxorubicin or DAPT but with the same volume of DMSO. Cells were then incubated at 37^{o}C for a further 48 hours or 96 hours before the MTT assay was performed. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (M2128; Sigma-Aldrich) was diluted in DPBS to a working concentration of 5mg/ml. The media in each well was replaced with 50µl of the MTT solution, and incubated in the dark for 3 hours at 37^{o}C. The MTT solution was then replaced with 250µl of propan-1ol, and 100µl of the resulting solution was transferred onto a well of a 96 well plate. The optical density was measured using a microplate reader at 570nm (Opsys MRTM Dynex technologies Ltd, UK), where the optical density readings from wells containing medium only was subtracted. The resulting IC (inhibitory concentration) 10, 25, and 50 doses of doxorubicin were determined for MCF7 and T47D cell lines. The cell lines were also treated with DAPT alone to examine its effect on cell growth/survival at lower doses of up to 10uM. Finally, MCF7 and T47D cells were incubated with or without the three IC doses of doxorubicin and with increasing doses of DAPT (range; 1nM to 100uM) for 2 or 4 days.

3.10 Western blots

3.10.1 Protein extraction and quantification

Proteins were extracted from adherent cultures in 25cm^{2} tissue culture flasks (430639; Corning life sciences). Medium was removed, and cells were washed twice with DPBS. Once the DPBS was removed, 200µl of RIPA buffer (see Appendix Section 1 for recipe) with protease inhibitors (G6521; Promega) was added to each flask. Cell scraping was performed immediately, and the lysis buffer/cell mix was placed on ice for 15 minutes. The resulting mixture was then centrifuged at 14,000g for 10 minutes at 4^{o}C to separate the cell debris from protein. The supernatant was then transferred to a

new microcentrifuge tube for assessment of protein concentration using the Bio-Rad DC™ protein assay (500-0116; Bio-Rad). This assay uses the principle that differential colour change of a dye is proportional to protein concentration. Bovine serum albumin (BSA) was serially diluted in PBS to create a range of concentrations (2mg/ml to 0.125mg/ml) to generate a standard curve. The corresponding concentration of each lysate was extrapolated from this curve. 5µl of the lysate sample was added per well of a 96 well plate to 25µl of reagent A, followed by the addition of 200µl of reagent B. Absorbance was measured after 15 minutes using a Dynex OpSys MR™ microplate photometric reader (Dynex, Chantilly, VA) at 630nM.

3.10.2 Gel electrophoresis and immunoblotting

The lysate volume required for either 20µg or 35µg protein loading was determined for each sample. 5:1 mixture of NuPage® LDS 4x sample buffer (NP0007; Invitrogen) and 2- β mercaptoethanol (Sigma-Aldrich) was prepared. This mixture was combined with the lysate and with additional RIPA buffer as appropriate to standardise the volumes of the samples. Samples were denatured at 105^{o}C for 5 minutes and transferred to ice for 5 minutes. The samples were then centrifuged at 8000g for 30 seconds, re-suspended and placed on ice.

NuPage® Novex 4-12% Bis-Tris 1.0mm x 10 well gels (NP0321BOX; Life Technologies) were used for electrophoresis. Gels were slotted into XCell SureLock™ Mini-cell electrophoresis system (Invitrogen, Carlsbad, CA), which was filled with a 1x NuPage® MOPS SDS running buffer (NP0001; Invitrogen). Samples were loaded into the wells along with a Precision Plus Protein™ Dual Color Standards (161-0374; Bio-Rad). Electrophoresis was performed at 180V for 1 hour. Polyvinylidene fluoride (PVDF) membrane (88518; Thermo Scientific) was cut to match the size of the gel, and

activated in methanol for 30 seconds. This was rinsed for 5 minutes in running water thrice and soaked in 1X NuPage® transfer buffer (NP0006-1; Invitrogen). The pre-cast gel was removed and placed onto the activated membrane, flanked by Mini Trans-blot filter papers (170-3932EDU; Bio-Rad) and sponges soaked in transfer buffer. The resulting sandwich was loaded into the XCell II Blot Module (Invitrogen, Carlsbad, CA), and slotted into the mini-electrophoresis system filled with the transfer buffer. The outer chamber was filled with de-ionised water, and protein transfer was performed at 30V for 90 minutes. The PVDF membrane was removed from the system and Ponceau-S was used to confirm the success of protein transfer. Blocking of non-specific antibody binding was achieved by incubating the membrane with 1% or 5% milk powder solution dissolved in TBST for 1 hour. Following a 5 minutes wash with TBST, primary antibody incubation was performed for 16 hours at 4^{o}C. This was performed by submerging the membrane in a Corning® 50ml centrifuge tube (430828; Corning®) containing the primary antibody, which was diluted in 1% milk powder solution dissolved in TBST. Three 10 minutes wash in TBST was performed, followed by HRP conjugated polymer secondary antibody incubation (Dako, Glostrup, Denmark) at room temperature for 1 hour at concentration of 1/2000. Following further three 10 minute washes in TBST, chemiluminescence detection was performed using the SuperSignal® West Pico Trial kit (34079; Thermo Scientific) or the West Femto Trial kit (34094; Thermo Scientific). The images were acquired using a Bio-Rad Gel Doc Imaging system and Image Lab^{TM} software (version 4.0.1).

3.10.3 Antibody optimisation for western blots

For western blots, the following monoclonal antibodies were used; C219 for Pgp, MRPr1 for MRP1, and BXP21 for BCRP. Variables for optimisation were denaturing versus non-denaturing protocol, primary antibody concentration, quantity of protein loading, and method of blocking (Table 3.5). Antibody optimisation was attempted

using UIC2 and QCRL1 monoclonal antibodies for Pgp and MRP1 respectively. This proved to be unsuccessful and hence different antibodies to those used for immunohistochemistry were used for immunoblotting. The following table describes the optimised conditions for immunoblotting, where beta actin was used as a loading control.

Antigen	Antibody	Concentration	Denature	Protein load	Block
Pgp	C219 (Mouse mAb, 517310, Calbiochem)	1/100 overnight	No heating	35µg	1% milk
MRP1	MRPr1 (Rat mAb, ab3368, Abcam)	1/100 overnight	No heating	35µg	1% milk
BCRP	BXP21 (Mouse mAb, ab3380, Abcam)	1/250 overnight	Heating	20µg	1% milk
Notch1	Anti-activated Notch1 antibody (Rabbit pAb, ab8925, Abcam)	1/2000 overnight	Heating	20µg	5% milk
Beta actin	Anti-β-actin antibody (mouse mAb, A5441, Sigma-Aldrich)	1/100,000 1 hour	Heating	20µg	5% milk

Table 3.5: Conditions for use of antibodies for western blot

3.11 DNA extraction

3.11.1 DNA extraction from cell lines

Cells were harvested using trypsin (adherent cells) or centrifugation (suspension cells). The cells were centrifuged for 10 minutes at 10^{o}C (290g). The supernatant was discarded, and the pellet was re-suspended in 10ml of DPBS and washed twice. The resulting pellet was re-suspended in 10ml of DNA buffer (see Appendix Section 1) and centrifuged for 10 minutes. After removing the supernatant, the pellet was re-suspended in 3ml of DNA buffer with 125µl of proteinase K (10mg/ml) and 400µl of 10% SDS. The resulting solution was incubated for 16 hours at 45^{o}C. 3.6ml of phenol, chloroform, and isoamyl alcohol was subsequently added and mixed thoroughly by vortexing, and the resulting mixture was centrifuged for 10 minutes at 10^{o}C (1811g). The supernatant was transferred into a new tube, and 3.6ml of chloroform/isoamyl alcohol was added and mixed thoroughly. The resulting mixture was again centrifuged, and 3ml of the supernatant was added to 300µl of 3M sodium acetate (pH 5.2) and 9ml of 2-propanol to enable DNA precipitation. The resulting precipitate was washed in 70% ethanol, and the remaining dry precipitate was dissolved in 500µl of nuclease free water (P119E; Promega).

3.11.2 DNA extraction from formalin-fixed paraffin-embedded breast tissue blocks

Macrodissection of FFPE tissues were performed to isolate tumour-rich regions with an active effort to limit stromal contamination. From the NAET cohort, 46 biopsy tissues were available, compared to 51 resection tissues. The tumour-rich regions were identified and marked on the stained sections after extensive training from breast pathologist EV. Guided by this, macrodissection was performed on adjacent sections of 10µm thickness using a sterile surgical scalpel blade number 11 (0503; Swann-Morton

Ltd). The resulting tissue was collected in 1.5ml microcentrifuge tubes (Eppendorf, Hamburg, Germany). For the larger resection tissues, 3 sections of 10µm were dissected. For the smaller biopsy tissues, 5 sections of 10µm were dissected. DNA extraction was performed using the QIAamp® DNA FFPE tissue kit (56404; Qiagen) and the manufacturer's protocols. In brief, paraffin was removed from material using xylene and ethanol. Material was resuspended and digested with Proteinase K and then incubated at 90^{o}C in order to reverse the formalin cross-linking partially. The resulting DNA was then bound to silica-based membranes, and washed before elution. A Nanodrop spectrophotometer (Nanodrop® Technologies) was used to quantify the amount of DNA in the resulting solution for the cell line samples as well as the FFPE samples (µg/µl). The DNA concentration in the biopsy tissues ranged 6.1 to 139.1 ng/µl, compared to 9 to 958.1 ng/µl in the resection tissues (Appendix Section 2, Table S3). This was in comparison to DNA concentrations of 58.2 ng/µl in H929, 138.1ng/µl in MCF7, 193.6ng/µl in HL60, and 392.3ng/µl in HB2 cell lines.

3.12 Bisulphite conversion of extracted DNA

Bisulphite conversion of the extracted DNA was performed using the Epitect® bisulfite kit (59104; Qiagen) and the manufacturer's protocols. For bisulphite conversion of extracted DNA from FFPE tissue, 500ng of DNA was used where there was sufficient sample volume (n=71), otherwise the entire sample volume was used for bisulphite conversion (n=26). The latter strategy was mainly required for the core tissues (21/26 cases). There was sufficient sample volume to convert 500ng of DNA extracted from cell lines. In brief, DNA solutions containing 500ng of extracted DNA were added to "DNA protect" buffer, which prevents DNA fragmentation during the bisulphite treatment. The resulting solution was added to the bisulphite mix in ABgene low profile PCR tube strips (AB-0776; ThermoScientific). Bisulphite-mediated conversion of unmethylated cytosines was carried out using a Bio-Rad PTC-220 DNA engine Dyad™

Peltier thermal cycler. The thermal cycling program outlined below (Table 3.6) denatures DNA and facilitates sulfonation and cytosine deamination.

The converted single-stranded DNAs were bound to membranes of EpiTect spin columns, were desulfonated on the membranes, and were eluted in 20µl of nuclease free water. In the case of DNA from FFPE tissue, carrier RNA was added after the thermal cycling incubation in order to enhance binding of DNA to EpiTect spin-column membranes.

Step	Time	Temperature
Denaturation 1	5 minutes	95^{o}C
Incubation 1	25 minutes	60^{o}C
Denaturation 2	5 minutes	95^{o}C
Incubation 2	85 minutes	60^{o}C
Denaturation 3	5 minutes	95^{o}C
Incubation 3	175 minutes	60^{o}C
Hold	Indefinite	20^{o}C

Table 3.6: Thermal cycler condition for bisulphite conversion of extracted DNA

3.13 Amplification of target DNA after bisulphite conversion

Two sets of primers were used targeting specific regions of the BCRP (*ABCG2*) promoter, as described in previous studies in myeloma (Turner *et al*., 2006) and pancreatic cancer cell lines (Chen *et al*., 2012a). The primers from the former study

have been designated as Turner *et al* and the latter primers designated as Chen *et al* (Table 3.7).

Turner *et al* ABCG2 forward	5'-GGATAATATTAGGTAAGGTTGAGTAA-3'
Turner *et al* ABCG2 reverse	5'-TCAAAATAACTCCCTCCAAACAAAAC-3'
Chen *et al* ABCG2 forward	5'-AATGAGYGTTTGGTGATTTT-3'
Chen *et al* ABCG2 reverse	5'-ATTTCCCCAAATCRAAATTC-3'

Table 3.7: Primers for BCRP promoter regions

5µl out of total of 20µl of the bisulphite-modified DNA was added to a mixture (35µl) containing 8µl GoTaq flexi buffer (M890A; Promega), 4µl of 10mM dNTPs (U151; Promega), 0.5µl each of the 100µM forward and reverse primers (Sigma-Aldrich), 0.5µl of GoTaq Hot Start polymerase (M500B; Promega), 2µl of 25M $MgCl_2$ (A351H; Promega), and 19.5µl of nuclease free water. The following thermal cycling program was used for amplification (Table 3.8). Gradient PCR was performed for each set of primers to determine optimal annealing temperature (54.7^{o}C to 61.4^{o}C) for PCR: 56^{o}C for the Turner *et al* primer and 55^{o}C for the Chen *et al* primer (Appendix Section 3, Figs S5 and S6). These conditions were used for subsequent PCR on all cell lines which confirmed the optimal conditions (Appendix Section 3, Fig S7).

1	Denaturation	95^{o}C	3 minutes
2	Denaturation	95^{o}C	30 seconds
3	Annealing	56^{o}C for the Turner *et al* primer and 55^{o}C for the Chen *et al* primer	30 seconds
4	Extension	72^{o}C	30 seconds
5	Repeat	Step 2 to 4 were repeated 34 times	
6	Extension	72^{o}C	45 seconds
7	Hold	10^{o}C	Indefinitely

Table 3.8: PCR thermal cycler conditions used to amplify BCRP promoter region sequences

10µl of the PCR product was subsequently analysed by gel electrophoresis to confirm the amplification of the correct sized product. 1% agarose / TAE gels, containing ethidium bromide, were cast in gel trays containing 20 well combs and allowed to set at room temperature (30 min). Gels were submerged in an electrophoresis system (Sub-cell GT cell®; Bio-Rad) filled with TAE buffer. Each sample was mixed with 6x Loading Dye (#R 0611; Fermentas), and loaded into the wells. The first well was reserved for the DNA ladder (#SM1331; Fermentas), which contains markers ranging from 75 to 20,000 base pairs. DNA was visualised using the Bio-Rad Gel Doc Imaging system, and Image Lab™ software.

3.14 Molecular cloning and sequence analysis of promoter products

The remaining 30µl of the PCR product was separated from residual primers by gel electrophoresis and purified from the gel using a Zymoclean™ gel DNA recovery kit and the manufacturer's protocol (D4001; Zymo Research). Gel electrophoresis was performed as stated in the previous section with the exception of using 10µl of

GelGreenTM nucleic acid gel stain (41005; Biotium) instead of ethidium bromide. Minimal ultraviolet light exposure enabled visualisation of the DNA fragments, which were excised from gel with sterile surgical scalpel blades and transferred into 1.5ml microcentrifuge tubes. Agarose dissolving buffer was used to ensure full dissolution of the gel. The resulting solutions were placed into spin columns to enable DNA binding to membrane, and elution of the purified DNA was achieved after washing with 70% ethanol. The resulting DNA was used for both direct Sanger sequencing and molecular cloning followed by sequencing. The resulting sequences were aligned to a reference bisulphite-converted sequence (Fig 3.3).

5'AATGAGCGTTTGGTGATTTTCGTAGTTAATTATTTTGGTTTATTTCGTTCGATTTC
GGAGGCGGGAGTGTTTGGTTTGTTTTTGCGTGTTACGGTAGGGTGATTTTAGTTT
CGAGGGAGGGCGGTGGTATTAGTTTTGTTGGCGGTTTAGCGCGGTAGGATACGT
GTGCGTTTTTAGTCGGGTCGTAGGGCGTTTATCGCGGTTCGGTAGTCGGGGTTAC
GTTTTATTTTCGTTCGCGAATTTCGATTTGGGGAAAT3'

Figure 3.3: Bisulphite-converted reference sequence for the Chen *et al* amplicon. The resulting amplicon contains 257 base pairs and 27 CpG sites which are highlighted in green, assuming all CpG sites are methylated. The primer sequences are underlined.

Molecular cloning was performed using the TA Cloning® kit with pCR™2.1 vector (K2020-40; Invitrogen) and the manufacturer's protocol. Ligation reactions consisted of PCR product (sufficient to achieve 1:1 vector:insert ratio), ligation buffer, pCR™2.1 vector, and DNA ligase in a total volume of 10µl and were incubated at 14^{o}C for 16 hours. MAX Efficiency® DH10B™ competent *E. Coli* cells (18297-010; Invitrogen) were transformed by the addition of ligated DNA, heat shocking the cells for 45 seconds at 43^{o}C, and subsequent addition of the S.O.C. medium. The resulting mixture was incubated at 37^{o}C for 1 hour and spread onto the Lysogeny Broth (LB) agar plates

containing ampicillin for 16 hour incubation at 37^{o}C. Colonies were screened by colony PCR. M13 forward (5´-GTAAAACGACGGCCAG-3´) and reverse (5´-CAGGAAACAGCTATGAC-3´) primers were used to enable amplification of insert. 20µl reactions contained 4µl of GoTaq flexi buffer, 2µl of 10mM dNTPs, 0.25µl of the forward and reverse 100µM M13 primer, 0.25µl of GoTaq Hot Start polymerase, 1µl of 25M $MgCl_2$, and 9.75µl of nuclease free water. Each colony was harvested using a fine pipette tip, dipped into the PCR reaction, and subsequently dipped into a 15ml centrifuge tube (430790; Corning®) containing 5ml of ampicillin-treated LB solution. The following thermal cycling program was used for colony PCR (Table 3.9).

1	Denaturation	98^{o}C	3 minutes
2	Denaturation	94^{o}C	30 seconds
3	Annealing	60^{o}C	30 seconds
4	Extension	72^{o}C	30 seconds
5	Repeat	Step 2 to 4 were repeated 29 times	
6	Extension	72^{o}C	10 minutes
7	Hold	10^{o}C	Indefinitely

Table 3.9: Thermal cycler conditions used for colony PCR

Gel electrophoresis was performed as stated in section 3.13. The PCR products were visualised, and colonies selected that showed product sizes representative of plasmids containing an appropriate plasmid insert (Appendix Section 3, Figs S8 to S11). The 5ml cultures of these specific colonies were incubated at 37^{o}C for 16 hours in a shaker. Minipreps were performed using the Qiagen plasmid buffer set. Bacterial cultures were

centrifuged and the supernatant discarded. Bacteria were re-suspended in P1 re-suspension buffer (1047015; Qiagen) and lysed by the addition of P2 lysis buffer (1014935; Qiagen). Cell debris was precipitated by addition of P3 neutralisation buffer (1014958; Qiagen). The resulting mixture was centrifuged, and the DNA in the supernatant was precipitated by addition of propan-2-ol. The DNA was pelleted and the pellet washed in 70% ethanol and then re-suspended in 50µl of nuclease free water. The resulting DNA was diluted to 500ng/µl for Sanger sequencing (Source BioScience plc, Nottingham, United Kingdom). The resulting sequences from individual clones were successfully aligned to the reference sequence using ApE software (v2.0.32; copyright © 2003-2009 by M. Wayne Davis). Methylation analysis was performed using CpGviewer software (http://dna.leeds.ac.uk/cpgviewer/) (Carr *et al*., 2007) designed by Dr Ian Carr (Leeds Institutes of Molecular Medicine, University of Leeds) and checked manually.

3.15 Pyrosequencing

In pyrosequencing (Doyle *et al.*, 2011), nucleotide incorporation during the synthesis of a complementary strand results in the release of pyrophosphate, which subsequently generates ATP. This provides the energy for the luciferase-mediated conversion of luciferin to oxyluciferin. This chemical reaction generates visible light, where the degree of luminescence is proportional to the number of incorporated nucleotides. The four nucleotides are added sequentially to enable base calling and provides quantitative analysis of each CpG sites. Pyrosequencing can perform direct sequencing of bisulphite-modified DNA and examine multiple CpG sites. Hence, it is ideal for examining methylation status of clinical samples and has been used extensively using FFPE tissues (van Bemmel *et al.*, 2012, Christians *et al*., 2012, Tuononen *et al.*, 2012, Baba *et al.*, 2010). PyroMark CpG assay was used to examine 5 CpG sites (Fig 3.4) within the Chen *et al* amplicon (PM00111321; Hs_ABCG2_01_PM PyroMark CPG

assay, Qiagen). This assay was performed by Dr Philip Chambers (Leeds Cancer Research UK Centre, Genomics Facility) to determine if pyrosequencing analysis mirrored the methylation analysis from the cloned sequences.

5'AATGAGCGTTTGGTGATTTTCGTAGTTAATTATTTTGGTTTATTTCGTTCGATTTC
GGAGGCGGGAGTGTTTGGTTTGTTTTTGCGTGTTACGGTAGGGTGATTTTAGTTT
CGAGGGAGGGCGGTGGTATTAGTTTTGTTGGCGGTTTAGCGCGGTAGGATACGT
GTGCGTTTTTAGTCGGGTCGTAGGGCGTTTATCGCGGTTCGGTAGTCGGGGTTAC
GTTTTATTTTCGTTCGCGAATTTCGATTTGGGGAAAT3'

Figure 3.4: Bisulphite-converted reference sequence for the Chen *et al* amplicon highlighting the sites examined for pyrosequencing. The resulting amplicon contains 257 base pairs and 27 CpG sites which are highlighted in green, assuming all CpG sites are methylated. The primer sequences are underlined. The five CpG sites examined by pyrosequencing are highlighted in red.

This approach resulted in successful methylation analysis for the cell line samples which mirrored the cloned sequences, but not for the FFPE samples (Appendix Section 3, Figs S12 and S13), perhaps reflecting the amplicon size of 228 base pairs. Therefore, alternative primer sets were designed using PyroMark Assay Design SW 2.0 (Qiagen) by Dr Philip Chambers (Leeds Cancer Research UK Centre, Genomics Facility) (Fig 3.5) with an aim to design primers with an amplicon size of less than 150 base pairs (Patterson *et al.*, 2011). This resulted in 2 assays which examined 2 CpG sites each. The designed assays resulted in amplicons of 62 and 144 base pairs.

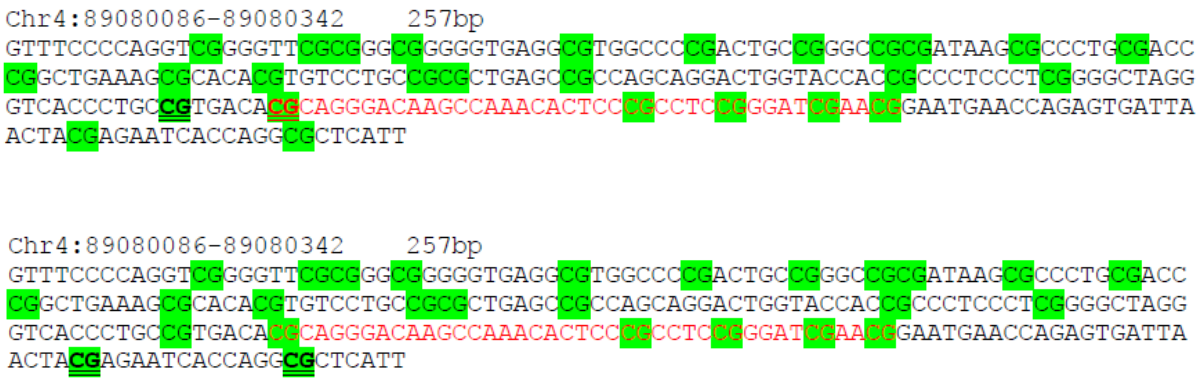


Figure 3.5: Two sets of primers designed for pyrosequencing assay. The 257 base pair sequence is the amplicon of the Chen *et al* primers. The CpG sites are highlighted in green. The 5 CpG sites previously examined using the PyroMark assay is highlighted in red. Two sets of primers were designed to amplify a 62 (top row of sequences) and 144 (bottom row of sequences) base pair products analysing two CpG sites each (highlighted in bold and double underlined). The 4 CpG sites have been labelled as position 165 and 172 on the top row of sequences, and as position 236 and 250 on the bottom row of sequences. The cloned sequences were examined to ensure that 4 CpG sites selected were representative of the whole region.

3.16 Statistical analysis

Statistical analyses were performed using SPSS v16.0 (SPSS, Chicago, USA), GraphPad Prism v6.0 (GraphPad, California, USA), and MedCalc v12.4.0 (MedCalc software, Ostend, Belgium).

4.0. Neoadjuvant chemotherapy induces expression levels of Breast Cancer Resistance Protein that predict disease-free survival in breast cancer

4.1. Abstract

My aim in this chapter was to investigate tumour expression of Pgp, MRP1, and BCRP before and after neoadjuvant chemotherapy (NAC) to determine whether levels define response to NAC or subsequent survival. The NAC regime consisted of anthracyclines with or without taxanes. Paraffin embedded tissue was collected representing matched pairs of core biopsy (pre-NAC) and resection specimens (post-NAC) from 45 patients with invasive ductal carcinomas, and expression of the three markers was examined using immunohistochemistry. A computer-aided scoring protocol was developed and validated against 2 independent observers (intra-class correlation coefficients 0.83 and 0.82). Pgp and MRP1 expressions were significantly up-regulated after exposure to NAC ($p=0.0024$ and $p<0.0001$; Wilcoxon signed-rank test). BCRP expression showed more variation in response: cases showed either down- (41%) or up-regulation (59%) after NAC, but no overall significant difference was observed. Pre- or post-NAC expression of Pgp, MRP1 or BCRP did not correlate with clinical (MRI-determined) response to NAC. Pre-NAC expression of all three markers, and post-NAC expression of Pgp and MRP1 did not correlate with disease free survival. However, high post-NAC BCRP expression independently predicted for poorer disease free survival (hazard ratio of 4.04; 95% confidence interval 1.3-12.2; $p=0.013$). Therefore, NAC-induced BCRP expression has potential value in predicting survival in breast cancer patients treated with NAC, whilst Pgp and MRP1 expressions have little predictive value.

4.2. Introduction

Given the known function of the xenobiotic transporters, it is reasonable to expect that high expression of them within tumour cells may contribute to relative chemotherapy resistance, which may be reflected in poor responses to therapy. In the context of NAC, both pre- and post-NAC expression levels can be assessed, as tissue from both pre-treatment biopsies and post-treatment resections would potentially be available. Either of these measures, or the change in expression levels during treatment, may be informative in terms of predicting response to treatment or subsequent survival. Given the overlap in substrate specificities of the xenobiotic transporters, analyses of the relative expressions of multiple xenobiotic transporters is likely to be more informative than individual analyses. In this study, I aimed to determine whether Pgp, MRP1, or BCRP expression pre-NAC or post-NAC has a predictive role, whether their expression is altered by NAC, and whether any change in expression during treatment has clinical relevance. Together, these proteins can efflux more than 80% of chemotherapeutic agents that are currently in use (Kuo, 2007).

Only six studies, involving a total of 176 patients, have utilised tissues from patients treated with NAC to study the relevance of xenobiotic transporter protein expression in breast cancer (Leonessa and Clarke, 2003). However, most of these immunohistochemistry studies have focused on Pgp expression only, and examined whether Pgp expression is induced by NAC. Combined analyses of these studies show that Pgp expression was detected pre-NAC by immunohistochemistry in 42.9% of patients, compared to 63.9% post-NAC, suggesting an NAC-dependent increase in expression. For example, 36.8% of the cases where Pgp expression was initially negative were positive following NAC (Leonessa and Clarke, 2003). This suggests that Pgp could potentially contribute to acquired chemotherapy resistance. Since then, further studies have examined combined expression of xenobiotic transporters in

breast cancer patients treated with NAC. A notable study by Rudas *et al* showed that Pgp and MRP1 expression were both significantly up-regulated after exposure to NAC (Rudas *et al*., 2003), and the absence of pre-NAC MRP1 expression predicted for longer DFS. However, the cohort used for this study was heterogeneous, including both patients with ductal carcinomas and those with lobular carcinomas. This is important since published data suggest that lobular carcinomas respond poorly to NAC, and that the breast conserving surgery rate in this group is not increased after NAC (Boughey *et al*., 2009), therefore the results in Rudas *et al* may combine differential responses in the ductal and lobular groups. Tanei *et al* found BCRP expression, but not Pgp expression, was up-regulated after exposure to NAC (Tanei *et al*., 2011). No correlation to clinicopathological parameters were detected for Pgp or BCRP expression. The NAC regimes in the Tanei *et al* study involved 12 cycles of paclitaxel, followed by 4 cycles of fluouracil, epirubicin, and cyclophosphamide and therefore comparisons to typical current NAC regimen of 6 cycles only are difficult. No previous studies have examined Pgp, MRP1, and BCRP together in breast cancer patients treated with NAC. More importantly, previous studies were not designed to limit tumour heterogeneity in terms of cohort selection.

Breast cancer recurrences are typically distant metastases and it is these that are responsible for subsequent cancer-related deaths (Redig and McAllister, 2013). If expression levels of xenobiotic transporters within the primary tumour reflect survival, it can be assumed that this is because these levels also reflect the levels in metastatic deposits. Occult distant metastatic cells are not available to test this assumption, since metastases are by definition sub-clinical in these patients with primary cancers, and even in patients with metastatic recurrences samples are typically not available since distant metastases of breast cancers are seldom biopsied or resected. However, the resected axillary lymph nodes at the time of surgery can potentially be examined, which

may reflect the xenobiotic transporter expression in metastatic deposits. This approach has been used previously to examine Pgp and MRP1 expression, but not BCRP expression, in the axillary lymph nodes of breast cancer patients (Zochbauer-Muller *et al*., 2001). Pgp and MRP1 expression was determined by immunohistochemistry using a cohort of 32 patients. The authors determined that Pgp expression was lower in the lymph nodes compared to the corresponding primary tumours, but MRP1 expression was higher in the lymph nodes compared to the corresponding primary tumours. However, patients were not treated with NAC. Lymph node tissue sampling pre-NAC is frequently performed by fine needle aspiration yielding cytological material rather than the histopathological material available after core biopsies or surgical resections. As a result, no lymph node tissue samples were available for analysis pre-NAC. This study will examine whether xenobiotic transporter expression in the lymph nodes post-NAC reflect the equivalent expression in the matching primary tumour sample post-NAC.

4.3 Results

4.3.1 Expression of Pgp and MRP1 were significantly up-regulated after NAC, but BCRP expression responded more variably

I aimed to determine expression levels of Pgp, MRP1, and BCRP both pre- and post-treatment in the tumours of a cohort of breast cancer patients treated with NAC. A cohort of 39 patients was assembled who had matching pre-NAC core biopsy tissues and post-NAC resection tissues, and a further 6 patients with pre-NAC core biopsy tissues but no matched post-NAC tumour material as these individuals had complete pathological responses (Table 3.1). Protein expression was determined using immunohistochemistry and quantified using semi-automated histoscores. As described in the methods (Section 3.6), quantification of expression was initially performed

manually, and then digitally using the validated semi-automated scoring protocol. This enabled determination of protein expression for the 45 core tissues and 39 matched resections for Pgp, MRP1, and BCRP (full data set: Table S4). The three proteins mainly displayed cytoplasmic/membrane staining pattern that was specific to epithelial cells, with some accentuation of cell nuclei. Representative staining patterns are illustrated in Figure 4.1 and quantified staining levels are shown in Figure 4.2.

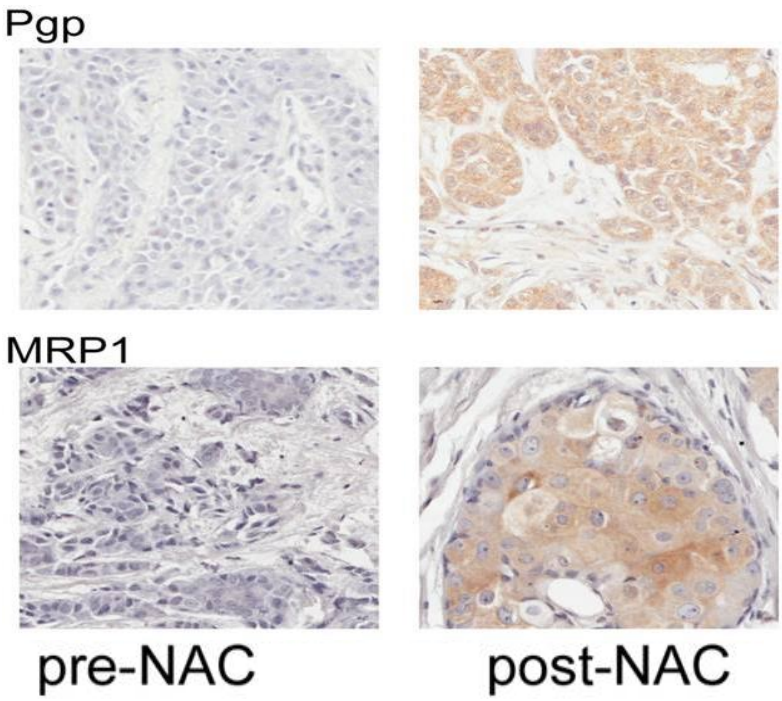


Figure 4.1a: Representative immunohistochemistry images for Pgp and MRP1 (x20 magnification) in matched pre-NAC (left) and post-NAC (right) breast cancer samples. In both cases, very little/no staining is visible in the core tissues (pre-NAC), as opposed to positive staining in the resection tissues (post-NAC). These images represent the significant up-regulation in Pgp and MRP1 expression after exposure to NAC (Kim *et al*., 2013).

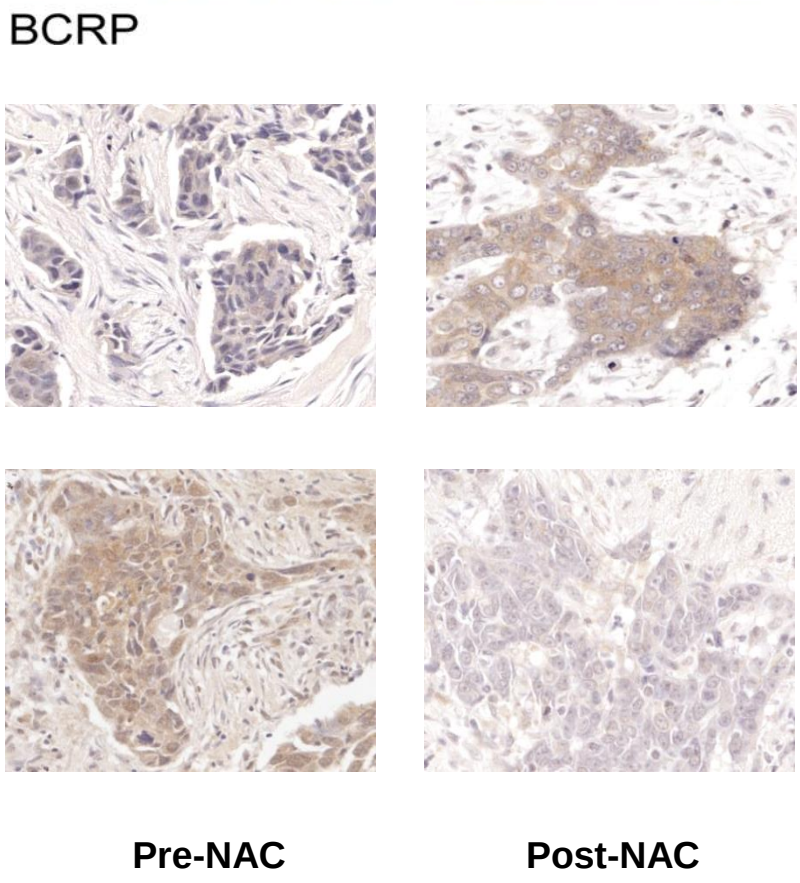


Figure 4.1b: Representative immunohistochemistry images for BCRP (x20 magnification) in matched pre-NAC (left) and post-NAC (right) breast cancer samples from two individuals (top and bottom). Different individuals showed weak (top) or strong (bottom) staining pre-NAC. The top image shows up-regulation in BCRP expression post-NAC, whereas the bottom image shows down-regulation in BCRP expression (Kim *et al*., 2013).

Pre-NAC expression was variable for Pgp (histoscores range: <0.1-161.7) and BCRP (histoscores range: 0.2-143.5), compared to MRP1 expression, which was very low in almost all core biopsy samples (histoscores range: <0.1-35.4). Post-NAC expression

was variable for all three proteins; Pgp (histoscores range: 0.2-171.4), MRP1 (histoscores range: 0.2-202.9), and BCRP (histoscores range: 4.3-161.3).

Pgp and MRP1 expressions were frequently up-regulated post-NAC (Figures 4.1a and 4.2a), but changes in BCRP expression were more variable (Figures 4.1b and 4.2a), showing frequent up-regulation or down-regulation. Up-regulation was observed in 29/39 cases for Pgp (74%), 36/39 cases for MRP1 (92%), and 23/39 cases for BCRP (59%) (Figure 4.2a). Significant up-regulation was observed for Pgp and MRP1 (Wilcoxon signed-rank tests: Pgp, p=0.0024; MRP1, p<0.0001) (Figure 4.2b). The up-regulation seen in for MRP1 was particularly striking, with very low expression seen in the core tissues in almost all the cases. However, a significant up- or down-regulation was not observed for BCRP (Fig 4.2b).

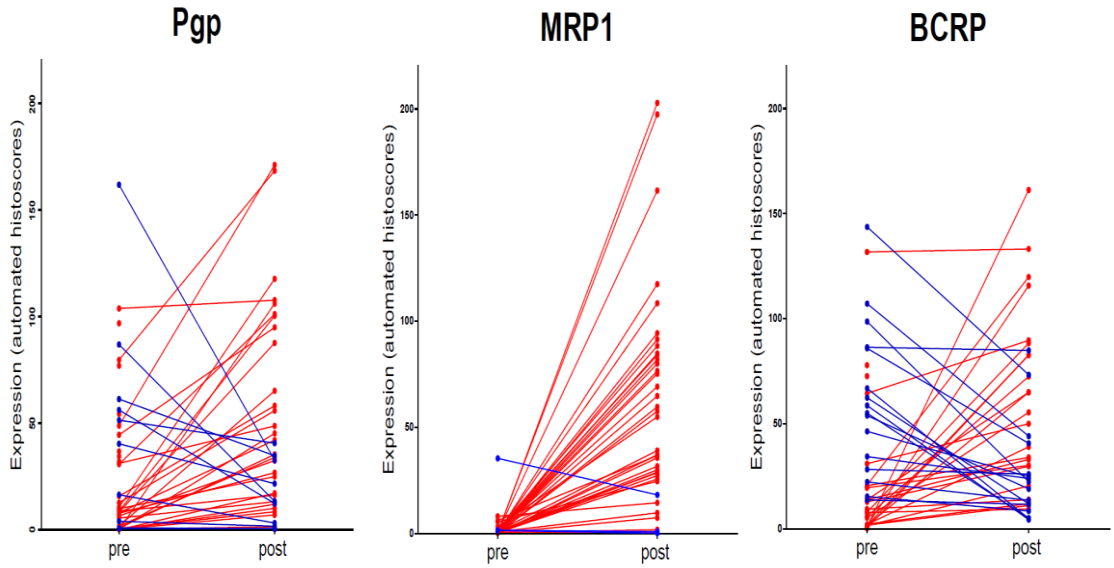


Figure 4.2a: Pgp and MRP1 were up-regulated whilst BCRP responded variably (red: up-regulation, blue: down-regulation). Matched pre-NAC and post-NAC breast cancer samples were stained using immunohistochemistry for Pgp, MRP1 and BCRP. Expression within tumour cells was quantified as histoscores of 0 – 300. Expression levels in matched samples are linked by lines coloured red or blue so as to indicate an increase or decrease in expression respectively.

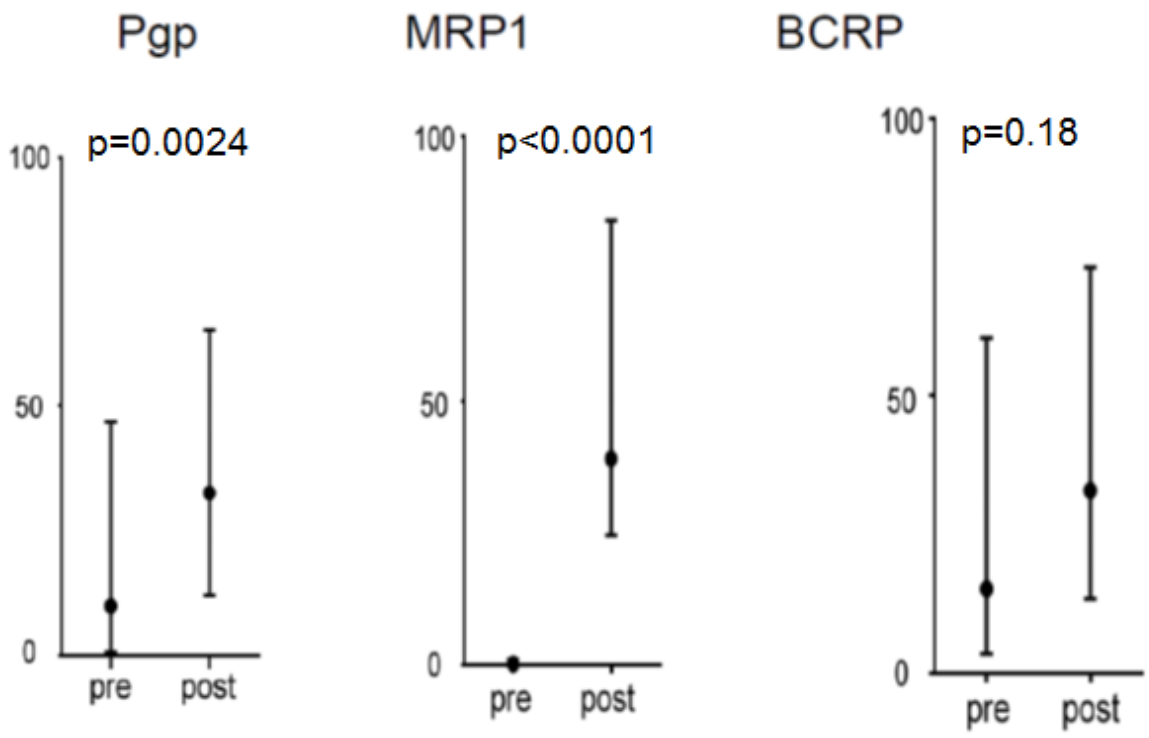


Figure 4.2b: Pgp and MRP1, but not BCRP, were significantly up-regulated by NAC. Pre- and post-NAC expressions of Pgp, MRP1, and BCRP are shown as median histoscore values with interquartile range; n=39 (pre-NAC samples lacking matched post-NAC samples were excluded). Statistically significant up-regulation was observed for Pgp and MRP1 ($p=0.0024$ and $p<0.0001$ respectively; Wilcoxon signed-rank tests). No statistically significant up-regulation was observed for BCRP ($p=0.18$; Wilcoxon signed-rank test).

4.3.2 Post-NAC expression of Pgp, MRP1, and BCRP in the axillary lymph nodes reflects expression of the primary tumours post-NAC

If post-NAC expression levels of these transporters in the primary tumours were to be of relevance for cancer outcomes in terms of likelihood of metastatic recurrences and therefore disease-specific survival, this would suggest that primary tumour expression could be representative of expression in metastatic cells. In order to test this

hypothesis, I aimed to compare the expression of Pgp, MRP1, and BCRP in tumour cells in the axillary lymph nodes, when available, to the corresponding primary breast cancer tissue expression post-NAC. 13 lymph node blocks were identified, and immunohistochemistry was performed for the three proteins using same experimental conditions as for the primary breast cancer tissues. As previously, the semi-automated scoring protocol was used to quantify expression. Representative staining patterns are shown in Figure 4.3, demonstrating epithelial-specific staining pattern that is mainly cytoplasmic/membraneous.

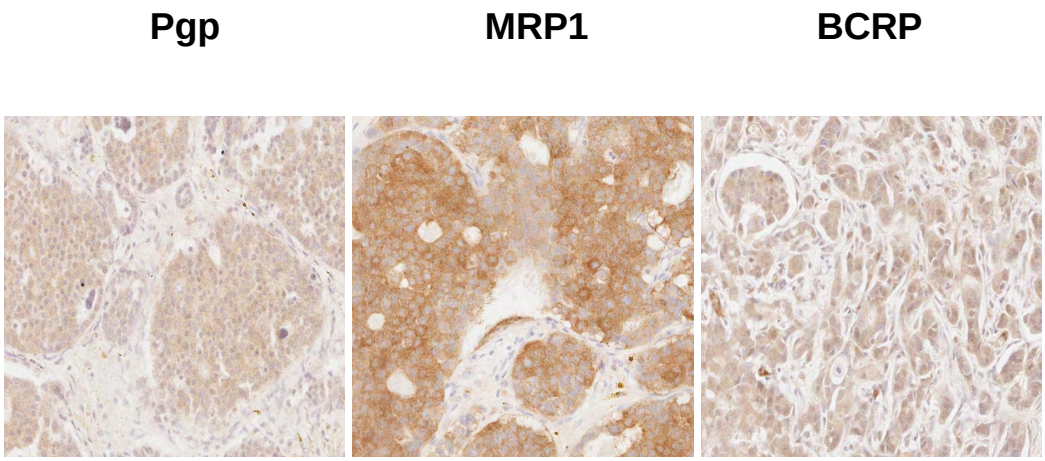


Figure 4.3: Pgp, MRP1, and BCRP expression in axillary lymph nodes. Post-NAC axillary lymph node tissues were stained using the same immunohistochemistry protocol as per primary tumour. The images show positive staining in tumour cells with weak or no staining in the surrounding stroma.

Post-NAC expression in the axillary lymph nodes for the 13 patients were highly variable for Pgp (histoscores range: 21.6-108.7), MRP1 (histoscores range: 0.5-167.1), and BCRP (histoscores range: 3.6-105.7). This variability was similar to that seen in post-NAC in the primary tumours of the same 13 patients; Pgp (histoscores range:

10.1-168.7), MRP1 (histoscores range: 0.9-197.5), and BCRP (histoscores range: 4.3-119.9). There were no statistically significant differences between the lymph node expression and the primary tumour expression post-NAC (Fig 4.4) (Mann-Whitney test: Pgp, p=0.57; MRP1, p=0.75; BCRP, p=0.26). This suggests that post-NAC primary tumour xenobiotic transporter expression is potentially reflective of that in metastatic deposits. However, no statistically significant correlation was found when post-NAC primary tumour expressions were compared to the matching lymph node expressions for Pgp, MRP1, and BCRP using Spearman's rho analyses (Fig 4.5; Pgp, rho=0.007, p=0.99; MRP1, rho=0.13, p=0.68; BCRP, rho=0, p>0.99).

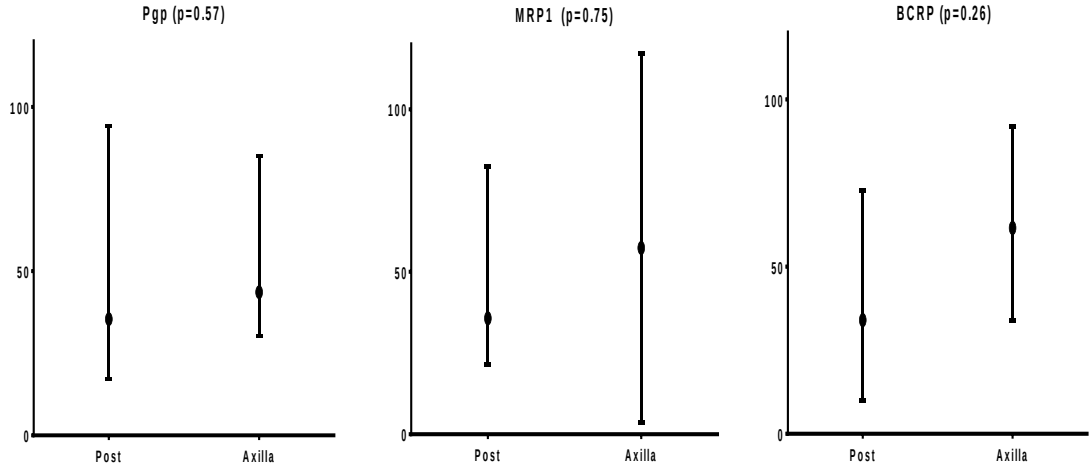


Figure 4.4: Comparison of xenobiotic transporter expression in the axillary lymph nodes and the corresponding primary tumour post-NAC (median histoscore values with interquartile range). There were no statistically significant differences between the lymph node expression and the primary tumour expression post-NAC (Mann-Whitney test: Pgp, p=0.57; MRP1, p=0.75; BCRP, p=0.26).

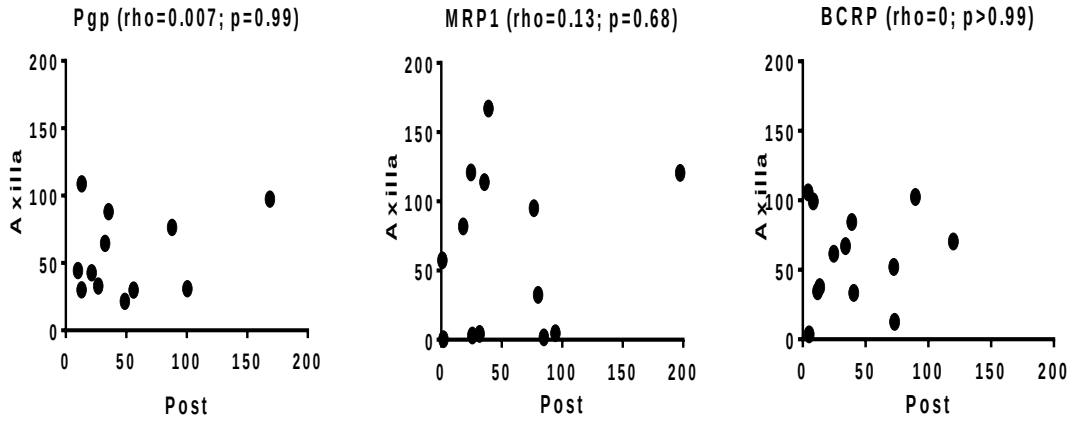


Figure 4.5: Correlation of post-NAC lymph node expression with corresponding post-NAC primary tumour expression. Protein expressions quantified by histoscores are shown on the y (post-NAC axilla) and x axis (matched post-NAC primary tumour) for Pgp, MRP1, and BCRP. No significant correlations were seen, which also remained the case when all three markers were examined together.

4.3.3 Analyses of correlations between expression of Pgp, MRP1, and BCRP

Next, I wished to examine whether expression levels of Pgp, MRP1 or BCRP either pre- or post-NAC, or the changes in expression of these markers between pre- and post-NAC, were related to each other. This would potentially reveal evidence of co-regulation of these transporters. To compare the change in protein expression after exposure to NAC, pre-NAC expression was subtracted from matching post-NAC expression and positive values were assigned as up-regulation, whereas negative values were assigned as down-regulation.

I analysed these relationships using Spearman's rho analyses (Table 4.1). The pre- and post-NAC levels showed some significant correlations to the changes in expression levels of the same proteins, as would be expected since these values are

directly related. In addition, for Pgp the post-NAC level positively correlated with the pre-NAC level, suggesting that post-NAC expression depends to some extent on the initial level. The changes in expression levels of the proteins were not related to each other, providing no evidence of co-regulation or mutually exclusive induction. Interestingly a significant and moderately strong negative correlation was seen between the pre-NAC Pgp expression and the change in BCRP expression (rho coefficient -0.5; p=0.01), as well as the post-NAC BCRP expression (rho coefficient -0.34; p=0.03).

	Pgp pre n=45	Pgp post n=39	Pgp Δ n=39	MRP1 pre n=45	MRP1 post n=39	MRP1 Δ n=39	BCRP pre n=45	BCRP post n=39	BCRP Δ n=39
Pgp pre n=45	-								
Pgp post n=39	**0.44 (0.005)**	-							
Pgp Δ n=39	-0.15 (0.36)	**0.75 (<0.01)**	-						
MRP1 pre n=45	0.09 (0.56)	0.07 (0.67)	0.11 (0.5)	-					
MRP1 post n=39	-0.11 (0.51)	-0.29 (0.078)	-0.15 (0.37)	-0.2 (0.23)	-				
MRP1 Δ n=39	-0.12 (0.47)	-0.28 (0.079)	-0.28 (0.08)	-0.25 (0.13)	**0.99 (<0.01)**	-			
BCRP pre n=45	0.27 (0.071)	0.07 (0.69)	-0.13 (0.43)	0.24 (0.11)	-0.03 (0.88)	-0.05 (0.78)	-		
BCRP post n=39	**-0.34 (0.032)**	-0.16 (0.35)	0.05 (0.75)	-0.17 (0.29)	0.14 (0.40)	0.14 (0.39)	-0.12 (0.46)	-	
BCRP Δ n=39	**-0.5 (0.01)**	-0.15 (0.37)	0.16 (0.32)	-0.15 (0.36)	0.12 (0.48)	0.13 (0.42)	**-0.77 (<0.01)**	**0.63 (<0.01)**	-

Table 4.1: Spearman's correlation coefficients demonstrating relationships between expression pre-NAC or post-NAC, or change in expression (Δ) for Pgp, MRP1 and BCRP (p values are denoted in brackets).

4.3.4 Do pre-NAC Pgp, MRP1, and BCRP expression levels predict complete pathological response?

Next, I examined whether pre-NAC expression levels predicted whether patients would achieve pCR. There were 6 patients with pCRs leaving 39 in the non-pCR group. There were no significant differences in the MRP1 and BCRP expression levels between the pCR group versus the non-pCR group (pCR vs non-pCR, MRP1: medians 0.1 vs 0.4, p=0.4; BCRP: medians 6.35 vs 19.5, p=0.43). However, for Pgp, the expression level in the pCR group (median score 45.7) was significantly higher compared to the non-pCR group (median score 8.3, Mann-Whitney U test: p=0.013) (see discussion below for comments on this surprising finding).

4.3.5 Correlation of xenobiotic transporter expression with clinico-pathological parameters

My next aim was to analyse potential correlations between pre- and post-NAC xenobiotic transporter expression levels as well as the change in expression levels, and the clinico-pathological parameters (outlined in Table 3.1) using Spearman's rho analyses (Table 4.2). Due to the large number of parameters being tested, a more strict threshold of $p<0.01$ was used to indicate statistical significance (Cleophas and Zwinderman, 2006). This is a more pragmatic approach to reduce the risk of type I error, which is the risk of parameters achieving significance of commonly stated p value of less than 0.05 by chance due to the large number of parameters being tested. This approach of using a lower p value has been adopted in a number of studies (Kim *et al*., 2013, Morenos *et al.*, 2014). An alternative approach would have been to adopt Bonferroni correction, which has potential limitations. For example, it is a stringent test with a conservative approach which has potential to overcorrect for type I error and thereby potentially lead to type II error (Pocock, 1997).

No significant correlations were observed for MRP1 and BCRP expression against the clinico-pathological parameters. Similar findings were observed for post-NAC Pgp expression, as well as the change in Pgp expression. However, a positive and weakly strong correlation between the pre-NAC Pgp expression and patient age (rho coefficient 0.41; p=0.005) was detected. Correlation coefficient values of 0.2, 0.5, and 0.8 were defined as cut-off values for categorising the strength of correlation as weak, moderate, and strong respectively (Zou *et al*., 2003).

	Pgp pre	Pgp post	Pgp Δ	MRP1 pre	MRP1 post	MRP1 Δ	BCRP pre	BCRP post	BCRP Δ
Age at diagnosis	**0.41 (0.005)**	0.31 (0.057)	0.04 (0.81)	0.1 (0.5)	-0.13 (0.45)	-0.13 (0.44)	0.08 (0.62)	-0.18 (0.25)	-0.33 (0.04)
Tumour factors determined pre-NAC:									
Grade	0.19 (0.21)	-0.16 (0.33)	-0.3 (0.06)	0.16 (0.28)	-0.02 (0.91)	0.01 (0.95)	-0.09 (0.54)	0.04 (0.81)	0.04 (0.82)
T Stage	0.06 (0.69)	-0.09 (0.57)	-0.13 (0.42)	-0.14 (0.36)	0.29 (0.07)	0.27 (0.1)	-0.26 (0.08)	-0.16 (0.34)	0.04 (0.82)
ER status	-0.08 (0.6)	-0.17 (0.29)	-0.12 (0.48)	0.24 (0.11)	0.13 (0.43)	0.11 (0.5)	0.18 (0.23)	-0.07 (0.7)	-0.06 (0.74)
Her2 status	-0.03 (0.85)	0.21 (0.19)	0.33 (0.04)	-0.18 (0.23)	0.01 (0.97)	0.01 (0.94)	0.01 (0.96)	-0.02 (0.91)	0.03 (0.86)
Tumour factors determined post-NAC:									
T stage	-0.36 (0.017)	-0.15 (0.38)	0.04 (0.83)	0.22 (0.15)	0.14 (0.39)	0.1 (0.55)	0.14 (0.38)	0.05 (0.79)	0.05 (0.78)
Lymphovascular invasion	-0.35 (0.41)	-0.1 (0.53)	0.04 (0.8)	0.04 (0.79)	0.23 (0.15)	0.24 (0.14)	0.17 (0.26)	-0.38 (0.02)	-0.23 (0.16)
Axillary metastasis	-0.07 (0.66)	0.19 (0.24)	0.3 (0.07)	0.38 (0.011)	-0.1 (0.53)	-0.11 (0.49)	0.36 (0.02)	-0.08 (0.62)	-0.26 (0.11)

Table 4.2: Spearman's correlation coefficients demonstrating relationships between expression pre-NAC or post-NAC, or change in expression (Δ) for Pgp, MRP1 and BCRP with clinico-pathological parameters (p values are denoted in brackets).

Expression levels and the change in expression levels were then examined against the response to NAC. The latter was defined in three separate ways: the change in tumour stage after NAC (TNM staging; see Section 1.2), qualitative response as assessed by

MRI scan, and as a quantitative change in tumour size derived from comparison of resection pathology to pre-NAC MRI scans (Table 4.3). No significant correlations were observed at the threshold of $p<0.01$.

	Pgp pre	Pgp post	Pgp Δ	MRP1 pre	MRP1 post	MRP 1 Δ	BCRP pre	BCRP post	BCRP Δ
Δ T stage	-0.25 (0.1)	-0.08 (0.63)	0.11 (0.52)	0.19 (0.22)	0.09 (0.57)	0.06 (0.72)	0.1 (0.53)	0.15 (0.38)	0.07 (0.69)
MRI response	0.2 (0.19)	0.07 (0.69)	0.004 (0.98)	-0.28 (0.06)	0.04 (0.8)	0.08 (0.62)	0.03 (0.85)	-0.09 (0.61)	-0.2 (0.23)
Δ in tumour size	-0.25 (0.1)	-0.16 (0.34)	-0.02 (0.92)	-0.003 (0.98)	0.15 (0.37)	0.16 (0.32)	0.17 (0.28)	0.37 (0.02)	0.21 (0.21)

Table 4.3: Spearman's correlation coefficients demonstrating relationships between expression pre-NAC or post-NAC, or change in expression (Δ) for Pgp, MRP1 and BCRP with tumour response (p values are denoted in brackets).

4.3.6 Disease free survival analysis of xenobiotic transporter expression

Next, expression levels and change in expression levels of the transporters were analysed for correlations with disease free survival (DFS), defined as survival post-surgery, using Kaplan-Meier survival analyses. Receiver Operating Characteristic (ROC) curve analysis was performed to dichotomise expression into high and low groups for both pre- and post-NAC levels. This was performed by comparing the presence or absence of disease recurrence against protein expression as quantified by histoscores. This generated a ROC curve with sensitivity value on the y-axis and one minus specificity value on the x-axis for each histoscore value. From this curve, the cut-off providing the highest combined sensitivity and specificity for prediction of DFS was selected (Fig 4.6). For the change in expression, we dichotomised into groups with up-regulation after NAC or with down-regulation.

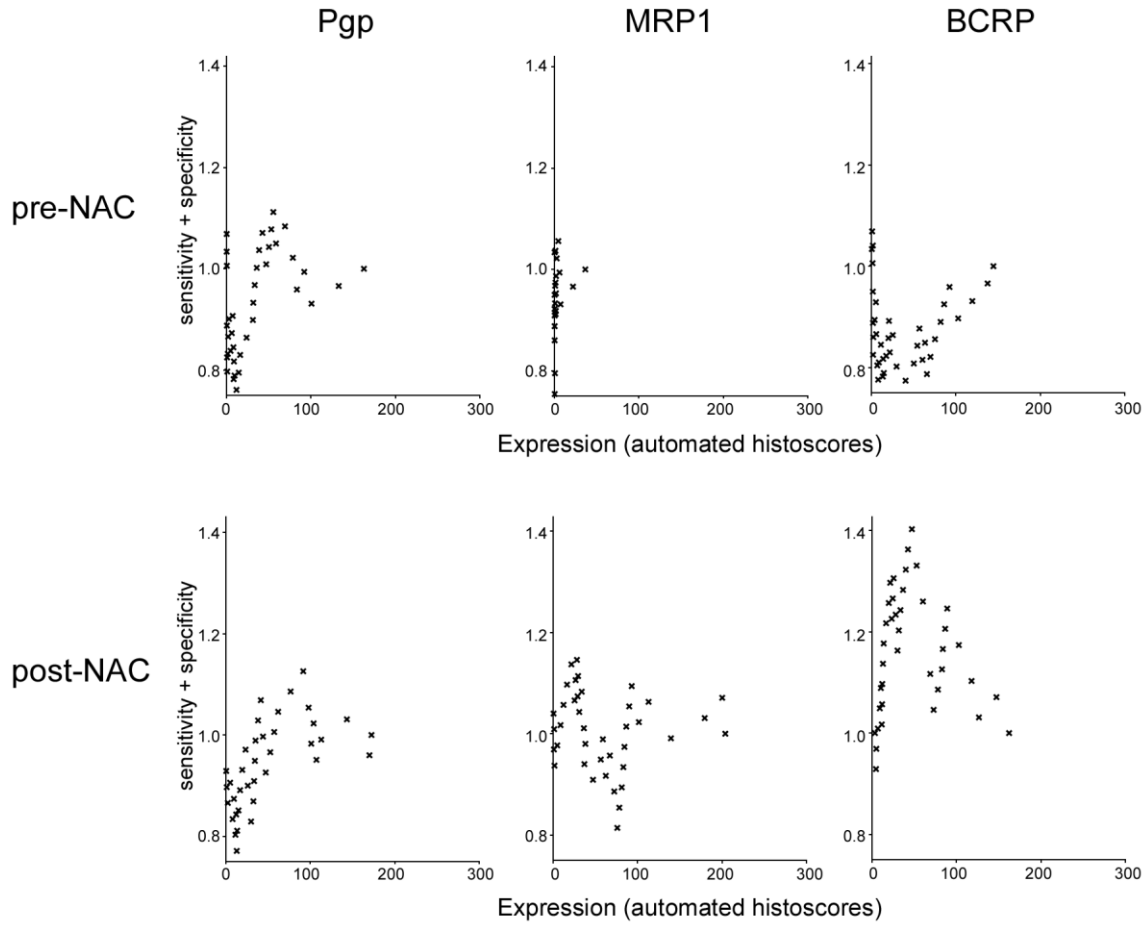


Figure 4.6: ROC curve analysis for Pgp, MRP1, and BCRP; for pre-NAC histoscores, the respective cut-offs were 55 for Pgp, 4 for MRP1, and 1 for BCRP. For post-NAC histoscores, the respective cut-offs were 90 for Pgp, 21 for MRP1, and 47 for BCRP.

There was no significant relationship between the DFS and the pre-NAC expression levels for any of the three transporters (Fig 4.7). Similarly, no significant relationship was observed between DFS and the change in expression levels (Fig 4.8). It is worth noting that down-regulation was a rare event for MRP1, occurring in only three patients none of whom suffered from recurrences, therefore this particular analysis is limited by the small numbers in this group.

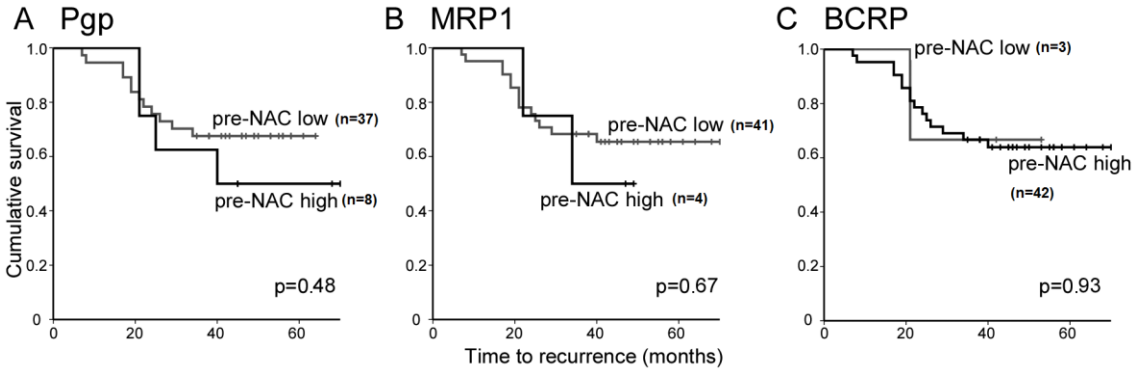


Figure 4.7: Kaplan-Meier survival analyses for pre-NAC xenobiotic transporter expression versus disease free survival.

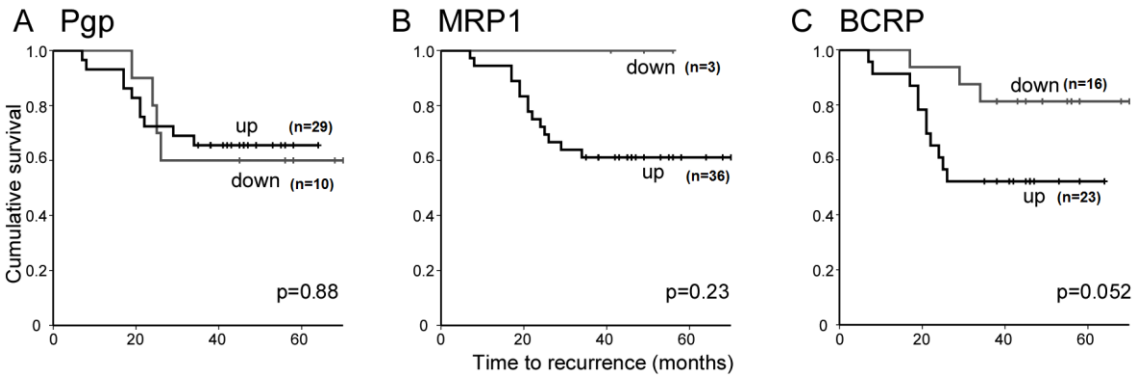


Figure 4.8: Kaplan-Meier survival analyses for the change in xenobiotic transporter expression versus disease free survival.

For post-NAC Pgp and MRP1 expression levels, no significant relationship was seen for DFS (Fig 4.9). For BCRP however, post-NAC expression was significantly correlated with DFS (Log rank: p=0.007). Patients with high BCRP expression had 5-year survival of 40%, compared to 80% for those with low BCRP expression (Fig 4.9). Multivariate Cox regression analysis was performed taking into account post-NAC BCRP expression and the pathological factors typically regarded as having prognostic impact, including tumour grade, receptor status, axillary metastasis, tumour stage, and lymphovascular invasion. This showed that only BCRP expression post-NAC independently predicted DFS, with high expression giving a hazard ratio of 4.04 (95% confidence interval, 1.3-12.2; p=0.013).

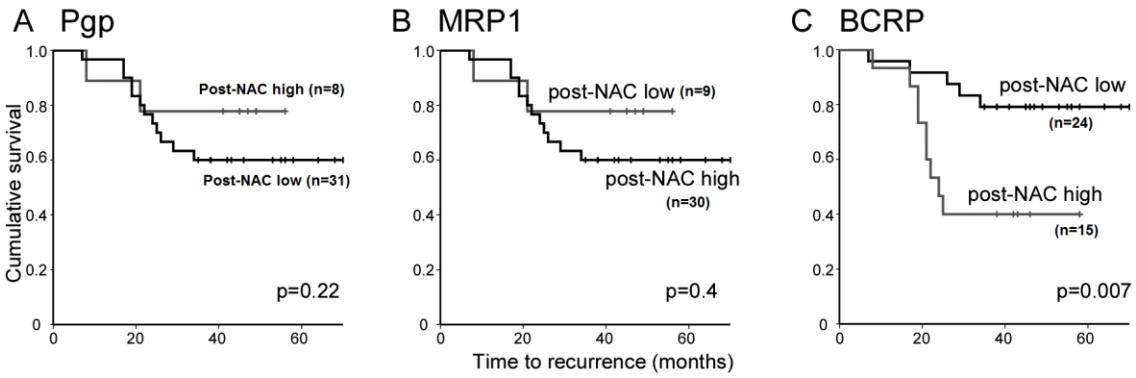


Figure 4.9: Kaplan-Meier survival analyses for post-NAC xenobiotic transporter expression versus disease free survival. No significant correlation was detected for Pgp and MRP1. However, high BCRP expression post-NAC significantly predicted for poorer disease free survival.

4.4 Discussion

Methodology

This is the first published study to examine Pgp, MRP1, and BCRP expression using immunohistochemistry in the context of breast cancer patients treated with NAC (Kim *et al*., 2013). Immunohistochemistry was used since protein expression is more likely to reflect the function and efflux activity of the transporters than analysis of transcript levels. Also, immunohistochemistry provides microanatomical details and hence tumour epithelium can be readily distinguished and examined specifically. However, subjectivity in quantifying protein expression can be a problem in interpreting results. Another potential method would have been to examine mRNA levels of Pgp, MRP1, and BCRP in clinical samples using RT-PCR. Although this would have potentially provided a more quantitative measure of expression, quantity and quality of nucleic acids extracted from FFPE tissues can be variable. Moreover, unless tumour cells are isolated by macrodissection or laser capture microscopy, results from RT-PCR need to

be interpreted with caution due to potential contamination by non-tumour cells. Burger *et al* examined mRNA levels of MDR1 (Pgp), MRP1, and BCRP using fresh frozen tumour resection tissues from 59 patients, where 30/59 went onto receive adjuvant chemotherapy. The authors found that MDR1 mRNA levels were inversely correlated with tumour response and progression free survival, but not MRP1 or BCRP mRNA levels. No active effort was made to isolate tumour cells in this study, and the survival analysis did not include Cox regression analysis performed in our study (Burger *et al*., 2003). A further study by Faneyte *et al* examined MRP1 mRNA expression in tissue samples of 30 breast cancer patients treated with NAC. However, the tissue availability was limited in their study with 23 samples available after neoadjuvant chemotherapy, and only 3 patients had matching pre- and post-NAC tissue samples available for analysis. They found that MRP1 mRNA expression did not correlate with clinico-pathological parameters or patient survival (Faneyte *et al*., 2004). Furthermore, as eluded in section 1.8.4, post-transcriptional regulation, amongst other complex mechanisms, may play an important role in regulation of ABC transporters and hence mRNA levels may not reflect functional activity.

To overcome the potential issues in subjective quantification of protein expression in immunohistochemistry, we have used a novel method of placing core tissues on the same slide as the resection tissues, thereby limiting potential inter-experimental variation. Furthermore, we quantified protein expression using the objective computer-aided scoring protocol, which is being increasingly used in other studies (Mohammed *et al*., 2012) with the potential advantages of reproducibility and increased objectivity. Our scoring protocol also enabled quantification of protein expression as a continuous variable, and hence enabled more precise comparison of change in expression after exposure to NAC, as well as selection of appropriate cut-offs for survival analysis. This is in comparison to change in protein expression simply quantified as positive/negative

as adopted in another NAC study (Tanei *et al*., 2011). As eluded to in section 1.5, previous studies regarding potential predictive markers to NAC have shown that significant correlations are only present in selected tumour subtypes, suggesting that tumour heterogeneity plays an important role. Therefore, unlike previous studies mentioned in section 4.1, we have made an active effort to limit tumour heterogeneity during the patient cohort selection.

Marker expression levels

My results show that Pgp and MRP1 are up-regulated after exposure to NAC. These findings are in keeping with previously published studies (Rudas *et al*., 2003, Chung *et al*., 1997). Interestingly we found that MRP1 expression was uniformly low pre-NAC, which is in contrast to study by Rudas *et al* in which pre-NAC MRP1 expression was detected in 62% of their study cohort. Up-regulation of MRP1 expression occurred in 92% of our cohort, compared to 57% in their cohort. The differences in finding may be due to the difference in the cohort characteristics or in experimental protocol, such as the monoclonal antibody used to detect MRP1 expression. Tanei *et al* found that BCRP expression was significantly increased after NAC with positive BCRP expression seen in 15/72 patients pre-NAC, compared to 26/72 patients post-NAC. However, the limitation of this study in terms of quantifying change in expression as positive/negative rather than as a continuous variable highlights the limitations of their study. BCRP expression was rather variable in our study with up-regulation observed in 59%, compared to the respective values of 74% for Pgp and 92% for MRP1.

This study showed that Pgp, MRP1, and BCRP expression in the axillary lymph nodes post-NAC was not significantly different to that of primary breast cancer post-NAC. However, no correlation in expression pattern was found for all three transporters,

perhaps reflecting the fact that only 13 lymph node tissues were examined. Our finding is different to the previously mentioned study in section 4.1 where Pgp and MRP1 expressions in the lymph nodes differed significantly from those of the corresponding primary tumours (Zochbauer-Muller *et al*., 2001). As our study examined lymph node expression post-NAC, as opposed to untreated in Zochbauer-Muller *et al*, it is difficult to compare the results and further studies with larger cohort sizes are required.

We have also found that in tumours with low pre-NAC Pgp expression, there is a greater increase in the BCRP expression after NAC. One possible explanation is that high Pgp expression results in sufficient efflux of chemotherapeutic agents, and hence the stimulus for BCRP up-regulation is reduced. However, this is unlikely since MRP1 up-regulation was observed in 92% of the cases, suggesting that the stimulus for up-regulation remains. Alternatively, regulatory cross-talk between the two transporters may enable tumours with low Pgp expression to respond by compensatory up-regulation of BCRP. A study by Bark *et al* supports this hypothesis of regulatory cross-talk between Pgp and BCRP (Bark *et al*., 2008). Their study reported that Pgp down-regulated the expression of BCRP in a doxorubicin-resistant lung cancer cell line.

Interestingly for Pgp, pre-NAC expression level in the pCR group was significantly higher compared to the non-pCR group (Mann-Whitney U test: $p=0.013$). This is a paradoxical finding that requires further investigation, and may potentially be explained by single nucleotide polymorphism (SNP) in these patients attenuating the efflux activity of Pgp. George *et al* examined SNP 3435 C>T in exon 26 of the *MDR1* gene in 76 breast cancer patients treated with NAC. The authors found that patients with 3435TT genotype with low Pgp expression had improved overall response to NAC than 3435CC genotype with high Pgp expression. However, the difference was not significant (George *et al*., 2009). This study is further supported by meta-analysis by

Wang *et al* who showed association between the *MDR1* C3435T polymorphism and risk of breast cancer based on 10 case-control studies involving 5282 breast cancer cases and 7703 controls. TT versus CC genotypes was associated with the most significant increased risk of breast cancer with an odds ratio of 1.45 (Wang *et al*., 2013b). I do not have data concerning the genotypes of patients in my cohort. For MRP1 and BCRP, no statistically significant difference was detected between the pCR group and the non-pCR group. Therefore, these findings also suggest that pre-NAC xenobiotic transporter levels do not predict response to NAC, and that post-NAC expression may be more informative. This is reflected by the significance of the post-NAC BCRP expression.

Significance of expression in terms of survival

Pre-NAC BCRP expression had no predictive value in terms of survival, and our study shows that BCRP expression levels vary widely after exposure to NAC. However, this variably induced-change is of potential relevance since high BCRP expression post-NAC correlated with poor DFS. BCRP expression has also been shown to significantly correlate inversely with survival in patients with acute myeloid leukaemia (Damiani *et al*., 2006, Benderra *et al*., 2004, Benderra *et al*., 2005). Our study finding and the transferrable methodology of using objective computer-aided scoring protocol requires examination in a larger independent cohort for validation. Based on the Kaplan-Meier survival analysis of post-NAC BCRP expression versus disease free survival, post hoc power analysis was performed to examine whether the study cohort size was sufficient to identify this association. IBM SPSS SamplePower v3.0 (SPSS, Chicago, USA) was used for this analysis. Based on a two-tailed test with alpha value of 0.05, the cohort size of 39 patients resulted in a power value of 0.78. Further analysis showed that a cohort size of 44 patients would have achieved the commonly accepted power value of 0.8.

Patients with high BCRP expression post-NAC may benefit from further adjuvant therapies to attempt to improve disease free survival. However, the adjuvant therapy may not be in the form of further chemotherapy as these patients will already have high BCRP expression level within any remaining tumour cells. Alternative approaches include investigation of mechanisms that regulate BCRP expression to potentially improve the efficacy of NAC, which is the focus of chapter 6.

Pre-NAC MRP1 expression did not predict survival in this study, which is in contrast to the findings of the Rudas *et al* study (Rudas *et al*., 2003), which found that high pre-NAC MRP1 expression predicted poor survival. However, the cohort used in this work included patients with lobular carcinoma, a different monoclonal primary antibody, and a scoring protocol which quantified expression in a discrete manner rather than our continuous data. In our study, MRP1 expression pre-NAC was uniformly low, and up-regulation occurred in 92%, compared to 57% in their study. Investigating potential mechanism responsible for this striking up-regulation of MRP1 expression is the focus of the next chapter.

5.0 NAC up-regulates Notch1 and MRP1 expression, but Notch1 inhibition does not potentiate chemotherapy efficacy

5.1 Abstract

My data in chapter 4 demonstrated that MRP1 expression was up-regulated in 36/39 cases after NAC. Evidence within the literature suggested that MRP1 up-regulation may be driven by activated Notch1. Therefore, my first aim in this chapter was to determine whether activated Notch1 expression correlated with MRP1 expression. Activated Notch1 expression was determined in the same NAC cohort tissue samples as used in chapter 4 using immunohistochemistry. Similarly to MRP1, Notch1 expression was significantly up-regulated after NAC ($p=0.0003$). Moreover, a significant correlation was observed between post-NAC Notch1 and MRP1 expression (rho coefficient 0.6; $p=0.0008$), suggesting that Notch may indeed drive MRP1 up-regulation after NAC. The hypothesis that inhibition of Notch signaling, and therefore of MRP1 up-regulation, increases the sensitivity of breast cancer cells to chemotherapeutics was developed from these observations. This hypothesis was tested by assessing survival/proliferation of breast cancer cell lines using MTT assays after treatment with combinations of the chemotherapeutic doxorubicin and the Notch inhibitor DAPT. Minor additive effects on survival/proliferation were seen with combinations of doxorubicin and DAPT, but no synergistic effect was observed. Further investigation using immunoblotting revealed that under these conditions doxorubicin did significantly up-regulate MRP1 expression, but activated Notch1 expression was not significantly up-regulated. Immunohistochemistry findings from this study add further weight to the potential role of Notch1 in regulating MRP1 expression. However, further *in vitro* studies and alternative experimental strategies are required to determine whether abrogating Notch1 expression results in improved efficacy of chemotherapy.

5.2 Introduction

In the previous chapter I showed that treatment of breast cancers with NAC was associated with dramatic up-regulation of MRP1 expression within the tumour cells. I was interested to investigate the molecular mechanisms potentially responsible for this up-regulation. A considerable body of work exists concerning the pathways that regulate MRP1 expression in normal physiology as well as in cancer (Bakos and Homolya, 2007, Haimeur *et al*., 2004).

Regulation of MRP1 expression in normal physiology

MRP1 has a role in protection against xenobiotics and endogenous toxic metabolites. Lorico *et al* used knock-out mice to investigate the physiological role of MRP1 (note, the authors refer to the murine gene as *mrp*). No physiological abnormalities were observed between *mrp*(+/+) and *mrp*(-/-) mice up to 4 months of age, perhaps indicating functional redundancy amongst the xenobiotic transporters. However, the *mrp*(-/-) mice displayed increased sensitivity to the chemotherapeutic agent etoposide, which resulted in greater bone marrow toxicity (Lorico *et al*., 1997). MRP1 is highly expressed in lung, testis, kidney, heart, and placenta (Bakos and Homolya, 2007). Pascolo *et al* showed that MRP1 expression increased with placental maturation, supporting the notion that MRP1 contributes to protection of the fetus from toxic compounds. They showed that *MRP1* mRNA expression was increased four-fold in third compared to first trimester human placental tissue samples (Pascolo *et al*., 2003). Qian *et al* showed that high expression of MRP1 in Leydig cells of testis has potential roles in protecting testes from the feminising side effects of endogenous oestrogen conjugates. They used MRP1-transfected HeLa cells to show that conjugated oestrogen was efficiently transported by MRP1 (Qian *et al*., 2001).

Regulation of MRP1 expression in cancer

As well as the aforementioned studies in section 1.8.4, the following studies elaborate further on regulation of MRP1 expression in cancer, providing evidence of transcriptional regulation. Under wild-type conditions, the p53 tumour suppressor protein represses transcription of MRP1. In cancer, mutant p53 loses the ability to repress MRP1 transcription, thereby resulting in up-regulation of MRP1 expression (Wang and Beck, 1998). A further transcriptional control is exerted by the oncogenic transcription factor c-jun, potentially via an AP-1 site within the MRP1 promoter. Cripe *et al* showed that transfection of a multidrug resistant leukaemic cell line, HL-60/ADR, with a vector to over-express dominant negative c-jun resulted in reduced MRP1 expression and abrogation of MRP1-dependent efflux, and hence increased sensitivity to daunorubicin (Cripe *et al.*, 2002).

My work described in the previous chapter demonstrated that MRP1 expression was up-regulated by exposure to NAC in breast cancer tissue samples (Kim *et al*., 2013). Two further publications were of specific interest in the context of determining factors potentially responsible for NAC-dependent up-regulation of MRP1. First, a study in which a potential link between the transcription factor Notch1 and MRP1 was examined (Cho *et al*., 2011). The authors showed that MRP1 was a direct transcriptional target of Notch1 in the multi-drug resistant breast cancer cell line, MCF7/VP. They showed that down-regulation of Notch1 activity, using the gamma-secretase inhibitor DAPT, led to subsequent down-regulation of MRP1 expression as detected by western blots. Furthermore, transfection of MCF7/VP cells with a luciferase reporter allowing measurement of activity of the *ABCC1* promoter (*ABCC1* being the gene that codes for MRP1), showed that *ABCC1* transcriptional activity was reduced by treatment with DAPT. Finally, siRNA-mediated knock down of MRP1 expression in Notch1-overexpressing cells resulted in increased sensitivity to etoposide. Overall, this study

convincingly demonstrates a regulatory link between Notch1 and MRP1, and that targeting of Notch1 with DAPT or siRNA could be used to modulate MRP1 expression. The second relevant publication demonstrated that Notch1 activity was up-regulated by NAC in clinical breast cancers. Analysis of gene expression profiles of clinical breast cancer samples treated with NAC (Gonzalez-Angulo *et al*., 2012) revealed over-expression of targets of Notch signalling. This study compared gene expression data of pre- and post-NAC tissues from 21 breast cancer patients. In non-basal-like subset of breast cancers, enriching of Notch signalling targets was observed in differentially expressed genes. Thus, from bioinformatics it was inferred that NAC may activate Notch signalling in the non-basal (mainly luminal) breast cancers. By combining the findings of these two highlighted studies, my hypothesis was that the up-regulation of MRP1 by NAC that I demonstrated in the previous chapter is mediated, at least in part, by Notch signalling.

Notch proteins and Notch signalling

Notch proteins are transmembrane receptors that interact with ligands of the delta or jagged families, which are mostly present on adjacent cells. Notch proteins are initially translated as precursor forms, which are then processed by an S1 cleavage in the Golgi network, resulting in the formation of the mature Notch proteins, which consist of extracellular, transmembrane, and cytoplasmic domains. Extra-cellular ligand binding to the Notch protein leads to further cleavage in two successive steps. The first proteolytic cleavage (S2) is performed at the extracellular domain by the metalloprotease ADAM17 (A Disintegrin And Metalloproteinase) and TACE (TNF-α converting enzyme). The second proteolytic cleavage (S3) is carried out by gamma secretase at the transmembrane domain, generating the free Notch intracellular domain (NICD) in the cytoplasm (Yin *et al*., 2010). The NICD is translocated to the cell nucleus, and acts as a transcription factor potentially regulating cell proliferation,

differentiation, and apoptosis through a large range of target genes. NICD binds to a transcriptional repressor CSL [CBF-1 (C-promoter binding factor 1), Suppressor of Hairless and Lag-1], which leads to the displacement of the co-repressor complex and recruitment of co-activators such as mastermind-like (MAML) and p300 to activate transcription of Notch target genes, including the Hes and Hey family genes. This in turn can lead to the regulation of transcriptional targets, cyclin D1 (involved in cell-cycle regulation), c-Myc (transcription factor), and HER2 (growth factor receptor) (Guo *et al*., 2011).

There are four Notch receptors; Notch1 to 4, which are encoded by separate genes. The Notch pathway can have oncogenic as well as tumour suppressive effects. For example, mouse models have demonstrated that Notch1 overexpression results in mammary gland tumours (Hu *et al*., 2006) and immunohistochemistry studies have shown that high Notch1 expression is associated with poorly-differentiated breast cancers (Li *et al*., 2010). Furthermore, Reedijk *et al* showed that high *Notch1* mRNA expression was associated with poor overall survival in breast cancer patients (Reedijk *et al*., 2005); 5-year OS for patients with high Notch1 expression was 49% versus 64% for low Notch1 expression. In contrast, Parr *et al* examined Notch2 expression in 97 breast cancer specimens using immunohistochemistry, and showed that high Notch2 levels correlated with improved survival in breast cancer patients and well-differentiated tumours (Parr *et al*., 2004).

<u>Notch signalling and NAC – a potential therapeutic opportunity?</u>

My initial hypothesis was that Notch signalling contributes to the up-regulation of MRP1 by NAC. Should this hypothesis be correct, my aim would then be to attempt to inhibit the chemotherapy-dependent up-regulation of MRP1 using inhibitors of Notch1 activity. I will test whether this might be used to increase the efficacy of chemotherapy, by

reducing MRP1-dependent chemotherapy resistance (Fig 5.1). A caveat to this therapeutic strategy is the fact that MRP1 expression did not demonstrate a significant relationship with patient survival in the work in the previous chapter, therefore MRP1-dependent chemotherapy resistance may be relatively unimportant. However, high MRP1 expression consistently showed a trend to be associated with poor survival and MRP1 showed the most consistent NAC-dependent up-regulation, therefore interfering with this *potential* pathway of chemoresistance remains attractive.

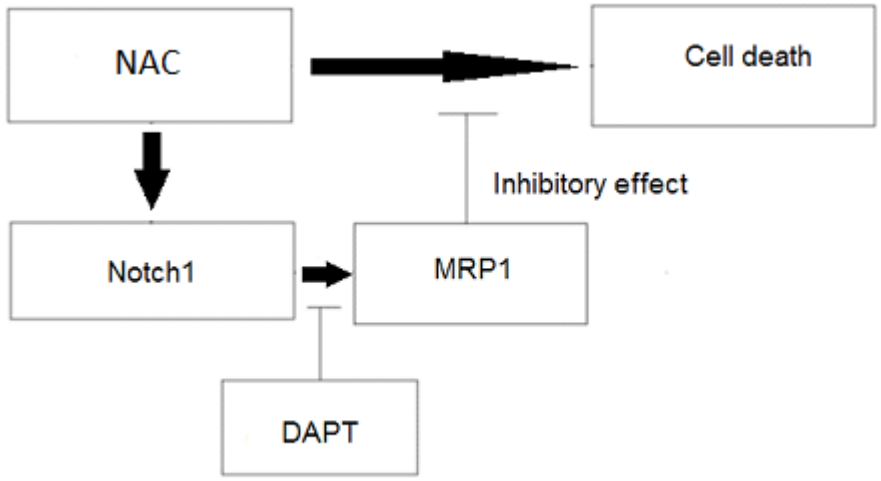


Figure 5.1: Proposed pathway for enhancing the efficacy of chemotherapy. The aim of NAC is to kill cancer cells but MRP1 has a role in protecting cancer cells by effluxing chemotherapeutics and hence reducing their efficacy. If up-regulation of MRP1 is mediated by Notch1 signalling, inhibition of Notch1 using DAPT would result in reduction of MRP1 levels in cancer cells, thereby improving the efficacy of NAC.

Gamma-secretase inhibitors (GSI), such as DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), have been developed to inhibit conversion of mature full-length Notch to its active NICD form. Hallahan *et al* used DAPT to induce this Notch inhibition in medulloblastoma cell lines and mouse models. They found that

DAPT caused dose-dependent decreases in the number of viable cancer cells (Hallahan *et al*., 2004). Farnie *et al* cultured ductal carcinoma in situ (DCIS) cells to form mammospheres, and showed that mammosphere-forming efficiency, calculated by dividing the number of mammospheres formed by the original number of single cells seeded, was attenuated by DAPT (Farnie *et al*., 2007). Therefore, DAPT was chosen to inhibit Notch expression for my study. GSIs are currently being evaluated in phase I clinical trials as a monotherapy (Groth and Fortini, 2012). However, they have been shown to have significant side-effects including gastro-intestinal bleeding, and immunosuppression. Other approaches, such as combination therapies, may be useful to improve the efficacy of GSI, potentially allowing use at reduced doses or lengths of exposure thereby reducing side-effects. Osipo *et al* showed that inhibition of HER2 function by Herceptin led to up-regulation of Notch1 activity in a panel of breast cancer cell lines. This up-regulation was attenuated by GSIs, and the combination of GSIs with Herceptin led to enhanced rates of apoptosis compared to GSIs alone in both Herceptin resistant and Herceptin sensitive cells (Osipo *et al*., 2008). Hence, ErbB2 inhibition activates Notch1 and leads to increased sensitivity to GSI. Moreover, Nefedova *et al* showed that inhibition of Notch signalling enhanced sensitivity to chemotherapeutics in multiple myeloma cells (Nefedova *et al*., 2008). Combining doxorubicin with GSI led to a greater rate of apoptosis in a myeloma cell line, and also led to significant tumour size reduction in a mouse model, compared to each drug alone.

Therefore, in this chapter I aimed to examine expression of activated (S3 cleaved) Notch1 in the same cohort as studied for MRP1 in the previous chapter and test the relationships between Notch 1 and MRP1, and between Notch1 and clinico-pathological parameters including NAC response. Finally, I aimed to treat breast

cancer cells with combinations of chemotherapeutics and inhibitors of Notch function to test whether the drugs exhibit synergistic cancer cell killing.

5.3 Results

5.3.1 Activated Notch1 expression was up-regulated post-NAC and correlated with post-NAC MRP1 expression

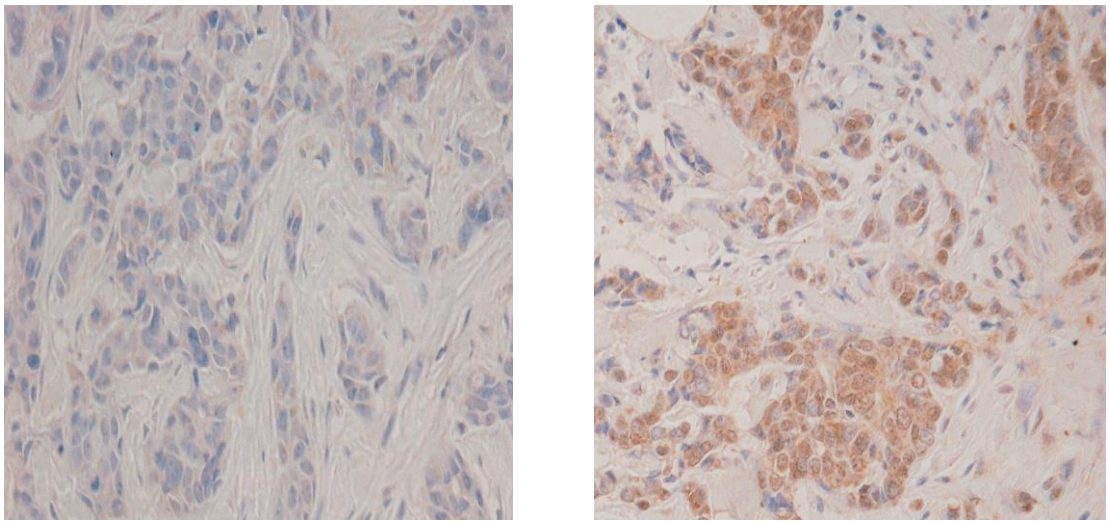
I aimed to determine the expression of activated Notch1 using immunohistochemistry in the same cohort of breast cancer patients treated with NAC as was used in Chapter 4, and then compare activated Notch1 expression patterns to MRP1 expression. However, further biopsy core tissues was available for analysis of Notch1 NICD expression in only a subset of this initial cohort, and therefore the matched analysis was possible in pre- and post-NAC tissues from only 29 of the 39 original cases. The clinico-pathological characteristics of this reduced cohort are illustrated in Table 5.1.

Characteristic	Categories	No. of patients (%) n=29
Age	<45	12 (41.4)
	>45	17 (58.6)
Grade (pre-NAC)	2	9 (31)
	3	20 (69)
Stage (pre-NAC)	T2	19 (65.5)
	T3	10 (34.5)
Stage (post-NAC)	T1	9 (31)
	T2	15 (51.7)
	T3	5 (17.3)
Tumour size change	Increase	6 (20.7)
	Decrease	23 (79.3)
MRI response	Minimal	9 (31)
	Partial	20 (69)
NAC regimen	Epirubicin + cyclophosphamide (EC)	5 (17.2)
	EC + taxanes	24 (82.8)
Lymphovascular invasion	Positive	11 (37.9)
Axillary metastasis	Positive	15 (51.7)
Oestrogen receptor	Positive	17 (58.6)
Her2	Positive	3 (10.3)
Surgery	breast conserving	11 (37.9%)
	Mastectomy	18 (62.1%)
Follow up	median: 4.2 years (range 3-5.7 years)	
Recurrence		11 (37.9)
Death		5 (17.2)

Table 5.1: The clinico-pathological characteristics of the patient cohort for which Notch1 NICD expression was examined

Immunohistochemistry for Notch1 NICD was performed on these matched pre- and post-NAC samples, using core biopsy and resection samples mounted on the same slide as before. Staining was observed in the nucleus and cytoplasm in epithelial cells only, with strong accentuation of the cell nuclei. Representative staining patterns are illustrated in Figure 5.2. Active Notch1 NICD is functionally active in the nucleus, unlike MRP1, therefore in order to score this specific active compartment a different scoring protocol was required from the semi-automated method used for MRP1. Manual Allred scoring of nuclear expression was used to quantify Notch1 NICD expression, involving assessment of both staining intensity (from 0 to 3) and the proportion of cells staining

positively (classes enumerated as 0 to 5) (Tacca *et al*., 2007). Two independent scorers quantified expression and inter-scorer reproducibility was assessed as excellent (Kappa values 0.78 for core tissues and 0.9 for resection tissues; see section 3.7); the mean value of the two scores (overall kappa value of 0.89) was taken as the final assessment.



Pre-NAC **Post-NAC**

Figure 5.2: Immunohistochemistry for Notch1 NICD (x20 magnification) shows no nuclear staining in the core tissues (pre-NAC), as opposed to positive nuclear staining in the resection tissues (post-NAC). These images represent the significant up-regulation in Notch1 NICD expression after exposure to NAC.

Pre-NAC and post-NAC Notch1 NICD expression ranged from 0-4 (out of maximum score of 8), and 0–8 respectively (Fig 5.3). Up-regulation of Notch1 NICD expression after exposure to NAC was seen in 23/29 cases (Figure 5.3a) and, overall, the up-regulation was statistically significant (Figure 5.3b) (Wilcoxon signed-rank test: p=0.0003).

5.3a 5.3b

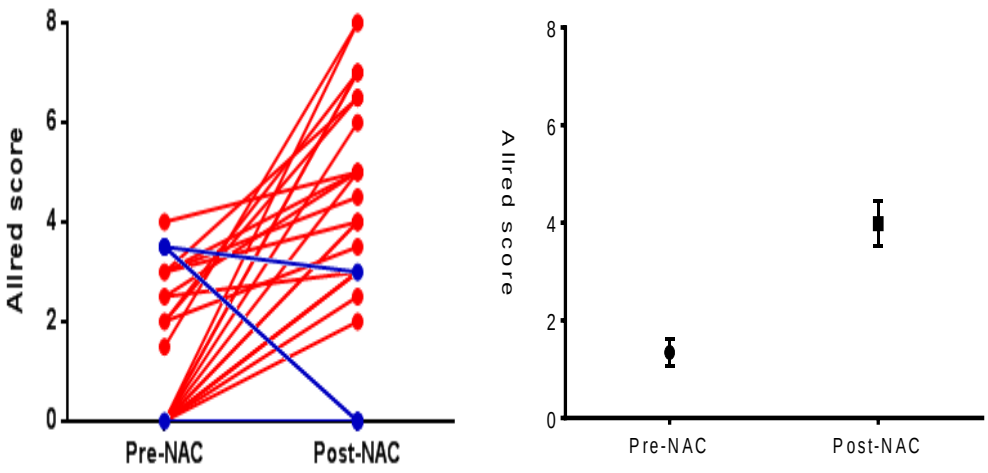


Figure 5.3a (left) and b (right): Notch1 NICD expression pre- and post-NAC (red: up-regulation, blue: down-regulation or no change) is shown on Figure 5.3a. Up-regulation was observed in 23/29 cases (79.3%). Matched pre- and post-NAC breast cancer samples were stained using immunohistochemistry for Notch1 NICD. Expression within tumour cells was quantified as Allred score of 0 to 8. Expression levels in matched samples are linked by lines coloured red or blue so as to indicate an increase or decrease in expression respectively. Figure 5.3b illustrates pre- and post-NAC expressions of Notch1 NICD shown as median Allred score values with interquartile range; n=29. Statistically significant up-regulation was observed for Notch1 NICD (p=0.0003; Wilcoxon signed-rank test).

The pre- and post-NAC Notch1 NICD expression was then analysed with the equivalent MRP1 expressions from the same samples using the Spearman's rho analysis. No significant correlation was observed pre-NAC between Notch1 NICD and MRP1 expression (rho coefficient 0.33; p=0.08; Fig 5.4a), although it should be noted that MRP1 expression was generally extremely low pre-NAC and these expression levels may be subject to a substantial contribution of non-specific noise (Fig 5.4a).

However, a significant correlation was observed between post-NAC Notch1 NICD and MRP1 expression (rho coefficient 0.6; p=0.001; Fig 5.4b). Pre- and post-NAC Notch1 NICD expressions were also compared to the equivalent Pgp and BCRP expressions. Notch1 NICD expression did not correlate with either Pgp or BCRP expression either pre- or post-NAC (pre-NAC Notch1 NICD and Pgp expression: rho -0.03, p=0.87; post-NAC Notch1 NICD and Pgp expression: rho 0.04, p=0.82; pre-NAC Notch1 NICD and BCRP expression: rho 0.03, p=0.88; post-NAC Notch1 NICD and BCRP expression: rho -0.02, p=0.91). These findings demonstrated that Notch1 NICD levels correlate with MRP1 specifically, rather than xenobiotic transporters generally.

5.4a (pre-NAC) 5.4b (post-NAC)

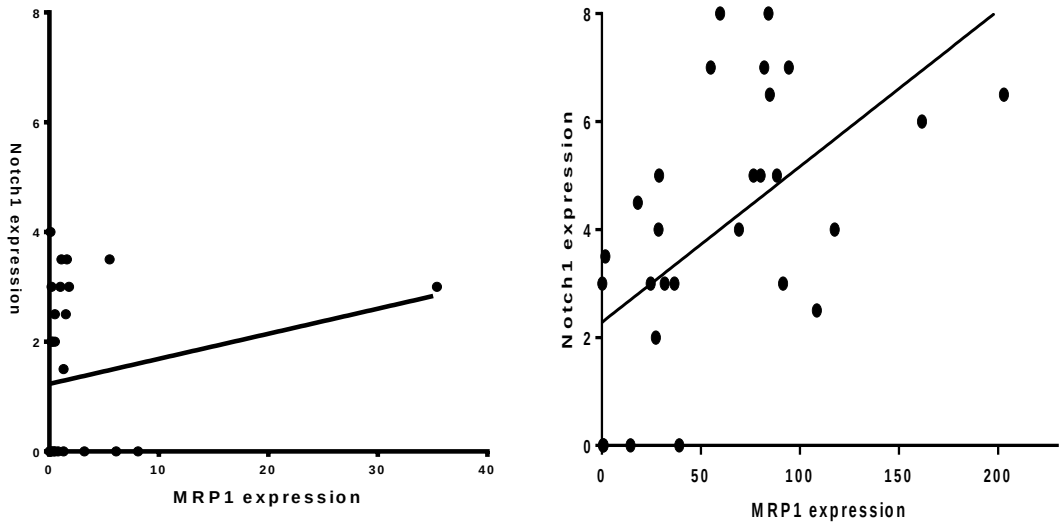


Figure 5.4a (left) and b (right): Scatter plots of Notch1 NICD expression versus MRP1 expression (n=29). Figure 5.4a illustrates pre-NAC Notch1 NICD expression plotted on the y-axis (Allred score of 0 to 8), and matching pre-NAC MRP1 expression plotted on the x-axis (histoscores). Due to low MRP1 expression pre-NAC, the x-axis is only shown up to histoscore of 40. No significant relationship was seen (Spearman's rho coefficient 0.33, p=0.08). Figure 5.4b illustrates post-NAC Notch1 NICD expression plotted on the y-axis (Allred score of 0 to 8), and matching post-NAC MRP1 expression plotted on the x-axis (histoscores; 0 to 300). A positive and significant correlation was observed between post-NAC Notch1 NICD and MRP1 expression (Spearman's rho coefficient 0.6; p=0.001).

5.3.2. Correlation of Notch1 NICD expression with clinico-pathological parameters and disease free survival.

My next aim was to examine pre- and post-NAC Notch1 NICD expression levels, as well as the change in expression of Notch1 NICD, against the clinico-pathological parameters (outlined in Table 5.1) using Spearman's rho analyses (Table 5.2). As stated in the previous chapter, $p<0.01$ was used to indicate statistical significance. A

statistically significant negative correlation was seen between tumour grade and pre-NAC Notch1 NICD expression level on univariate analysis, although this correlation was not significant after multivariate regression analysis. As previously (see Section 4.2.5), the expression levels and the change in expression levels were then examined against the response to NAC. No significant correlations were observed.

	Notch1 pre	Notch1 post	Notch1 Δ
Age at diagnosis	0.15 (0.43)	-0.003 (0.99)	0.097 (0.62)
Tumour factors determined pre-NAC:			
Grade	**-0.5 (0.006)**	-0.18 (0.34)	0.025 (0.9)
T Stage	0.25 (0.19)	0.32 (0.095)	0.26 (0.18)
ER status	-0.3 (0.12)	-0.089 (0.65)	0.083 (0.67)
Her2 status	-0.08 (0.68)	0.14 (0.46)	0.17 (0.37)
Tumour factors determined post-NAC:			
T stage	0.32 (0.87)	0.18 (0.34)	-0.044 (0.82)
Lymphovascular invasion	0.32 (0.09)	0.17 (0.38)	0.048 (0.8)
Axillary metastasis	0.4 (0.032)	0.075 (0.7)	-0.12 (0.54)
Tumour response			
Δ T stage	0.12 (0.53)	-0.088 (0.65)	0.14 (0.46)
MRI response	0.15 (0.43)	0.47 (0.011)	0.21 (0.28)
Δ in tumour size	-0.14 (0.46)	-0.005 (0.98)	-0.16 (0.41)

Table 5.2: Spearman's correlation coefficients demonstrating relationships between expression pre-NAC or post-NAC, or change in expression (Δ) for Notch1 NICD with clinico-pathological parameters (p values are denoted in brackets)

Next, expression levels and change in expression levels of Notch1 NICD were compared against disease free survival, using Kaplan-Meier survival analyses. Receiver Operating Characteristic (ROC) curve analysis was performed to dichotomise expression as previously. No significant correlations were detected between Notch1 NICD expressions pre- or post-NAC and disease free survival (Fig 5.5). To determine the change in Notch1 NICD expression, pre-NAC Notch1 NICD Allred score was subtracted from post-NAC Notch1 NICD Allred score. In 3 cases where the Allred score was 0 out 8 both pre- and post-NAC, these cases were excluded from the analysis.

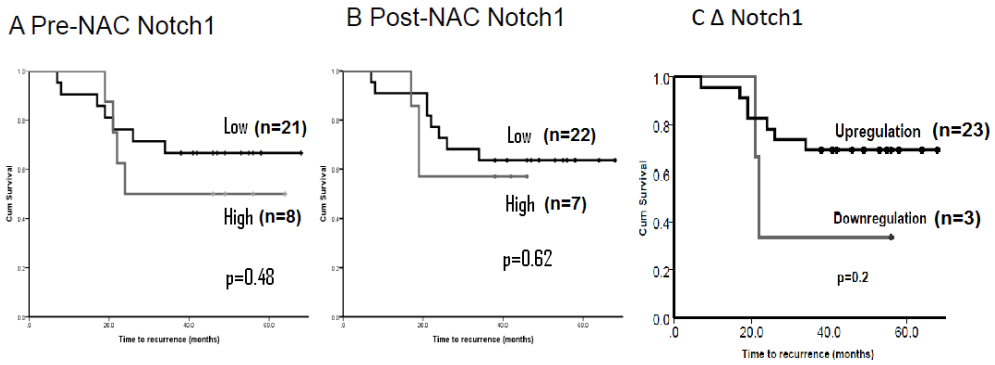


Figure 5.5: Kaplan-Meier survival analyses for Notch1 NICD expression versus disease free survival

5.3.3. Comparison of Notch1 expression in different breast cancer subtypes

The initial published report in which up-regulation of Notch signalling targets was demonstrated post-NAC found this to be true in non-basal-like breast cancers only (Gonzalez-Angulo *et al*., 2012). Therefore I aimed to compare the expression pattern of Notch1 NICD separately in basal-like and non-basal-like breast cancers in my cohort. This distinction was made by examining ER, PR, and HER2 status in each patient. Patients who had triple negative tumours (ER-, PR-, and HER2-) were categorised into a basal-like group (n=13). These who had ER+ and HER2- tumours were categorised

into luminal A-like group (n=13), and those with ER+ and HER2+ tumours into luminal B-like group (n=3). Luminal A and B-like groups were combined to form 16 luminal-like tumours. Notch1 NICD expression was not significantly different in luminal-like tumours as compared to basal-like tumours either pre- or post-NAC (Fig 5.6) (p=0.06 pre-NAC; p=0.66 post-NAC; Mann-Whitney test). Furthermore, Notch1 NICD expression was significantly up-regulated after NAC in both subtypes when analysed separately (Fig 5.7) (p=0.02 luminal-like; p=0.001 basal-like; Wilcoxon signed-rank test).

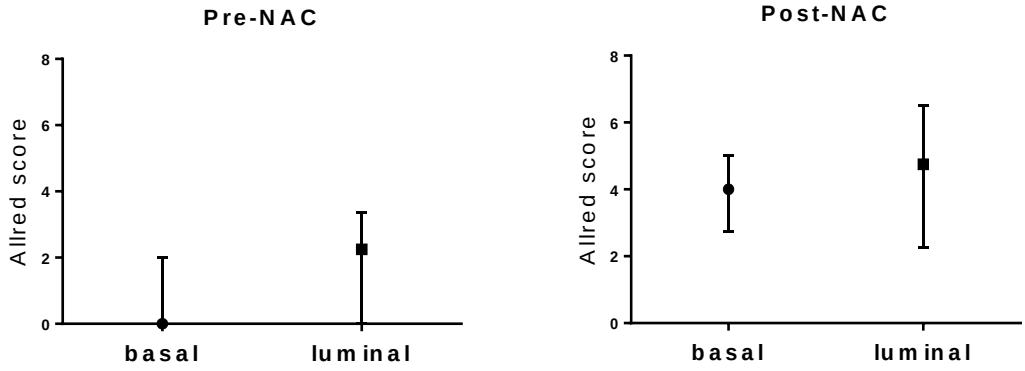


Figure 5.6: No difference in Notch1 NICD expression was observed between the basal- and luminal-like tumours pre- and post-NAC (p=0.06 pre-NAC; p=0.66 post-NAC; Mann-Whitney test). Notch1 NICD expression is shown on y-axis (Allred score 0 to 8) with median expression displayed with interquartile range for basal-like (n=13) and luminal-like tumours (n=16).

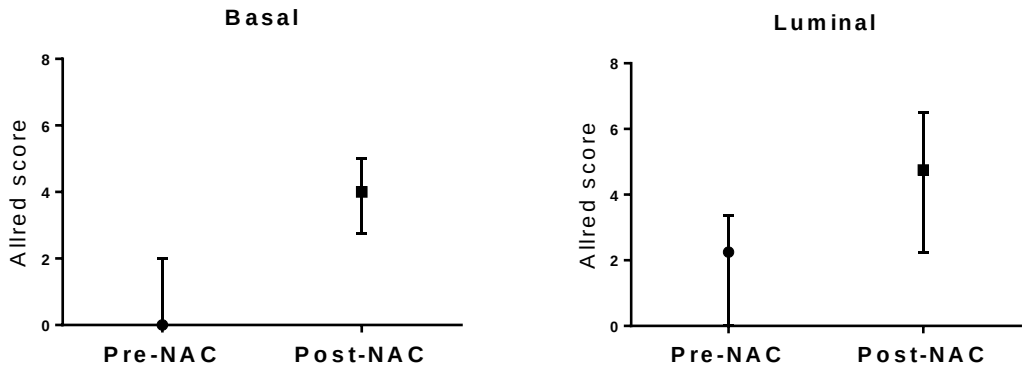


Figure 5.7: Significant up-regulation of Notch1 NICD expression was seen in both basal-like and luminal tumours upon exposure to NAC (p=0.001 basal-like; p=0.02 luminal-like; Wilcoxon signed-rank test). Notch1 NICD expression is shown on y-axis (Allred score 0 to 8) with median expression displayed with interquartile range for basal-like (n=13) and luminal-like tumours (n=16).

5.3.4. Do Notch inhibitors enhance the efficacy of doxorubicin?

Having determined that NAC exposure, including at least some component of anthracyclines, resulted in up-regulation of Notch1 NICD expression and MRP1 expression in many clinical breast cancers I wished to examine whether this would be recapitulated in cell line models, and whether treatment with chemotherapy agents would lead to induction of Notch1 activity and MRP1 expression. In accordance with this, data produced by other investigators in our laboratory demonstrated that treatment of T47D cells, a luminal breast cancer cell line, with doxorubicin induced Notch1 activation and MRP1 expression (Fig 5.8).

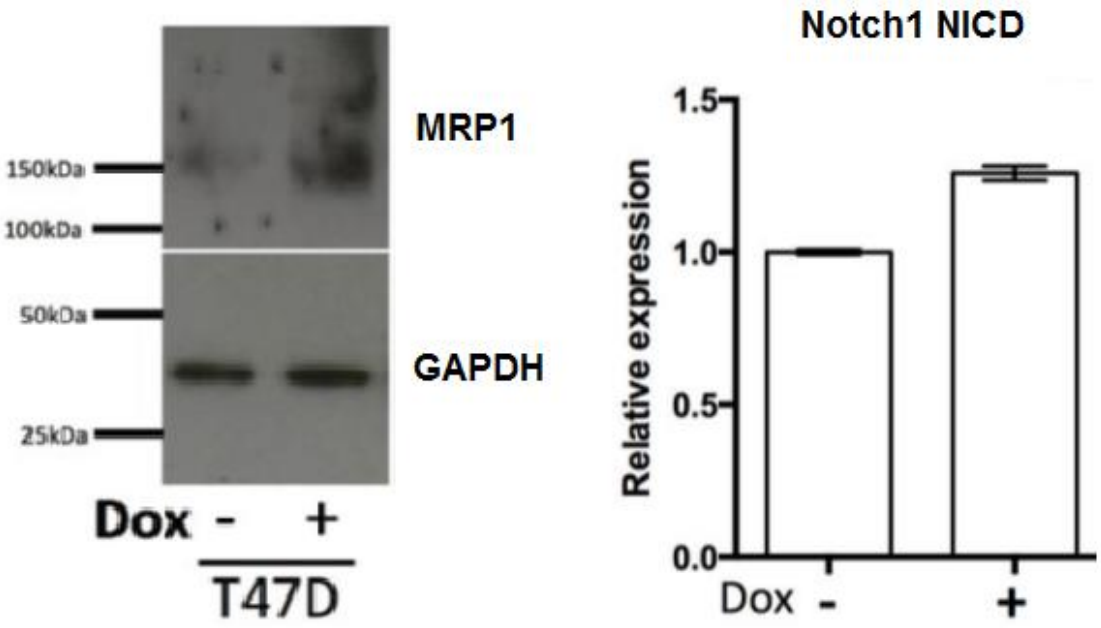


Figure 5.8: Doxorubicin induces MRP1 (left) and Notch1 NICD (right) expression *in vitro*. T47D cells were treated for 24h with 1μM doxorubicin or vehicle control. MRP1 expression was quantified by Western blot (left). Data for Western blot are representative of at least 2 independent biological replicates. The figure is courtesy of Dr James Thorne (LIMM). ELISAs (right) were used to quantify levels of Notch1 NICD with data shown representing 2 independent biological replicates with technical duplicates (error bars show SEM). The figure is courtesy of Dr Sam Stephen (LIMM).

Based on these findings, I aimed to determine whether treatment with DAPT, an inhibitor of Notch1 activation, would potentially sensitize cells to chemotherapy agents (see Figure 5.1) using MTT assays to determine cell survival/proliferation. First, I established suitable conditions for this assay, including doses of chemotherapy agent and length of treatment. I treated luminal breast cancer cell lines MCF7 and T47D *in vitro* with doses up to 1μM of doxorubicin, as a representative anthracycline chemotherapeutic, for up to 4 days and used MTT assays to measure relative growth/survival. Doxorubicin is an anthracycline that has similar clinical efficacy as epirubicin (Bontenbal *et al*., 1998), and has been used on MCF7 cell lines in other

studies (Zang *et al*., 2010). Addition of increasing concentrations of doxorubicin resulted in reduction of growth/proliferation of the luminal cancer cell lines in an exponential manner (Appendix Section 3, Fig S14). From the resulting dose-response curves, three inhibitory concentration (IC) values of doxorubicin were extrapolated for each cell line, and for MCF7 cells for treatment for both 2 and 4 days. The IC10, 25, and 50 values are shown in Table 5.3.

	IC10 (nM)	IC25 (nM)	IC50 (nM)
MCF7 (2 days)	10	25	70
MCF7 (4 days)	4	10	30
T47D (4 days)	10	25	60

Table 5.3: Inhibitory concentration values of doxorubicin for MCF7 and T47D cell lines

Next, I examined the influence of the inhibitor of Notch activation DAPT, using the same strategy, on MCF7 cell lines only. In contrast to doxorubicin, concentrations of up to 10μM DAPT had little influence on growth/proliferation of MCF7 cells after either 2 or 4 days of treatment (Appendix Section 3, Fig S15).

Finally, MCF7 cells were treated for either 2 or 4 days with doxorubicin, at either the IC_{10}, IC_{25} or IC_{50} doses (Table 5.3), combined with an initial range of doses of DAPT from 1nM through to 1uM, and cell survival/proliferation was determined as before. Data are shown for the IC25 shown in Fig 5.9a, while for the IC10 and 50 doses these are included in the appendix (Appendix Section 3, Fig S16a). As expected, doxorubicin alone caused a reduction in cell numbers appropriate for the dose (i.e. approximately reductions of 10%, 25% or 50%). The inclusion of doses of DAPT caused some minor

additive effects although a clear dose dependency was not seen, and overall there was no evidence for synergy between doxorubicin and DAPT. Next, the assay was repeated using a range of higher doses of DAPT, 1µM to 100µM (Fig 5.9b and Appendix Section 3, S16b). In this case, some toxicity from the highest doses of DAPT was seen, but it remained the case that there was no evidence of a synergistic effect from the drugs. These assays were also repeated in T47D cells at either IC_{10}, IC_{25}, or IC_{50} doses (Table 5.3) combined with DAPT doses ranging from 1nM to 100µM. The results for the IC25 are shown in Fig 5.10, while the results for IC10 and 50 doses are shown in Appendix Section 3, Fig S17. As previously, some minor additive effects were seen when doxorubicin was added to DAPT, but no synergistic effect was seen between doxorubicin and DAPT.

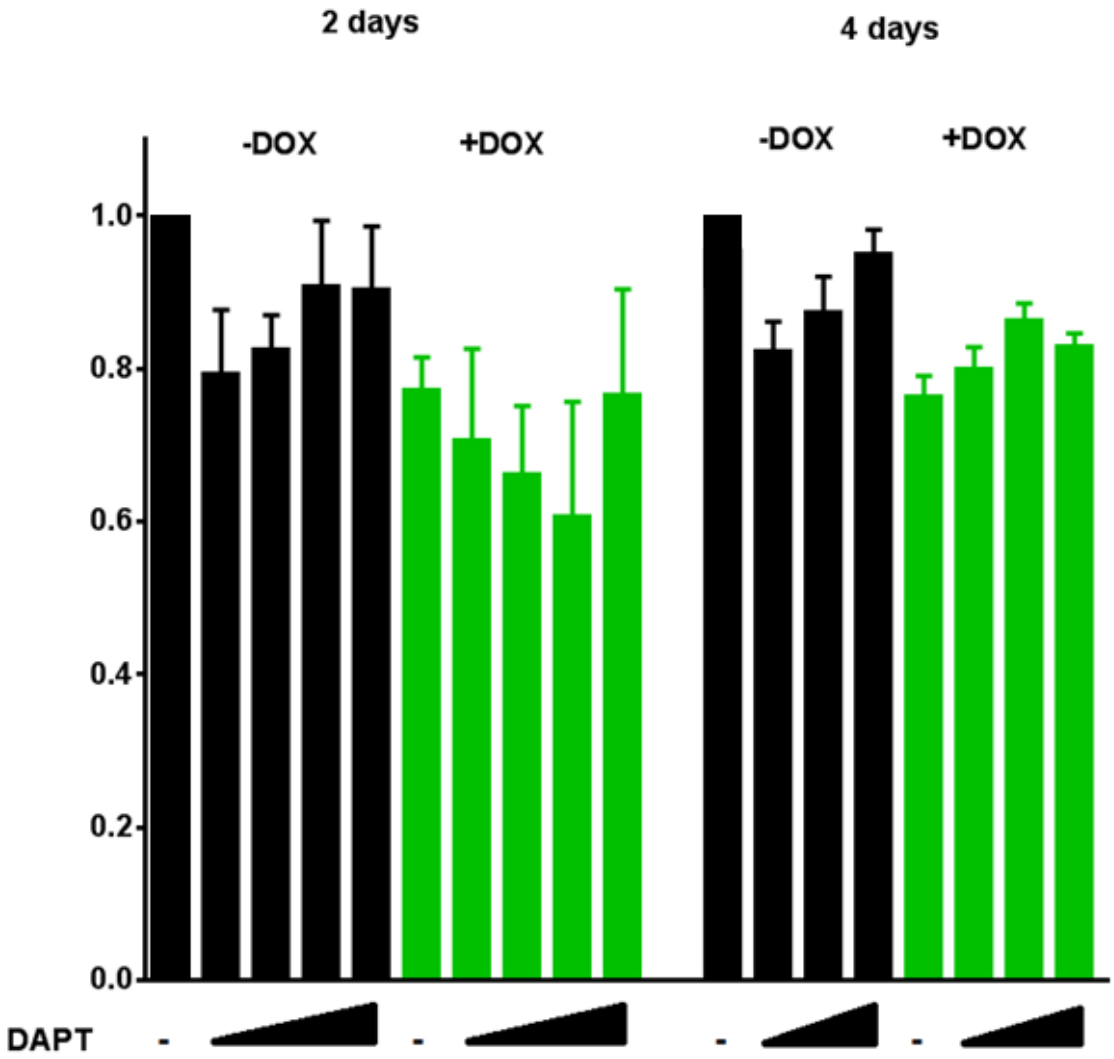


Figure 5.9a: 2 and 4 day combination assay using MCF7 cell lines for the lower DAPT concentrations (1nM to 1µM). Y- axis shows optical density reading normalised to untreated. The first black bar denotes the controls (-) with no DAPT or doxorubicin, with remaining black bars showing increasing DAPT alone. The first green bar denotes the IC25 value (25nM and 10nM for 2 and 4 day assays respectively) without DAPT (-), with the remainder showing IC25 doxorubicin (DOX) with increasing DAPT.

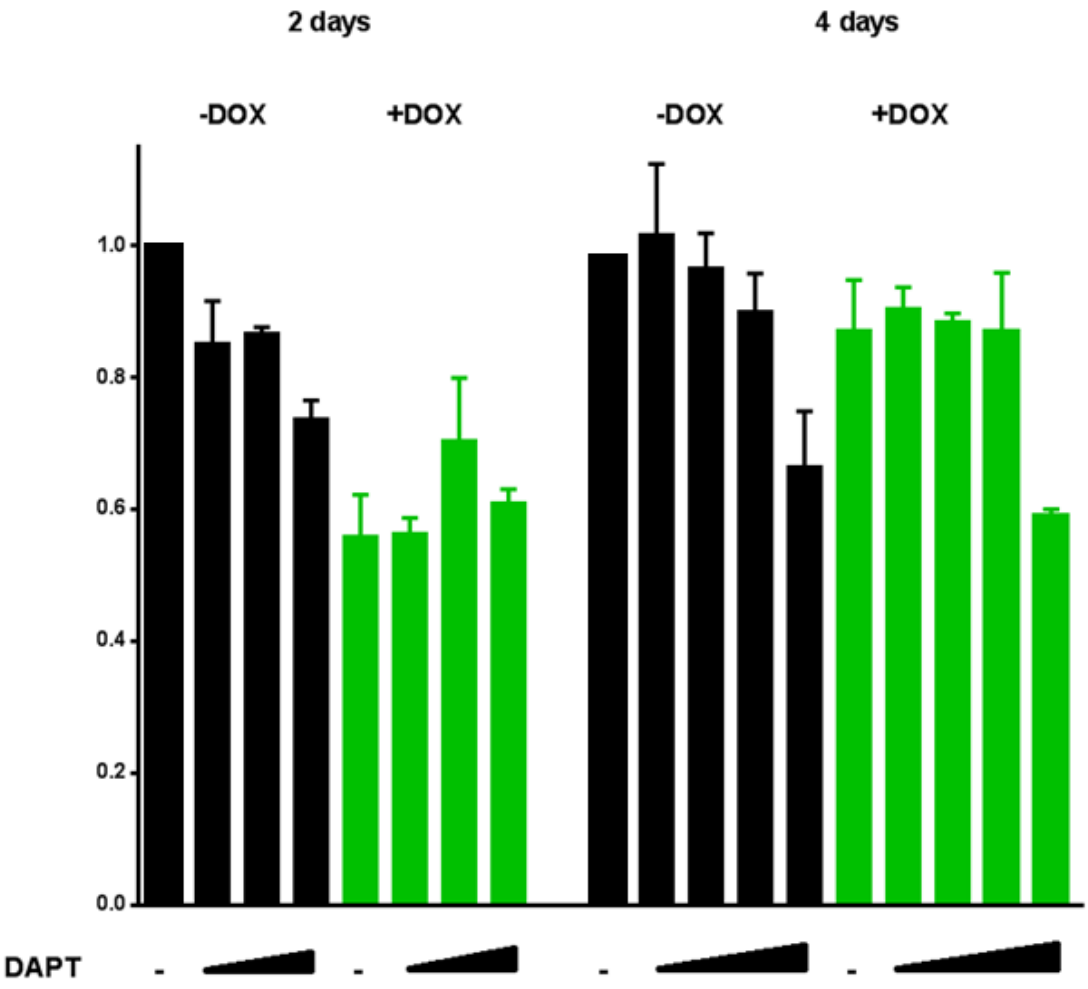


Figure 5.9b: 2 and 4 day combination assay using MCF7 cell lines for the higher DAPT concentrations (1µM to 100µM). Y- axis shows optical density reading normalised to untreated. The first black bar denotes the controls (-) with no DAPT or doxorubicin, with remaining black bars showing increasing DAPT alone. The first green bar denotes the IC25 value (25nM and 10nM for 2 and 4 day assays respectively) without DAPT (-), with the remainder showing IC25 doxorubicin (DOX) with increasing DAPT.

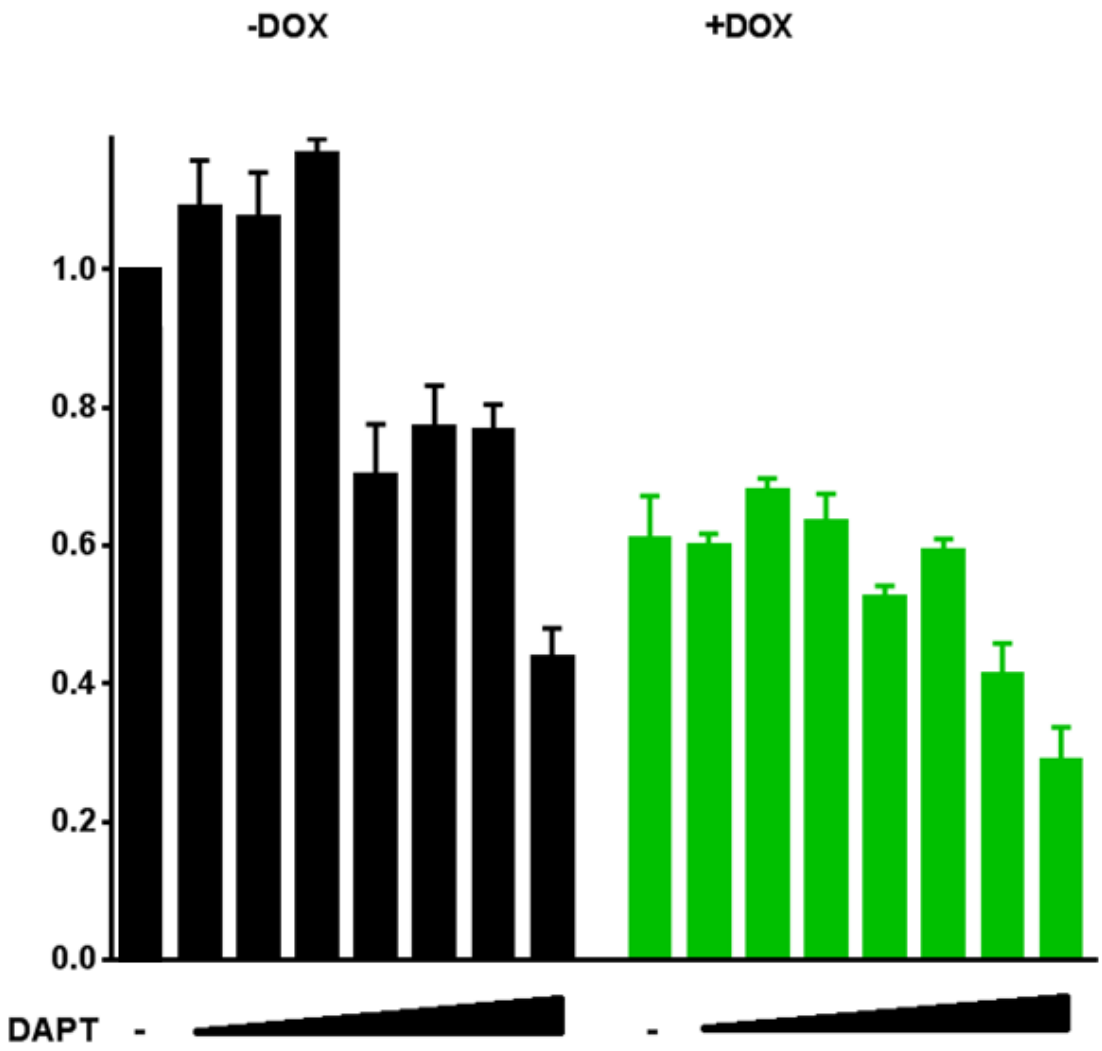


Figure 5.10: 4 day combination assay using T47D cell lines (DAPT concentrations of 1nM to 100µM). Y- axis shows optical density reading normalised to untreated. The first black bar denotes the controls (-) with no DAPT or doxorubicin, with remaining black bars showing increasing DAPT alone. The first green bar denotes the IC25 value (25nM) without DAPT (-), with the remainder showing IC25 doxorubicin (DOX) with increasing DAPT.

5.3.5. Notch1 NICD expression was not up-regulated in response to doxorubicin and DAPT did not inhibit Notch1 NICD or MRP1 expression at the doses used for survival assays

Contrary to our proposed model, combined treatments with doxorubicin and DAPT did not demonstrate synergy *in vitro.* Therefore, I next performed Western blot analysis (see Section 3.10 for methods) to determine whether MRP1 expression and Notch1 activation was changing as expected under the drug treatment conditions used, and also whether any changes in expression of the other xenobiotic transporters, Pgp and BCRP could be acting as confounding factors, since both are potentially able to efflux doxorubicin. Therefore, immunoblotting was performed to examine the protein expression for Notch1 NICD, MRP1, Pgp, and BCRP under essentially the same conditions used for the cell survival/proliferation analyses.

MRP1 and Notch1 NICD expressions were examined on western blots using MCF7 cell lysate (Fig 5.11). Basal MRP1 expression was low, and up-regulation of MRP1 expression was only observed at the lowest dose of doxorubicin treatment (IC10). In contrast, basal Notch1 NICD expression was considerable. Marginal up-regulation of Notch1 NICD expression was also observed at the lowest dose of doxorubicin treatment (IC10). Surprisingly, both MRP1 and Notch1 NICD expressions were down-regulated with increasing doses (IC25 and IC50) of doxorubicin treatment. DAPT treatment on its own resulted in up-regulation of MRP1 expression. Similarly, combination therapy of doxorubicin and DAPT also resulted in up-regulation of MRP1 expression. DAPT treatment on its own did not result in down-regulation of Notch1 NICD expression as expected. The combination therapy of Notch1 and DAPT resulted in down-regulation of Notch1 NICD expression however.

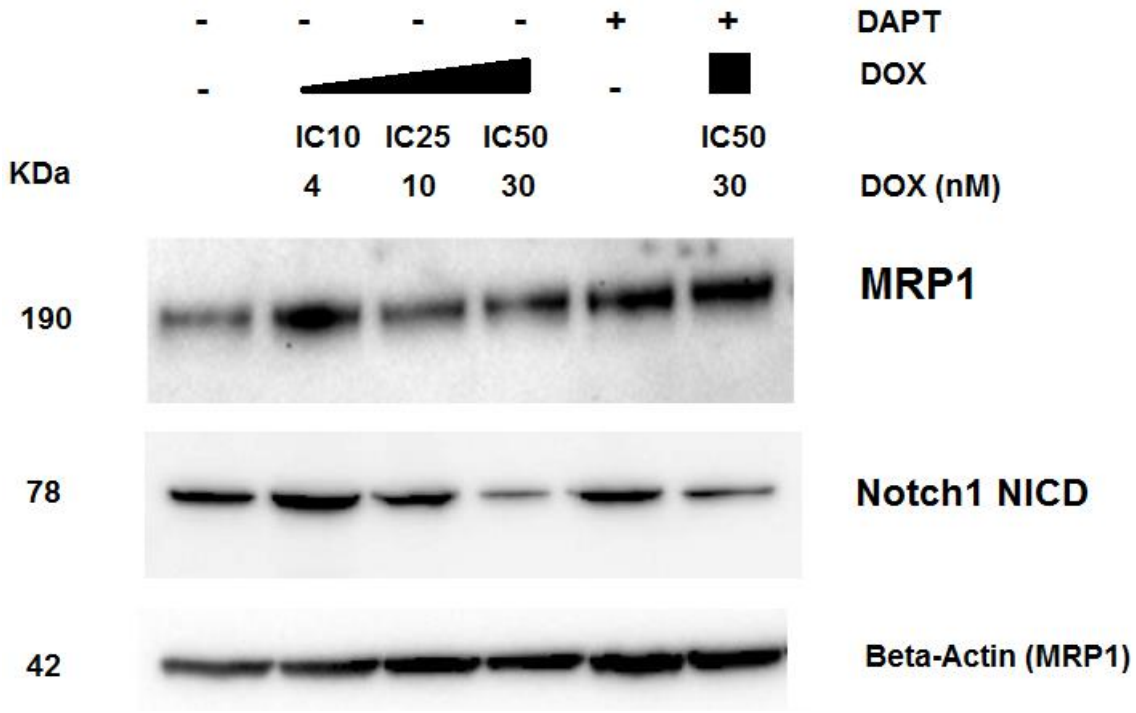


Figure 5.11: Western blot to examine MRP1 and Notch1 NICD expression using MCF7 cell lysates treated with doxorubicin +/- DAPT for 4 days. IC10 doxorubicin treatment led to up-regulation of MRP1 and Notch1 NICD expression. Note that basal Notch1 NICD expression was considerable without any drug treatment (control). However, subsequent IC25 and IC50 doxorubicin treatment led to down-regulation of MRP1 and Notch1 NICD expression. DAPT treatment (100μM) with or without doxorubicin led to up-regulation of MRP1 expression. Treating the cells with DAPT did not result in down-regulation of Notch1 NICD expression. However, the combination of doxorubicin and DAPT led to down-regulation of Notch1 NICD expression.

The western blots for MRP1 and Notch1 NICD were repeated twice more using different protein lysates harvested at different biological time points but using the same treatment conditions described so far, as well as further combination treatments with DAPT for IC10 and IC25 doxorubicin doses. The resulting immunoblots (Fig 5.12)

showed that MRP1 expression was up-regulated in a dose dependent manner. However, DAPT with or without doxorubicin did not result in down-regulation of MRP1 expression. Further MRP1 western blot was consistent with this finding (Appendix Section 3, Fig S18), and Notch1 NICD western blots reflected the findings on Figure 5.11 (Appendix Section 3, Fig S19 and S20). These data provided little support for the role of Notch1 in regulation of MRP1 under these conditions, and provided no evidence that doxorubicin-dependent MRP1 up-regulation could be inhibited using DAPT.

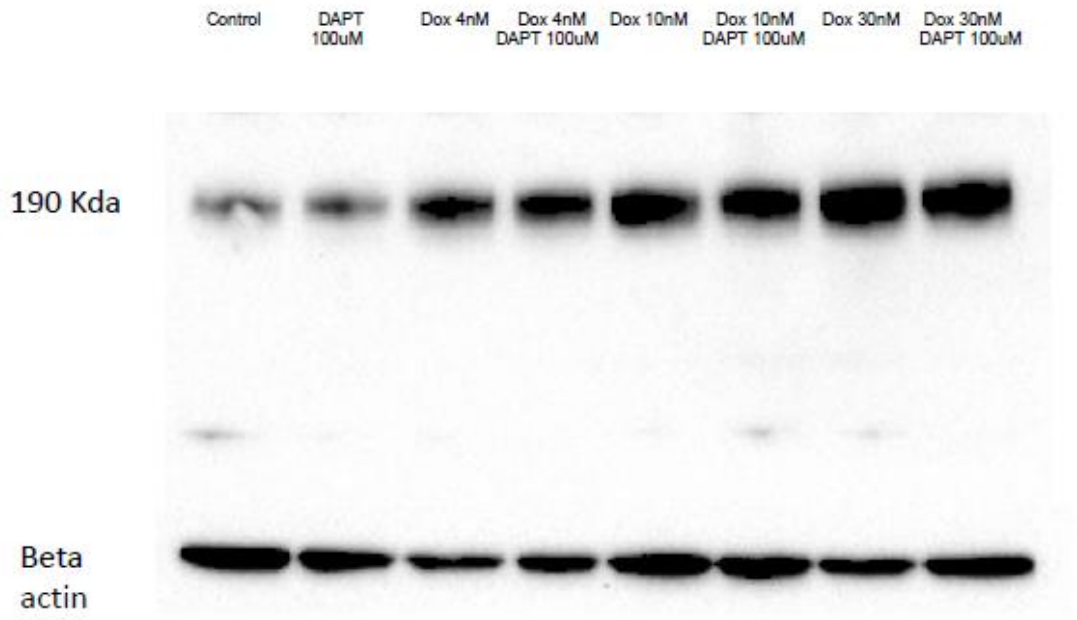


Figure 5.12: Western blot to examine MRP1 expression using MCF7 cell lysates treated with doxorubicin +/- DAPT for 4 days. Increasing doses of doxorubicin treatment led to up-regulation of MRP1 expression. DAPT treatment did not result in down-regulation of MRP1 expression however. Combination therapy of doxorubicin and DAPT did not cause significant down-regulation of MRP1 expression.

Finally, Pgp and BCRP expressions were also examined, to determine whether changes in expression of these pumps had potential to influence the effects on MRP1 and Notch1 induced by doxorubicin and/or DAPT. Pgp expression was up-regulated by doxorubicin treatment, although not in a dose-dependent manner (Fig 5.13). Interestingly Pgp expression was significantly up-regulated by DAPT treatment, either with or without doxorubicin, raising the possibility that enhanced Pgp function could interfere with the effective intra-cellular doses of either DAPT or doxorubicin in this experiment. BCRP expression did not alter significantly with doxorubicin or DAPT treatment (Fig 5.13).

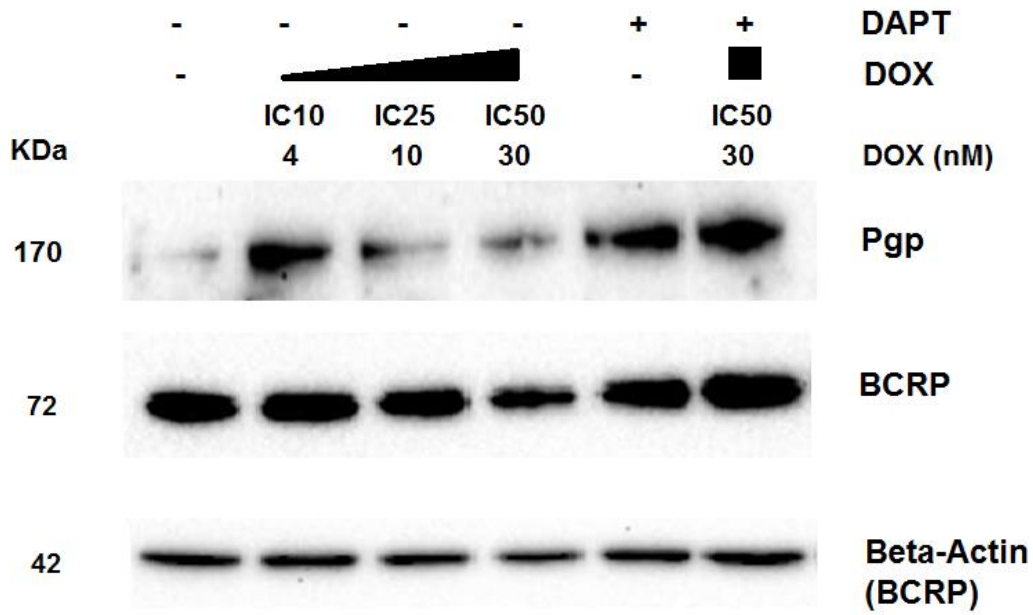


Figure 5.13: Western blot to examine Pgp and BCRP expression using MCF7 cell lysates treated with doxorubicin +/- DAPT for 4 days. Doxorubicin treatment (IC10; 4nM) up-regulated Pgp expression, but this up-regulation was not dose-dependent. DAPT treatment (100μM) with or without doxorubicin resulted in a significant up-regulation of Pgp expression. Doxorubicin or DAPT treatment did not cause a significant change in BCRP expression.

5.3 Discussion

This chapter contains data that can conveniently be divided up into clinical and tissue culture based analyses, and I will discuss these separately.

Notch and MRP in clinical cancers

The clinical findings demonstrate that Notch1 NICD expression and MRP1 expression were both significantly up-regulated after NAC in breast cancers. Furthermore, post-NAC MRP1 expression correlated significantly with post-NAC Notch1 NICD expression. As far as I am aware, this is the first study to examine the change in Notch1 NICD expression after NAC setting using immunohistochemistry. My data add further weight to the gene expression study mentioned previously (Gonzalez-Angulo *et al*., 2012), in which it was inferred that Notch activity was up-regulated after NAC in luminal-like tumours by the changes in expression of Notch target genes. However, unlike the Gonzalez-Angulo *et al* study, I found Notch1 NICD expression to be up-regulated by NAC in basal-like tumours as well as luminal-like tumours. Notch1 NICD expression or up-regulation did not significantly correlate with clinico-pathological parameters, response to NAC or subsequent survival on multivariate analyses, although it is clear that a cohort of only 29 patients is likely to be underpowered to detect these potential relationships. In contrast to my findings, some studies have revealed correlations between Notch1 expression and clinico-pathological features of tumours. For example, Wu *et al* examined total Notch1 expression in 44 patients diagnosed with cholangiocarcinoma using immunohistochemistry and found that Notch1 overexpression was more prevalent in patients with larger tumour size ($p=0.021$) (Wu *et al*., 2014b). Yao *et al* examined nuclear, cytoplasmic, and membrane Notch1 expression in 48 breast cancer patients. On multivariate analysis, high cytoplasmic Notch1 expression significantly correlated with nodal status (Yao *et al*., 2011). However, the literature are conflicting since Zardawi *et al* found that total Notch1

expression in 228 patients with operable breast cancer did not correlate significantly with clinico-pathological parameters or patient survival (Zardawi *et al*., 2010). Further investigations should clarify the relevance of Notch1 expression in breast cancer.

Notch and MRP1 in cell lines

The significant correlation I found between post-NAC Notch1 NICD and MRP1 expression supports the conclusions of the cell line-based study by Cho *et al*, in which MRP1 was found to be a direct transcriptional target of Notch1 (Cho *et al*., 2011) and allowed me to build the hypothesis that Notch-dependent up-regulation of MRP1 might be responsible for some degree of acquired chemotherapy resistance (see flow-chart Figure 5.1). I then proceeded to test this hypothesis using cell line models. Luminal breast cancer cell lines (MCF7 and T47D) were used in accordance with the findings from Gonzalez-Angulo *et al* study (Gonzalez-Angulo *et al*., 2012). Doxorubicin is an anthracycline that has similar clinical efficacy to epirubicin (Bontenbal *et al*., 1998), which is widely used in clinical practice. MRP1 does not efflux taxanes (Leonard *et al*., 2003), and hence were not used for this study. GSIs have been widely used in clinical trials in colorectal cancer (Strosberg *et al*., 2012) and in other solid tumours including breast cancer (Tolcher *et al*., 2012), providing credibility for its use in our study to achieve Notch inhibition. DAPT was used in this study to achieve Notch inhibition in line with other aforementioned *in vitro* studies mentioned in section 5.1 (Cho *et al*., 2011, Farnie *et al.*, 2007, Hallahan *et al*., 2004).

Three IC values of doxorubicin, IC10, 25 and 50, were chosen for use in combination with DAPT, itself at a wide range of potentially relevant conditions. Doxorubicin doses ranged from 4 to 70nM for MCF7 cell line, and 10 to 60nM for T47D cell line. These concentrations are comparable to concentration of doxorubicin detected by plasma

pharmacokinetic analysis by Chow *et al*. They showed that in 10 patients with solid tumours including breast cancer, the mean plasma end-of-infusion doxorubicin level was 83.8nM (range 59.1 to 106.9nM) (Chow *et al*., 2004). The physiological doses or plasma levels of DAPT in patients have not been well studied. However, concentration of 10mg/kg to 100mg/kg of DAPT has been used in mouse studies to achieve Notch inhibition (Lanz *et al*., 2003). The authors determined that these dosages resulted in plasma DAPT levels ranging from 140nM to 720nM. In our study, DAPT doses of 1nM to 1μM was initially used for the combination assay, which is comparable to the DAPT levels observed in the Lanz *et al* study. Furthermore, I also used higher DAPT doses of 1μM to 100μM in accordance with the *in vitro* study by Cho *et al* (Cho *et al*., 2011). However, these higher doses may be potentially harmful in clinical setting. Despite this, the combination therapy of doxorubicin and DAPT in luminal cell lines did not potentiate the efficacy of chemotherapy with additive effects seen at best.

Using western blots, I initially demonstrated activation of Notch1 NICD and MRP1 at only the lowest dose of doxorubicin (IC10). Further western blots at different biological time points showed that MRP1, but not Notch1 NICD, was up-regulated in a dose-dependent manner in response to doxorubicin treatment. This suggests that MRP1 up-regulation is not solely dependent on Notch. It is plausible that other regulators of MRP1 expression mentioned in section 1.8.4 and 5.1 may have a role in MRP1 up-regulation in response to treatment with doxorubicin. For example, Cyclo-oxygenase 2 (COX-2) activation can lead to up-regulation of MRP1 expression. Saikawa *et al* showed that COX-2 overexpression in colon cancer cell line, TR-5, led to up-regulation of *ABCC1* mRNA level and chemoresistance to cisplatin. Treatment with Cox-2 inhibitors subsequently enhanced chemosensitivity in this cell line (Saikawa *et al*., 2004). MicroRNA may also have a role in regulation MRP1 expression; Borel *et al* detected an inverse relationship between microRNA expression (miR-199a/b and miR-

296) and *ABCC1* mRNA expression (Borel *et al*., 2012). Marginal up-regulation of Notch1 NICD after doxorubicin treatment was observed which was not dose-dependent. This may potentially be explained by the difference in the cell density observed with varying dosage of doxorubicin treatment. For example, there is a greater density of viable cells after 4 days of IC10 treatment, compared to the IC50 treatment. Notch1 NICD is activated by extracellular ligands, and hence increased cell density may influence Notch1 NICD activation. This may also potentially explain the high basal Notch1 expression level in the control lysates.

It is important to note that MCF7/VP subline was used in the Cho *et al* study (Cho *et al*., 2011), which is etoposide-resistant. This is in contrast to the non-resistant MCF7 and T47D cell lines used in our study. An alternative approach may be to perform the combination assay on basal breast cancer cell lines, since the data from my immunohistochemistry study also showed up-regulation of Notch1 NICD expression in the basal-like breast cancers. This is in contrast to findings by Gonzalez-Angulo *et al* who found enriching of Notch signalling targets in non-basal-like breast cancers only (Gonzalez-Angulo *et al*., 2012).

Most concerningly, I was unable to demonstrate that DAPT achieved significant Notch1 NICD inhibition when cells were treated with DAPT alone. The western blot results showed that DAPT did not down-regulate Notch1 NICD or MRP1 expression in the MCF7 cell lysates. Cho *et al* showed that DAPT dosage of 50μM or greater was effective in achieving Notch1 NICD and MRP1 down-regulation. However, 100μM dosage of DAPT treatment in this study did not result in down-regulation of Notch1 NICD or MRP1 expression. This is perhaps explained by the significant up-regulation of Pgp expression with DAPT treatment, suggesting that Pgp may efflux DAPT to attenuate its effect on Notch activity. A potential solution may be to add a Pgp inhibitor

to the combination assay or to use siRNA to inhibit Notch1 expression at the transcriptional level. Other alternative methods of achieving Notch1 inhibition involve the use of monoclonal antibody against the Notch ligand TACE, or inhibitors that interfere with the S2 cleavage (Yin *et al*., 2010). It is also important to note that GSIs do not solely inhibit Notch. They have a range of other substrates, including E-cadherin and Erb-B4, which are involved in cell adhesion and apoptosis (Lleo, 2008), and therefore off-target effects may have a confounding influence.

Interestingly, subsequently to me completing my laboratory work, other researchers in my supervisor's group have continued with this *in vitro* work. They demonstrated that doxorubicin does indeed induce Notch activation and MRP1 expression and function. They also demonstrated that this can be inhibited by DAPT, leading to enhanced doxorubicin-induced cell death. Critically, however, they were only able to demonstrate this using short term assays, where cells were treated with doxorubicin for up to 24 hours, and by assessing induction of apoptosis in selected cell lines, rather than cell survival/proliferation. My data show that this combination treatment appears less promising in longer term assays and using cell survival as the end point, conditions that may potentially more accurately reflect chemotherapy in patients, where treatment lasts for many days and tumour cell survival is the most relevant measure of treatment failure or success.

Summary

The immunohistochemistry results from clinical tissue samples support previous *in vitro* studies that identified the significance of Notch signalling in regulating MRP1 expression and thereby chemoresistance in breast cancer. The MTT assays and the western blot results show that further investigations are required *in vitro* before Notch

inhibitors can be utilised to potentially improve the efficacy of chemotherapy in clinical setting.

6.0 Neoadjuvant endocrine therapy up-regulates Breast Cancer Resistance Protein expression but only pre-treatment levels predict survival

6.1 Abstract

The BCRP promoter has an oestrogen response element, and there is evidence that BCRP expression is regulated by oestrogen, with reports of oestrogen leading to either transcriptional up-regulation or post-transcriptional down-regulation. However, the relevance of this in oestrogen-dependent cancers remains unclear. Therefore, my aim in this chapter was to investigate whether BCRP expression in breast tumours is regulated by oestrogen and whether this impacts on outcome. 51 breast cancer patients receiving neoadjuvant endocrine therapy (NAET) were identified, and BCRP expression was examined by immunohistochemistry using matched pairs of core biopsy (pre-NAET) and surgical specimens (post-NAET). BCRP expression was significantly up-regulated after exposure to NAET ($p<0.0001$; Wilcoxon signed-rank test), with up-regulation seen in 48/51 cases. Neither pre- nor post-NAET BCRP expression correlated with tumour response to NAET. However, high pre-NAET BCRP expression independently predicted for poorer disease free survival (hazard ratio of 19.1; 95% confidence interval 1.05-348.2; $p=0.046$). Subsequent methylation sequencing analysis of cancer cell lines showed that the degree of methylation in the BCRP promoter region was inversely correlated to the protein expression of BCRP observed on immunoblotting. DNA was extracted from the paraffin embedded tissues used for immunohistochemistry and bisulphite-treated. Pyrosequencing was used to examine BCRP promoter methylation pattern. Comparison of protein expression and methylation levels in the clinical samples did not result in the significant negative correlation that was expected, and NAET did not consistently or significantly influence BCRP promoter methylation levels. The results from this chapter further highlight the significance of BCRP expression as a prognostic indicator. However, the significance

of DNA methylation in regulating BCRP protein expression requires further investigation.

6.2 Introduction

One of the main finding from Chapter 4 was that high BCRP expression post-NAC correlated with poor DFS. One hypothesis is that these induced high levels directly contributed to increased cancer cell survival. Therefore it would be of interest to investigate potential mechanisms responsible for defining these high BCRP expression levels, since modulation of these regulatory pathways could potentially be used to reduce BCRP expression and improve the efficacy of NAC. A considerable body of data concerning mechanisms responsible for regulation of BCRP expression is available – and this is summarised below. Potential regulation by oestrogen and at the level of promoter methylation are of particular relevance as these are investigated in this chapter.

Regulation of BCRP expression

Broadly, the mechanisms involved in deregulation of BCRP expression can be classified into DNA changes (gene amplification / mutation), transcriptional changes (use of multiple transcriptional start sites / histone modification / methylation of CpG sites), and post-transcriptional regulation by miRNAs amongst other mechanisms (Nakanishi and Ross, 2012).

The following studies elucidate the mechanisms involved in the regulation of BCRP expression mentioned above in order. Knutsen *et al* examined BCRP expression in breast cancer cell lines treated with chemotherapeutics. Using comparative genomic

and cDNA hybridisation, they determined that high BCRP expression was due to gene amplification (Knutsen *et al*., 2000). Imai et al examined *ABCG2* SNPs in 59 human tumour cell lines. cDNA sequence analysis showed that C421A mutation which substitutes Lys for Gln-141 was found in the breast cancer cell line MDA-MB-231. G34A mutation that substitutes Met for Val-12 was found in the MCF-7 cell line. BCRP proteins with coding changes encoded by these SNP variants proved to be expressed at lower levels than wild-type BCRP in murine fibroblast PA317 cell lines (Imai *et al*., 2002).

BCRP expression can also be influenced by alternative promoter usage. Zong *et al* identified three novel leader exons at the 5'-UTR region of mouse *ABCG2* mRNA. By using 5'-rapid amplification of cDNA ends (RACE), they showed that the three isoforms were differentially expressed from multiple promoters using different transcriptional start sites (Zong *et al*., 2006). Histone deacetylase inhibitors (HDIs) can increase chromatin acetylation, resulting in alteration of gene expression. Robey *et al* showed that treatment of kidney cancer cell lines with HDIs resulted in up-regulation of *ABCG2* mRNA expression (Robey *et al*., 2006).

Pan *et al* examined the role of miR-328 in post-transcriptional regulation of *ABCG2* in breast cancer cell lines. They showed that overexpression of BCRP protein was associated with reduction in miR-328 expression. Moreover, when breast cancer cells were transfected to over-express miR-328 BCRP protein expression was down-regulated. Furthermore, inhibition of miR-328 using a selective antagomir resulted in up-regulation of BCRP protein expression (Pan *et al.*, 2009).

The role of oestrogen in regulation of BCRP expression

Interestingly, oestrogen and its derivatives can regulate BCRP expression. Ee *et al* discovered a putative oestrogen response element (ERE) in the BCRP promoter region. They also showed that *ABCG2* mRNA expression was increased by the addition of 17β-oestradiol (E2) in the ER positive T47D and PA-1 breast cancer cell lines. This effect was reversed by the use of anti-oestrogen ICI 182,780. PA-1 cell lines were transfected with the BCRP promoter-luciferase reporter gene construct, along with an ERα expression vector. The subsequent luciferase assay showed that the promoter activity was enhanced in the presence of E2 (Ee *et al*., 2004). In contrast, Imai *et al* used western blots to show that BCRP protein expression was down-regulated in response to oestrogen in MCF-7 and T47D cell lines, and this effect was reversed by tamoxifen. They concluded that oestrogen caused post-transcriptional down-regulation of BCRP expression (Imai *et al*., 2005). Zhang *et al* treated MCF-7 cells with toremifene, an anti-oestrogen, and showed that both *ABCG2* mRNA and protein expressions were decreased by increasing doses of toremifene (Zhang *et al*., 2010). These studies show that oestrogen is likely to have a role in regulating BCRP expression. However, the study findings are conflicting as to whether oestrogen up-regulates of down-regulates BCRP expression. Moreover, these experiments are based on cell line models, and currently there is no existing literature on how BCRP expressions in the breast tumours of patients are modulated by the changes in oestrogen levels. The question is potentially of substantial clinical importance since any increases in BCRP expression caused by anti-oestrogen therapies would potentially increase subsequent tumour cell therapy-resistance.

The importance of methylation in regulating BCRP expression

The human BCRP gene is located on chromosome 4 at 4q22. It spans over 66 kb, and consists of 16 exons and 15 introns. BCRP promoter region was identified as the

region from -300 to -50 relative to the transcription start site, whose nucleotide position was found 529 base pairs upstream of the junction between exon 1 and 2 (Nakanishi and Ross, 2012). Bailey-Dell *et al* used bacterial artificial chromosome sequencing and Proscan, a promoter identification program, to identify the putative promoter region for the *ABCG2* gene. The functional importance of this putative promoter region was tested by luciferase reporter assay using MCF7 and human choriocarcinoma cell line known to have high endogenous BCRP expression. Transient transfection of these cell lines with series of deletion constructs, lacking segments of the putative promoter region, resulted in reduced transcriptional activity. This assay hence mapped the BCRP promoter region, which overlaps the adjacent CpG island (Bailey-Dell *et al*., 2001). CpG islands are regions with a high frequency of CpG sites. CpG sites are when a cytosine nucleotide occurs adjacent to guanine nucleotide (cytosine being 5 prime to the guanine nucleotide) in a linear fashion on the same strand (rather than the CG base pairing of cytosine and guanine between strands). The cytosine in CpG sites can be methylated to form 5-methylcytosine, which can lead to gene silencing in some cases, particularly within CpG islands. The methylated CpGs can prevent the binding of transcription factors, or recruit methyl-binding proteins which repress transcription.

Methylation of CpG islands is maintained by DNA methyltransferases (DNMT), and may be inhibited by 5-aza-2'-deoxycytidine (5-aza-dC) or by siRNAs specific for DNMTs, leading to reactivation of gene expression (Jung *et al*., 2007). Methylation status can be experimentally assessed by bisulphite treatment of DNA, which converts unmethylated cytosine to uracil, but leaves methylated cytosine intact. PCR amplification of the modified DNA then replaces uracil with thymine. The resulting product can be sequenced and aligned against a reference sequence to examine the methylation status of individual CpG sites – methylated sites are read as cytosine while unmethylated sites are read as thymine.

There is growing evidence that the methylation status of CpG islands in the *ABCG2* promoter region has a crucial role in regulating BCRP expression. Turner *et al* performed bisulphite sequencing analysis on multiple myeloma cell lines and determined that degree of methylation at the *ABCG2* promoter region was inversely correlated to the *ABCG2* mRNA levels. H929 cell lines were completely methylated in the 13 CpG sites examined, and subsequent 5-aza-dC treatment resulted in the up-regulation of *ABCG2* mRNA expression. Moreover, the cell line results were reflected in the patient samples where the percentage of methylated sites within the *ABCG2* promoter was inversely correlated to the *ABCG2* mRNA expression (Turner *et al*., 2006). Bram *et al* confirmed these findings when examining *ABCG2* promoter methylation patterns in breast, ovarian, and T-cell leukaemia cell lines (Bram *et al*., 2009). Furthermore, the increase in *ABCG2* mRNA levels observed after chemotherapy was due to site-specific demethylation, rather than global demethylation of CpG sites within the *ABCG2* promoter.

The above studies have examined the methylation status of BCRP promoter regions and found that the degree of methylation was inversely correlated to *ABCG2* mRNA expression levels. However, this has not been examined in detail in breast cancer cell lines or in clinical breast cancer samples. Furthermore, the above studies have not examined whether BCRP protein expression is influenced by the methylation status of BCRP promoter regions.

<u>Regulation of BCRP in NAET</u>

In the clinical setting, some patients are treated with NAET, which modulates oestrogen levels or its function (see Section 1.4). This provides an ideal setting for examining

BCRP expression levels before and after manipulation of oestrogen function and for investigating the regulatory mechanisms involved in defining these levels. When endocrine therapy is given in the adjuvant setting, patients who require chemotherapy are given this treatment first, followed by endocrine therapy. However, in NAET a subset of patients will subsequently receive adjuvant chemotherapy. Therefore, the impact of NAET on BCRP expression has potential therapeutic implications. For example, if NAET causes up-regulation of BCRP expression, this may potentially reduce the efficacy of adjuvant chemotherapy. Conversely, if NAET causes down-regulation of BCRP expression, this may support the use of concurrent chemotherapy and endocrine therapy. The latter is not currently recommended in clinical practice, although a phase II clinical trial has shown a marginal benefit in breast cancer patients randomised to letrozole plus cyclophosphamide, compared to letrozole alone (Bottini *et al*., 2006).

In this chapter, my aim was to investigate the effect of modulating oestrogen level or function on BCRP expression using clinical samples from patients treated with NAET. I then investigated whether BCRP promoter methylation was involved in defining BCRP expression in breast cancers. Initially, I used breast cancer cell lines for assay development, but I then applied assays to the same NAET clinical samples. This approach allowed examination of the relationship between methylation levels and protein expression in clinical samples. I also examined whether NAET influenced BCRP promoter methylation.

6.3 Results

6.3.1 BCRP expression was significantly up-regulated after NAET

I aimed to determine expression levels of BCRP both pre- and post-treatment in the tumours of a cohort of breast cancer patients treated with NAET. A cohort of 51 patients was assembled who had matching pre-NAET core biopsy tissues and post-NAET resection tissues (Table 3.2). Immunohistochemistry for BCRP was performed and quantified with the protocols used previously (Section 3.6), and I examined whether NAET modulates BCRP expression, and if expression levels have any significance in terms of response to NAET or subsequent survival. Detectable BCRP expression was mainly in epithelial cells, with localisation that was mainly cytoplasmic or membrane-associated with some accentuation of cell nuclei. This was similar to the staining patterns observed in the NAC cohort. Protein expression was successfully quantified in all 51 matched pairs of core and resection tissues; these data are shown in Appendix Section 1: Table S5. The following representative images demonstrate the changes in protein expression after exposure to NAET (Fig 6.1); BCRP expression was up-regulated post-NAET in majority of the cases; 48/51 cases (94%) (Fig 6.2), and this up-regulation was statistically significant (Wilcoxon signed-rank tests: $p<0.0001$) (Fig 6.2).

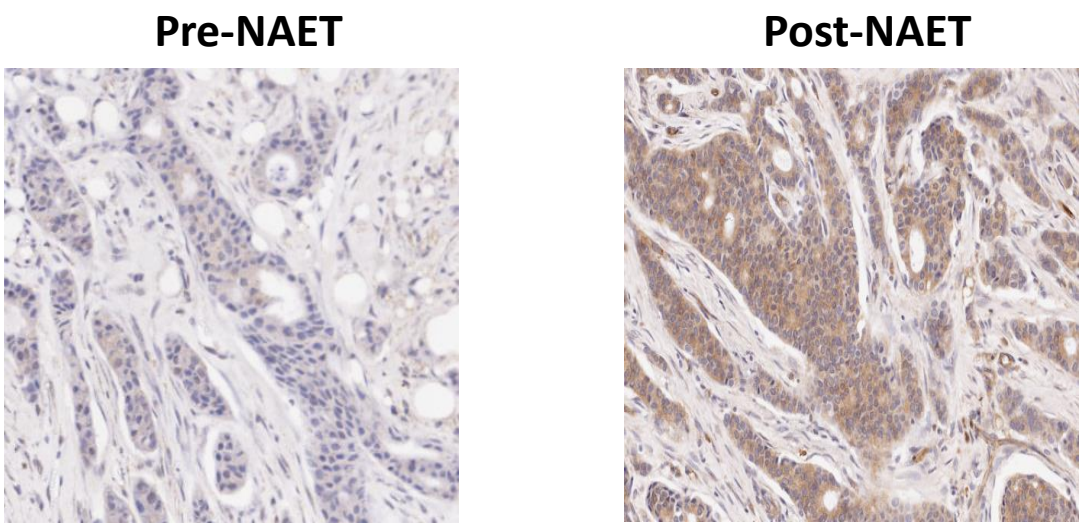


Figure 6.1: Immunohistochemistry for BCRP in the NAET cohort (x20 magnification). The representative matched pair of images show very little/no staining in the core tissues (pre-NAET), as opposed to stronger staining in the resection tissues (post-NAET). These images represent the significant up-regulation in BCRP expression.

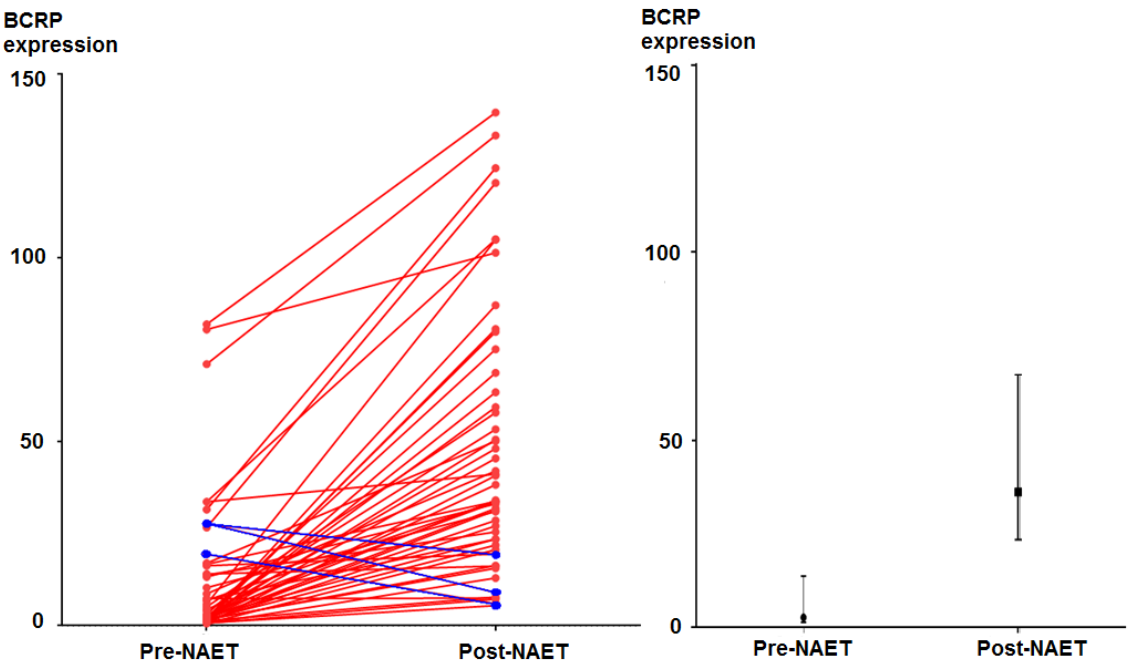


Figure 6.2: BCRP expression is up-regulated after exposure to NAET (red: up-regulation, blue: down-regulation; left). Matched pre-NAET and post-NAET breast cancer samples were stained using immunohistochemistry for BCRP. Expression within tumour cells was quantified as a histoscores of 0 – 300. Expression levels in matched samples are linked by lines coloured red or blue so as to indicate an increase or decrease in expression respectively. BCRP up-regulation was seen in 48/51 cases (94%). The right panel shows median histoscore values with interquartile range (n=51) and demonstrates a significant overall increase in BCRP expression after NAET exposure (Wilcoxon signed-rank tests: $p<0.0001$).

6.3.2. Correlation of BCRP expression with clinico-pathological parameters

My next aim was to analyse potential correlations between pre- and post-NAET BCRP expression levels, as well as the change in expression levels, against various clinico-pathological parameters (outlined in Table 3.2) using Spearman's rho analyses (Table 6.1). Change in BCRP expression level was determined by subtracting matched pre-NAET BCRP histoscores from post-NAET BCRP histoscores, with positive values

designated as up-regulation, and negative values designated as down-regulation. As stated in section 4.3.5, a more strict threshold of $p<0.01$ was used to indicate statistical significance. No significant correlations were observed for BCRP expression with the clinico-pathological parameters. The expression levels and the change in expression levels were then examined against the response to NAET. The latter was defined as the categorical changes in tumour stage (TNM staging) after NAET, or as the quantitative changes in tumour size derived from comparison of resection pathology tumour sizes to pre-NAET ultrasound tumour sizes (Table 6.1). No significant correlations were observed. NAET regimen and duration were also not significantly correlated to BCRP expression level pre- or post-NAET or the change in expression levels.

	BCRP pre	BCRP post	BCRP Δ
Age at diagnosis	0.16 (0.27)	0.1 (0.49)	0.06 (0.7)
Tumour factors determined pre-NAET			
Grade	0.12 (0.39)	-0.06 (0.68)	-0.05 (0.76)
T Stage	0.02 (0.87)	-0.21 (0.14)	-0.2 (0.16)
PR status	-0.17 (0.26)	-0.06 (0.68)	-0.03 (0.83)
Histological type	0.1 (0.5)	0.15 (0.3)	0.1 (0.5)
Tumour factors determined post-NAET			
T stage	0.07 (0.65)	-0.12 (0.39)	-0.08 (0.58)
Lymphovascular invasion	-0.04 (0.78)	-0.17 (0.24)	-0.12 (0.4)
Axillary metastasis	-0.05 (0.72)	-0.36 (0.011)	-0.3 (0.04)
Duration of NAET	-0.02 (0.89)	0.05 (0.71)	0.13 (0.35)
NAET regimen	-0.2 (0.17)	-0.09 (0.55)	-0.002 (0.99)
Tumour response			
Δ T stage	-0.09 (0.54)	0.05 (0.74)	0.14 (0.33)
Δ in tumour size	0.03 (0.84)	0.24 (0.11)	0.33 (0.02)

Table 6.1: Spearman's correlation coefficients demonstrating relationships between expressions pre- or post-NAET, or change in expression (Δ) for BCRP with clinico-pathological parameters and tumour response (p values are denoted in brackets).

6.3.3 Pre-NAET BCRP expression predicts disease free survival

Pre- and post-NAET BCRP expression levels as well as the change in expression levels were then analysed for correlations with DFS. For pre- and post-NAET expression levels, ROC curve analysis was performed to dichotomise expression into high and low expression groups. The cut-offs providing the highest combined sensitivity and specificity for prediction of DFS were selected; the respective cut-offs for histoscores were 13.6 for pre-NAET and 112.9 for post-NAET. For change in expression level, values were dichotomised into up-regulation or down-regulation. Kaplan-Meier Survival analyses were performed (Figure 6.3). There were no significant relationships between DFS and post-NAET BCRP expression levels or change in BCRP expression levels. However, pre-NAET BCRP expression levels significantly correlated with DFS (Log rank: p=0.027); patients with high BCRP expression had a relatively poor 5-year survival of 63.6%, compared to those with low BCRP expression for whom 5-year survival was an excellent 93.3% (Fig 6.3). Multivariate Cox regression analysis was performed taking into account the pathological factors typically regarded as having prognostic impact, including tumour grade, histological subtype, receptor status, axillary metastasis, tumour stage, receipt of adjuvant chemotherapy or radiotherapy and lymphovascular invasion. This showed that high BCRP expression pre-NAET independently predicted DFS, with a hazard ratio of 19.1 (95% confidence interval, 1.05-348.2; p=0.046).

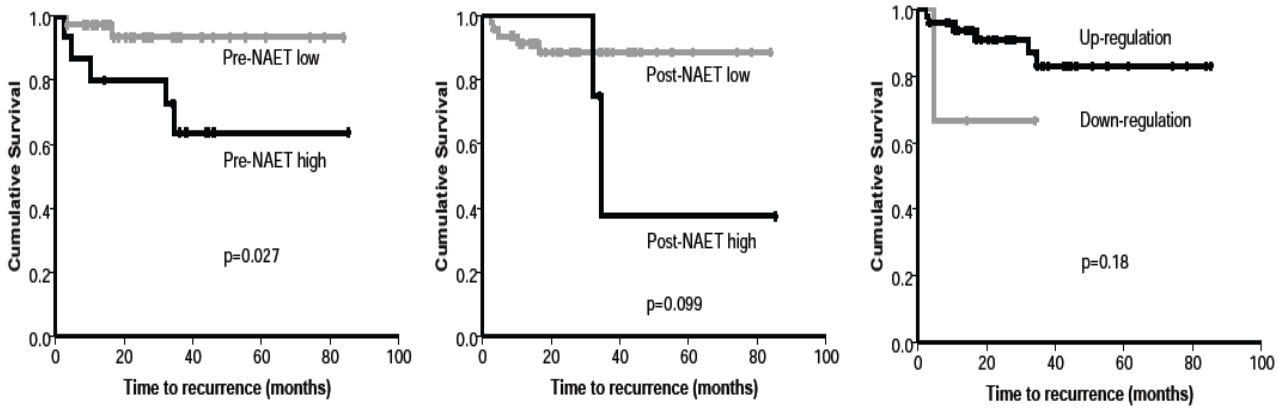


Figure 6.3: Kaplan-Meier survival analyses for BCRP expression levels and its change versus disease free survival. No significant correlation was detected for the change in expression levels or post-NAET expression level. However, high BCRP expression pre-NAET significantly predicted for poorer disease free survival.

6.3.4 Investigation of the role of BCRP promoter methylation in defining BCRP expression levels in breast cancers

My eventual aim was to investigate whether methylation status of *ABCG2* promoter region influenced BCRP protein expression observed in my cohorts of both NAC (see Section 4.3.1) and NAET (see Section 6.3.1) treated breast cancers and whether changes in methylation related to the changes in expression seen after treatment. In order to achieve this aim, *in vitro* experiments using cell lines were carried out initially to develop assays to assess BCRP promoter methylation. The cells lines used were the luminal breast cancer line MCF7, the non-neoplastic breast epithelial cell line HB2 and two multiple myeloma cell lines (H929 and HL60), for which BCRP promoter methylation levels have previously been reported (Turner *et al*., 2006). DNA was extracted from these cell lines and bisulphite treated before amplification of a 257bp BCRP promoter region that reportedly undergoes methylation that regulates BCRP expression (Chen *et al*., 2012a). Amplified DNA was cloned and individual clones were sequenced; this enabled examination of the methylation of 27 CpG sites for each

individual clone (see Section 3.11 to 3.14). Chen *et al* showed that the *ABCG2* promoter region was globally unmethylated in pancreatic cancer cell line SW1990 (Chen *et al*., 2012a). Turner *et al* showed that H929 cell line demonstrated infrequent methylation, while methylation was prevalent in HL60 cells (Turner *et al*., 2006). In accordance with published data (Chen *et al*., 2012a, Turner *et al*., 2006) both breast cell lines and H929 cell line demonstrated only infrequent methylation, while methylation was prevalent in HL60 cells (Fig 6.4).

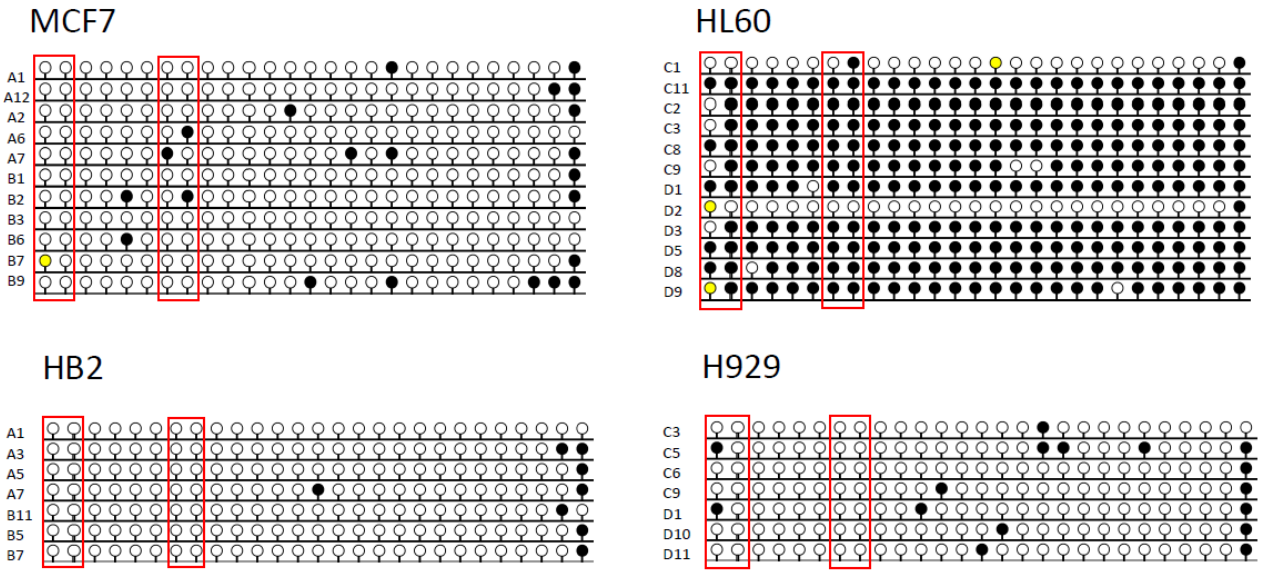


Figure 6.4: Examination of methylation status in the 27 CpG sites within the Chen *et al* amplicon. Black lollipops show methylated CpG sites, white lollipops show unmethylated CpG sites and yellow lollipops show data that were not interpretable. Each row represents individual clones. The majority of CpG sites were unmethylated in MCF7, HB2, and H929 cell lines. In contrast, CpG sites were globally methylated in HL60 cells. The red rectangles encompass the 4 CpG sites to be examined by pyrosequencing (see below).

Next, I examined whether BCRP protein expression correlated with these methylation levels. Western blots were performed using cell lysates from the four cell lines (see Section 3.10). Western blots showed that the level of BCRP expression was considerable in MCF7, HB2, and H929 cells, reflecting their unmethylated status. However, BCRP expression was attenuated in the HL60 cells, reflecting the methylated status (Fig 6.5). This demonstrates a potential inverse correlation between protein expression and methylation status of the BCRP promoter region, and implicated methylation as a key regulatory event.

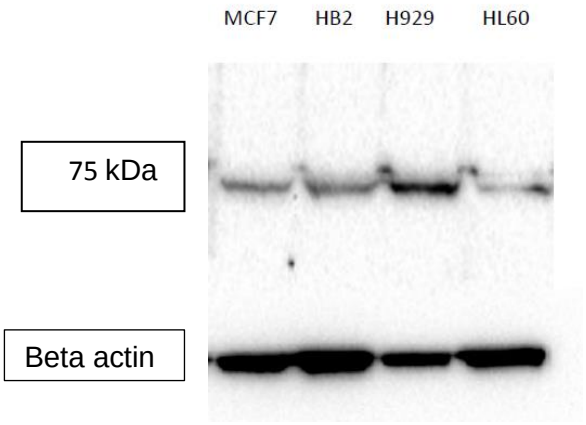


Figure 6.5: Western blot examining differential expression of BCRP in MCF7, HB2, H929, and HL60 cells.

Finally, I aimed to use pyrosequencing assays to determine methylation of specific CpG sites. This was because it was unlikely to be possible to amplify the relatively large amplicon described above from small, clinical FFPE samples, such as the breast biopsies I aimed to analyse ultimately. FFPE tissues have fragmented DNA, and for bisulphite modification and analysis the amplicon length should ideally be less than 150 base pairs (Patterson *et al.*, 2011). Therefore, this limits the number of CpG sites that can be examined. Given these constraints, two pyrosequencing assays were designed

within the Chen *et al* amplicon resulting in 4 representative CpG sites to be examined with pyrosequencing. The 4 CpG sites are highlighted within the red boxes on Figure 6.4; these were compatible with the criteria required for pyrosequencing and their methylation status appeared to be representative of the entire region for each cell line. Pyrosequencing analysis was performed for the 4 CpG sites in the 4 cell lines to confirm that pyrosequencing analyses reproduced the results obtained using bisulphite-treatment and molecular cloning (Table 6.2). This showed that both assays showed the same pattern of methylation between the cell lines and hence validated the use of the pyrosequencing assays.

CpG sites and degree of methylation	Position 165 (%)	Position 172 (%)	Position 236 (%)	Position 250 (%)
MCF7	0	2.95	1.77	8.63
HL60	60	59	58	63
HB2	0	0	1.72	6.51
H929	0	0	2.1	7.45

Table 6.2: Pyrosequencing analysis of the designated 4 CpG sites (see Fig 6.4) to be used for methylation analysis of clinical samples. As per molecular cloning analysis, high degree of methylation is seen in the HL60 cell line with barely detectable degree of methylation observed in the MCF7, HB2, and H929 cell lines. Therefore, this validated the use of pyrosequencing analysis for the clinical samples.

6.3.5 Pyrosequencing analysis of BCRP promoter methylation using clinical samples from patients treated with NAET

Having developed successful pyrosequencing assays that apparently provided insights into functionally-relevant BCRP promoter methylation, my next aim was to apply these assays to examine the methylation status of the BCRP promoter region in clinical samples from my neoadjuvant cohorts to determine if methylation status of BCRP promoter region influenced protein expression, and whether neoadjuvant therapy influenced the methylation status of the BCRP promoter region. Only the NAET cohort was available for this analysis, since I was unable to regain access to the clinical blocks for the NAC cohort. 46 core tissues and 51 resection tissues within the NAET cohort had sufficient tissue to attempt DNA extraction, bisulphite treatment, and pyrosequencing (see Section 3.11.2, 3.12, and 3.15). Data for the selected 4 CpG sites within the BCRP promoter region are shown in Appendix Section 1, Table S6. The analyses were successful for at least two CpG sites in only 36/46 pre-NAET core tissues, and for all four CpG sites in only 17/46 tissues. For the post-NAET resection tissues, pyrosequencing analysis was successful for at least two CpG sites in 48/51 cases and for all four sites in 33/51 cases.

The level of methylation varied from 0-40.7% in the pre-NAET samples, as opposed to 0-33.9% in the post-NAET samples. The medians and ranges of methylation for each CpG sites are shown in Table 6.3. This showed that methylation was barely detectable for the CpG sites at position 165, 172, and 236 pre- or post-NAET. The degree of methylation was more pronounced on position 250 however. Figure 6.6 shows that methylation levels did not change significantly after exposure to NAET. Although the change in methylation level in position 236 was statistically significant, the median change in methylation level was minimal (from 0 to 2.9%).

CpG sites and degree of methylation; median with range in brackets	Position 165 (%)	Position 172 (%)	Position 236 (%)	Position 250 (%)
Pre-NAET	2.5 (0-22)	0 (0-10.4)	0 (0-17.8)	10.1 (0-47.4)
Post-NAET	0 (0-12.3)	0 (0-7.8)	2.9 (0-16.9)	11.6 (0-33.9)

Table 6.3: Pyrosequencing analyses showing the degrees of methylation of 4 specific CpG sites. The degree of methylation is displayed for each site both pre- and post-NAET as percentages with mean and range.

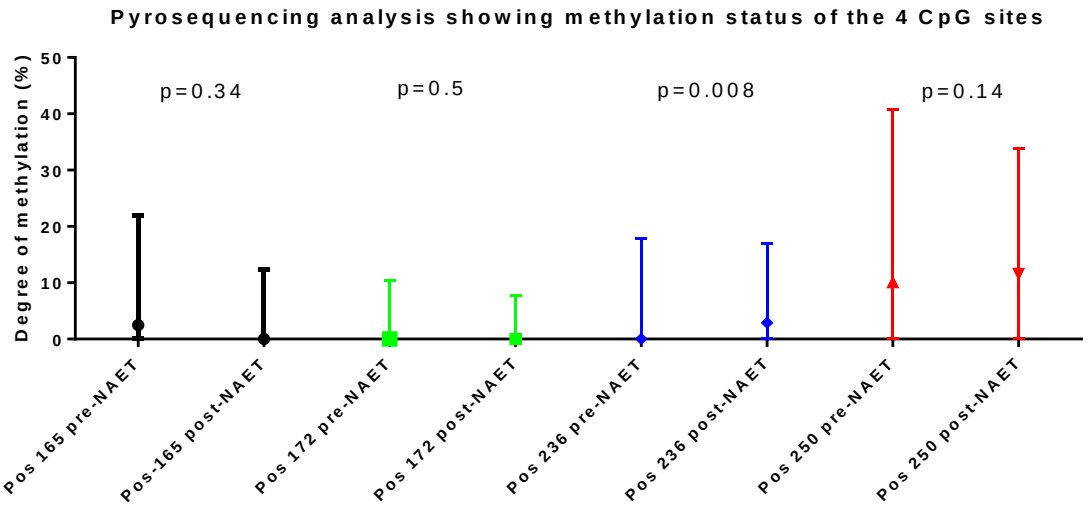


Figure 6.6: Analysis of pyrosequencing assay for the 4 CpG sites. Each CpG site is denoted in a different colour. The degree of methylation is displayed showing median with range. Apart from position 250, the remaining 3 CpG sites showed low degrees of methylation pre- or post-NAET. NAET did not result in change in methylation status of CpG sites shown by the p values (Mann-Whitney unpaired t-test) apart from position 236. However, the degree in change of median was minimal (from 0 to 2.9%) in position 236.

Examination of the change in methylation level after exposure to NAET for individual cases were possible in 32 cases for position 165 and 172, and for 13 cases for position 236 and 250. Increases in methylation were observed after NAET in 11/32 cases for position 165, 12/32 cases for position 172, 8/13 cases for position 236, and 7/13 cases for position 250 (Fig 6.7).

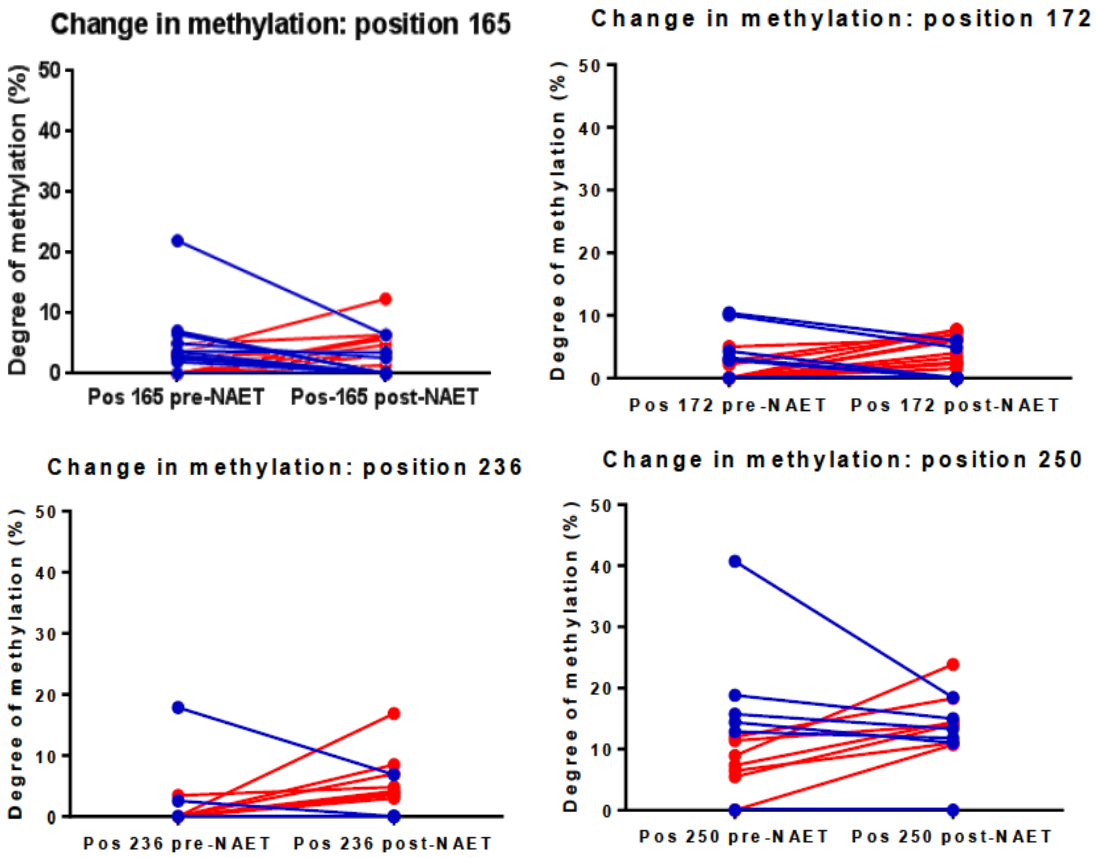


Figure 6.7: Matched comparison of methylation level after exposure to NAET. Each graph shows change in methylation level after NAET exposure for each CpG positions as determined by pyrosequencing; CpG positions 165 and 172 (n=32), and CpG positions 236 and 250 (n=13). Red lines denote increases in methylation level, and blue lines denote decreases in methylation level. Increases in methylation levels were observed in 34.3% and 37.5% for CpG positions 165 and 172. Increase in methylation levels were more frequently observed for CpG positions 236 and 250 (61.5% and 53.8% respectively).

6.3.6 Correlation of methylation levels in BCRP promoter region with protein expression

Using the data from the pyrosequencing analysis, I then examined whether methylation level of the CpG sites in the clinical samples correlated with the matching protein expression as determined in Section 6.3.1 by immunohistochemistry. This was performed using Spearman's rho analysis by comparing the degree of methylation as determined by pyrosequencing analysis with histoscore assessments of protein expression. Pre- and post-NAET histoscores were compared to pre- and post-NAET methylation levels respectively for the 4 CpG sites examined. The change in protein expression was determined by subtracting pre-NAET histoscore from the matching post-NAET histoscore. Similarly change in methylation level was determined by subtracting pre-NAET methylation level from the matching post-NAET methylation level. This enabled comparison of the change in methylation level with change in protein expression after exposure to NAET (Table 6.4). Pre- or post-NAET BCRP protein expression was not correlated with methylation level of the BCRP promoter region pre- or post-NAET respectively. Change in BCRP protein expression was subsequently compared to change in methylation levels. Again, no significant relationships were seen.

	Pre-NAET BCRP protein expression (n=51)	Post-NAET BCRP protein expression (n=51)	∆ BCRP protein expression (n=51)
Pos 165 pre n=36	0.17 (0.33)		
Pos 165 post n=48		-0.14 (0.35)	
Pos 165 ∆ n=32			0.1 (0.6)
Pos 172 pre n=36	0.08 (0.66)		
Pos 172 post n=48		-0.18 (0.22)	
Pos 172 ∆ n=32			-0.1 (0.59)
Pos 236 pre n=18	-0.04 (0.87)		
Pos 236 post n=36		0.32 (0.06)	
Pos 236 ∆ n=13			-0.03 (0.91)
Pos 250 pre n=18	0.05 (0.83)		
Pos 250 post n=36		0.11 (0.51)	
Pos 250 ∆ n=13			-0.35 (0.24)

Table 6.4: Spearman's correlation coefficients demonstrating relationships between protein expression pre- or post-NAET, or change in expression (∆) for BCRP versus methylation levels of 4 CpG sites pre- or post-NAET, or change in methylation (∆) of BCRP promoter region as determined by pyrosequencing (p values are denoted in brackets).

Finally, data concerning pre- and post-NAET samples were combined to determine whether methylation levels at each of the four CpG sites correlated with expression in up to 82 samples (Table 6.5). This analysis, surprisingly, showed that the degree of methylation on CpG position 236 was *positively* correlated with protein expression (rho=0.39; p=0.003). The methylation levels of the remaining 3 CpG sites did not show significant correlations with protein expression.

	Pre- and post-NAET combined methylation level			
	Pos 165 (n=82)	Pos 172 (n=82)	Pos 236 (n=54)	Pos 250 (n=54)
Pre- and post-NAET combined protein expression	-0.08 (0.5)	-0.02 (0.88)	**0.39 (0.003)**	0.18 (0.2)

Table 6.5: Combined analysis of protein expression versus methylation level. For each of the 4 CpG sites, the methylation levels pre- and post-NAET were combined and then compared against combined pre- and post-NAET protein expression using Spearman's rho analysis (p values are denoted in brackets). Methylation level of position 236 was positively correlated with protein expression (rho=0.39; p=0.003). No significant correlation was observed for the remaining 3 CpG sites however.

6.3.7 Comparison of BCRP promoter region methylation with clinico-pathological parameters

My next aim was to analyse potential correlations between pre- and post-NAET methylation levels of the 4 CpG sites within the BCRP promoter region as well as the change in methylation levels, and the clinico-pathological parameters (outlined in Table

3.2) using Spearman's rho analyses (Table 6.6). As stated in Section 4.3.5, a more strict threshold of $p<0.01$ was used to indicate statistical significance. The presence of axillary metastasis was positively correlated with the change in methylation of CpG site 250 (rho=0.83; p=0.001). Therefore, axillary metastasis was more likely to be detected in patients where the methylation level of CpG site 250 was increased after exposure to NAET. Multivariate linear regression analysis was performed to determine if change in methylation of CpG site 250 predicted the presence of axillary metastasis. The following clinico-pathological parameters were included in the analysis; tumour grade, pre- and post-NAET tumour stage, lymphovascular invasion, histological subtype, duration of NAET, and NAET regimen. The change in methylation of CpG site 250 still remained significant upon multivariate analysis in predicting the presence of axillary metastasis (p=0.013).

	Pos 250 pre	Pos 250 post	Pos 250 Δ	Pos 236 pre	Pos 236 post	Pos 236 Δ	Pos 172 pre	Pos 172 post	Pos 172 Δ	Pos 165 pre	Pos 165 post	Post 165 Δ
Age at diagnosis	0.35 (0.16)	0.02 (0.91)	0.24 (0.43)	0.44 (0.07)	0.21 (0.21)	0.19 (0.53)	0.01 (0.95)	0.05 (0.73)	0.11 (0.54)	0.14 (0.41)	0.15 (0.31)	-0.02 (0.9)
Tumour factors determined pre-NAET:												
Grade	0.29 (0.25)	-0.11 (0.53)	-0.42 (0.16)	0.2 (0.42)	0.03 (0.87)	0.35 (0.24)	-0.04 (0.82)	0.02 (0.91)	0.12 (0.51)	-0.21 (0.23)	-0.04 (0.78)	0.24 (0.19)
T Stage	-0.02 (0.92)	0.07 (0.71)	0.13 (0.67)	-0.23 (0.37)	-0.11 (0.53)	0 (1)	0.1 (0.58)	0.13 (0.4)	0.05 (0.79)	0.05 (0.8)	-0.03 (0.84)	-0.01 (0.95)
Histological subtype	-0.09 (0.74)	0.04 (0.81)	0.23 (0.45)	-0.37 (0.13)	-0.07 (0.7)	0.45 (0.12)	0.14 (0.43)	-0.02 (0.89)	-0.17 (0.36)	-0.04 (0.83)	-0.12 (0.42)	0.22 (0.23)
PR status	0.16 (0.52)	-0.04 (0.83)	-0.01 (0.98)	-0.18 (0.48)	-0.11 (0.54)	0.03 (0.92)	-0.17 (0.32)	-0.01 (0.96)	0.13 (0.48)	-0.06 (0.75)	-0.15 (0.36)	-0.07 (0.72)
Tumour factors determined post-NAET:												
T stage	-0.13 (0.6)	-0.16 (0.35)	0.34 (0.25)	-0.27 (0.27)	-0.13 (0.45)	0.26 (0.39)	0.31 (0.07)	0.25 (0.09)	0.09 (0.61)	0.29 (0.1)	-0.01 (0.95)	-0.08 (0.67)
NAET regime	0.03 (0.91)	0.16 (0.34)	0.18 (0.55)	0.2 (0.43)	0.08 (0.64)	-0.03 (0.93)	0.1 (0.57)	0.07 (0.62)	0 (1)	-0.16 (0.35)	-0.05 (0.73)	0.01 (0.97)
Duration of NAET	0.03 (0.91)	0.11 (0.51)	0.12 (0.69)	-0.32 (0.2)	0.29 (0.09)	0.16 (0.61)	0.23 (0.19)	0.11 (0.48)	0.04 (0.83)	-0.16 (0.35)	-0.19 (0.21)	0.1 (0.6)
Lympho-vascular invasion	-0.16 (0.54)	-0.02 (0.9)	0.29 (0.33)	-0.02 (0.95)	0.003 (0.99)	0.15 (0.63)	0.18 (0.31)	0.16 (0.29)	-0.09 (0.62)	-0.21 (0.22)	0.1 (0.53)	0.2 (0.29)
Axillary metastasis	-0.47 (0.05)	-0.19 (0.29)	**0.83 (0.001)**	0 (1)	-0.13 (0.48)	0 (1)	0.4 (0.02)	0.27 (0.08)	0 (1)	0.14 (0.45)	0.03 (0.87)	-0.004 (0.98)

Table 6.6: Spearman's correlation coefficients demonstrating relationships between methylation level pre- or post-NAET, or change in methylation level (Δ) with clinico-pathological parameters (p values are denoted in brackets).

Methylation levels and the change in methylation levels were then examined against the response to NAET. The latter was defined in two separate ways: the change in tumour stage after NAET (TNM staging), and as a quantitative change in tumour size derived from comparison of resection pathology to pre-NAET USS (Table 6.7). This analysis showed no significant correlation between pre- or post-NAET methylation levels and the change in methylation levels of the 4 CpGs sites with tumour response to NAET.

	Pos 250 pre	Pos 250 post	Pos 250 Δ	Pos 236 pre	Pos 236 post	Pos 236 Δ	Pos 172 pre	Pos 172 post	Pos 172 Δ	Pos 165 pre	Pos 165 post	Post 165 Δ
Δ T Stage	-0.07 (0.8)	0.08 (0.68)	0.32 (0.3)	0 (1)	0.19 (0.29)	0.39 (0.21)	0.26 (0.15)	0.01 (0.98)	-0.16 (0.39)	0.21 (0.24)	-0.06 (0.72)	-0.21 (0.27)
Δ in tumour size	-0.08 (0.77)	-0.13 (0.46)	0.1 (0.76)	0.04 (0.88)	-0.01 (0.95)	0.06 (0.86)	-0.11 (0.56)	-0.05 (0.77)	0.07 (0.73)	-0.03 (0.86)	-0.09 (0.58)	0.06 (0.76)

Table 6.7: Spearman's correlation coefficients demonstrating relationships between methylation levels pre- or post-NAET, or change in methylation levels (Δ) with tumour response (p values are denoted in brackets).

6.3.8 Comparison of methylation level of BCRP promoter region with disease free survival

Pre- and post-NAET methylation levels as well as the change in methylation levels were then compared against DFS using Kaplan-Meier Survival analyses, after dichotomisation as previously using ROC curve analysis (for pre-NAET and post-NAET levels; see selected cut-offs in Table 6.8) or simply into increased or decreased groups (for change in methylation).

	Pos 165	Pos 172	Pos 236	Pos 250
Cut-off methylation level pre-NAET	5.8	4.7	6.8	41.7
Cut-off methylation level post-NAET	1.8	12.4	11.9	28.9

Table 6.8: Cut-off values for methylation levels based on ROC curve analysis. Pre- and post-NAET methylation level cut-off were determined for the 4 CpG sites based on the highest point of combined sensitivity and specificity.

Subsequent Kaplan-Meier survival analyses showed that change in methylation levels after exposure to NAET did not predict DFS. High pre-NAET methylation levels in CpG positions 165 and 172 predicted for poor DFS (Log rank: $p=0.009$ and $p<0.01$ respectively). High post-NAET methylation levels in CpG positions 165, 172, and 250 also predicted for poor DFS (Log rank: $p=0.018$, $p=0.03$, and $p=0.003$ respectively (Fig 6.8). Multivariate Cox regression analysis was performed taking into account the pathological factors typically regarded as having prognostic impact, including tumour grade, histological subtype, receptor status, axillary metastasis, tumour stage, receipt of adjuvant chemotherapy or radiotherapy, and lymphovascular invasion. This showed that methylation levels pre or post-NAET did not predict DFS independently (Table 6.9).

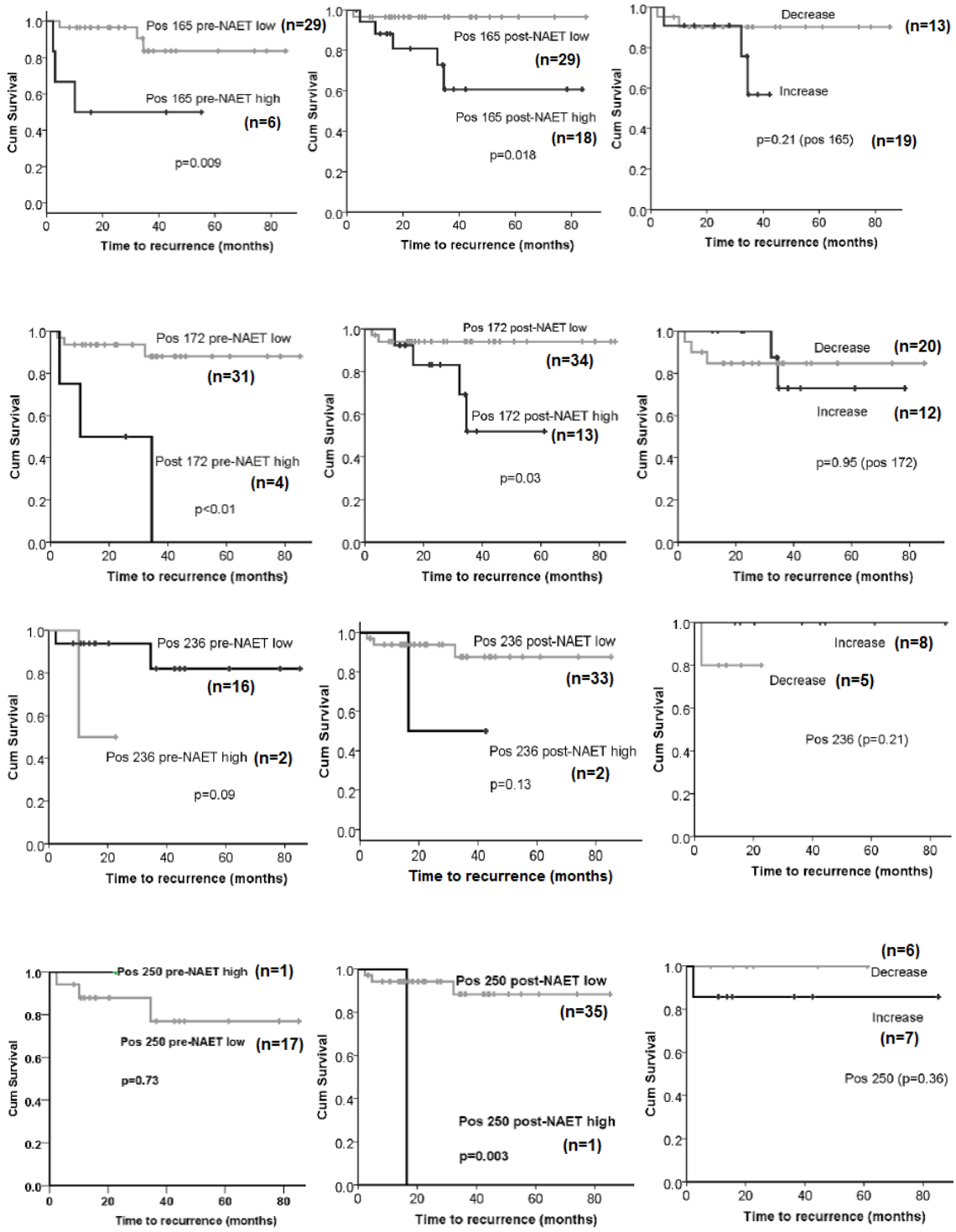


Figure 6.8: Kaplan-Meier survival analyses for BCRP methylation levels and its change versus disease free survival. Each row denotes each of the four CpG positions examined. For CpG position 165, high pre- and post-NAET methylation level predicted poor DFS (p=0.009 and p=0.018 respectively). For CpG position 172, high pre- and post-NAET methylation level predicted poor DFS (p<0.01 and p=0.03 respectively). For CpG position 250, high post-NAET methylation level predicted poor DFS (p=0.003).

CpG positions	Log rank p value	P value after multivariate Cox regression analysis
165 pre-NAET	0.009	0.21
172 pre-NAET	<0.01	0.22
165 post-NAET	0.018	0.24
172 post-NAET	0.03	0.26
250 post-NAET	0.003	0.93

Table 6.9: Results of multivariate Cox regression analysis on disease free survival versus methylation levels of the CpG sites examined. Although Kaplan-Meier analysis showed that the above methylation levels predicted for DFS on the Log rank p values, subsequent multivariate Cox regression showed that methylation levels of the examined CpG sites did not *independently* predict DFS.

6.3 Discussion

Impact of NAET on BCRP expression and its significance

In this chapter I first established that BCRP protein expression was up-regulated after exposure to NAET using immunohistochemistry performed on clinical FFPE samples. This has several significances. Firstly, this study clarifies the previous conflicting *in vitro* findings regarding the role of oestrogen in regulation of BCRP expression. Ee *et al* showed that *ABCG2* mRNA expression was enhanced by the addition of 17β-oestradiol, and this effect was reversed by the use of anti-oestrogen ICI 182,780. (Ee *et al*., 2004). In contrast, Imai *et al* used western blot experiment to show that BCRP expression was down-regulated in response to oestrogen, and this effect was reversed by tamoxifen (Imai *et al*., 2005). The results from immunohistochemistry in this study

concur with the latter study findings. This effect was irrespective of duration of NAET or the NAET regimen in this study. Secondly, given that BCRP expression is up-regulated after NAET, this raises the question regarding the efficacy of chemotherapy after NAET. It might be expected that administration of adjuvant chemotherapy would have reduced efficacy given the high BCRP expression post-NAET, although it remains unknown how long the raised BCRP levels would be maintained post-NAET. In the current study cohort, only 6/51 patients received adjuvant chemotherapy after completion of NAET, and no further tissues were available after adjuvant chemotherapy to further study BCRP expression. This question requires examination in a larger cohort to determine the role of sequential chemotherapy following NAET. Another key finding was that high BCRP expression *pre*-NAET independently predicted poorer disease free survival in patients. This has potential clinical significance in predicting which subset of patients may have poor disease free survival following NAET. These patients may benefit from adjuvant chemotherapy to potentially improve disease free survival, bearing in mind the potential question regarding the efficacy of chemotherapy post-NAET. It is difficult to envisage how the pre-NAET BCRP levels in tumour cells could have a direct functional link with response to therapy in these cases as these levels invariably change after NAET (and these changed levels were not associated with survival), and the main target for the aromatase inhibitors used in most of these patients is not even in the breast cancer cells themselves. It may be that BCRP levels are simply a marker of a more aggressive tumour. In support of this, high BCRP expression “side population” cells are known to be resistant to chemotherapy and radiotherapy, and have potential for colony formation and proliferation. These cells are enriched for cancer stem cells, characterised by their low accumulation of Hoechst dye, which is mediated by functional BCRP. They are also characterised by CD44 expression, and no or low CD24 expression (CD44+CD24-/low) (Nguyen *et al*., 2010). Therefore, high BCRP expression observed in this study may be a marker of cancer stem cell activity in breast cancer, and hence associated with higher rates of disease

recurrence. In any event, this finding requires further validation in an independent cohort. The semi-automated method of protein expression quantification in this study lends itself well for this purpose due to its reproducibility. It is worth noting that this study is the first to examine BCRP expression in an NAET cohort. Guidelines suggest NAET duration of 3 to 4 months (Chia *et al.*, 2010) although this is variable in clinical practice, as reflected by my study cohort. In any event, my study showed that NAET treatment duration did not have any impact on the degree of change in BCRP expression (see Table 6.1).

Investigation of methylation levels in BCRP promoter region *in vitro* and in clinical samples

An inverse relationship between BCRP promoter region methylation level and *ABCG2* mRNA and/or protein expression has been well established *in vitro*. Nakano *et al* treated human small lung cancer cell line, PC-6, with 5-aZa-dC to induce demethylation of the BCRP promoter region. This treatment resulted in increased expression at the mRNA and protein levels (Nakano *et al*., 2008). Similarly, our *in vitro* experiment results showed that hypomethylation seen in the MCF7 cell line was reflected in substantial BCRP protein expression seen on immunoblotting. However, no difference in the degree of methylation was observed between the MCF7 breast cancer cell line and the non-cancerous HB2 breast cell line. Similarly, Wu *et al* showed that *ABCG2* promoter region was hypomethylated in lactating mouse mammary gland (Wu *et al*., 2014a), supporting the cell line findings in this study that hypomethylation of *ABCG2* promoter region is not specific to cancer cells. The low levels of methylation in the four CpG sites examined in the clinical samples pre- and post-NAET mirror the low levels of methylation seen in the breast cell lines. Moreover, NAET exposure did not significantly change methylation levels in the matched FFPE tissue samples. This is a novel approach to examine the potential effect of endocrine therapy on methylation level of

BCRP promoter region. Other previous studies have examined the effect of DNA methyltransferase inhibitors on BCRP expression (Nakano *et al*., 2008, Turner *et al*., 2006), but not of therapeutic drugs. It is also worth bearing in mind that successful pyrosequencing analyses of all 4 CpG sites were possible in only 17 pre-NAET core tissues and 33 post-NAET resection tissues respectively out of total of 51 cases. This is due to the relative lack of core tissue availability compared to resection tissue. Although frozen tissue is superior in terms of DNA integrity, patients treated at LTHT do not routinely have frozen tissues available for research purposes. DNA extracted from FFPE breast tissue blocks has undergone formalin fixation, which cause degradation and cross-linking between proteins and protein/DNA bases, resulting in a lower DNA yield. In addition, bisulphite conversion also further degrades DNA.

Comparison of protein expression and methylation levels in the clinical samples did not result in the significant negative correlation that was expected. Although previous studies report an inverse relationship between *ABCG2* mRNA expression and level of methylation in the BCRP promoter region (Bram *et al*., 2009, Turner *et al*., 2006), no such direct correlation could be detected when protein expression was compared to methylation levels in the clinical samples. Therefore, other mechanisms stated on section 6.1 such as gene amplification, transcriptional regulation unrelated to DNA methylation, and post-transcriptional regulation by miRNAs amongst other mechanisms are likely to have further influences on BCRP protein expression (Nakanishi and Ross, 2012).

Comparison of methylation levels and their change showed that axillary metastasis was more likely to be detected in patients where the methylation level of CpG site 250 was increased after NAET exposure. This proved significant on multivariate analysis. A decrease in BCRP protein level might have been expected in cases where promoter

methylation levels increased after NAET. However, no such inverse correlation was detected between methylation level and protein expression in this study, and it may be that this correlation between BCRP promoter dynamics and breast cancer behaviour is not directly related to BCRP function, but is a marker of a different aspect of the biology of the cells. However, in practical terms, examining methylation levels at CpG sites within the BCRP promoter region offers potential prognostic insights. Pre- and post-NAET methylation levels in multiple CpG sites correlated with disease free survival on Log-rank univariate analysis, with higher methylation levels predicting poorer disease free survival. However, on multivariate analysis none of the methylation levels independently predicted disease free survival, demonstrating that these methylation levels mark aspects of cancer biology that are already encompassed within the routine clinico-pathological assessments.

Given that BCRP expression was shown to have prognostic significance in the NAC cohort, the same approach of promoter methylation analysis could have been performed – and indeed this was my intention although I was unable to access the samples for further analysis. This would have been of interest to study the potential effect of chemotherapy on methylation levels of BCRP promoter region, and whether methylation was involved in determining the post-NAC BCRP levels that were associated with survival.

7.0 Discussion

My work has focused on the analysis of xenobiotic transporter expression in the context of neoadjuvant systemic therapy (NST) in breast cancer. Pgp, MRP1, and BCRP, have been implicated in resistance to chemotherapy given their ability to efflux chemotherapeutics from cancer cells. The translational research carried out in this study aimed to identify potential molecular markers that predict tumour response or patient survival, and also examined potential underlying molecular mechanisms responsible for resistance to NST.

BCRP as a prognostic marker

This research strategy identified BCRP protein expression as having significance in independently predicting disease free survival in both NAC and NAET cohort. This has potential importance in the clinical setting since further adjuvant therapeutic strategies could be required to improve DFS in patients with high BCRP expression pre-NAET or post-NAC. It is interesting that BCRP expression has prognostic significance in both cohorts despite their considerable differences. In the NAET cohort, the majority of patients were post-menopausal with lower stage and lower grade breast cancers. All have high ER expression. In the NAC cohort, the majority of patients were pre-menopausal with higher stage and higher grade breast cancers. Exposure to NAET resulted in overall up-regulation of BCRP expression with up-regulation seen in 48/51 cases. In contrast, after exposure to NAC, no overall up-regulation of BCRP expression occurred with up-regulation seen in 23/39 cases. Despite these differences, BCRP expression remains a potential prognostic marker of disease free survival. This finding is further supported by other studies where BCRP expression was examined using immunohistochemistry in lung (Kim *et al*., 2009) and pancreatic cancer (Lee *et al*., 2012). Both studies have shown BCRP expression to be associated with poor DFS,

although this was not in the context of any neoadjuvant therapy. Tumour response to NST is categorised using the RESIST criteria which defines tumour response into four categories (Table 1.3). In our study BCRP expression in both cohorts did not correlate with tumour response, despite the significant correlations observed with DFS. This may have been due to the categorical nature of the RECIST criteria giving a non-continuous representation of response; therefore, actual change in tumour size was also compared to BCRP expression but this again did not detect any significant correlation. This raises the question about whether clinical tumour response to NST is representative of changes in subsequent DFS. So far studies have shown that only pCR is a marker of improved DFS (Cortazar *et al*., 2014).

Gene regulation of xenobiotic transporters: therapeutic implications

Two hypotheses concerning gene regulatory pathways were generated by examining the changes in xenobiotic transporter expression after NST. A key reason for attempting to understand these gene regulatory pathways better is to assess whether therapeutic strategies can be designed to manipulate the gene expression of key molecules, rather than their function as with traditional small molecule inhibitors. This is particularly relevant to the first hypothesis below. A second reason is to understand whether and how therapies already in clinic impact on expression of key molecules, and therefore whether therapy induced changes in expression should be taken into account when planning treatment.

Firstly, significant correlations between MRP1 and Notch1 NICD expressions observed after NAC, as well as the relevant study findings by Cho *et al* and Gonzalez-Angulo *et al*, resulted in the development of hypothesis that Notch1 NICD inhibition would result in abrogation of MRP1 activity and thereby enhance the efficacy of chemotherapeutics

(Cho *et al*., 2011, Gonzalez-Angulo *et al*., 2012). Chemoresistance continues to remain a challenge and alternative strategies are in clinical practice such as tailoring of NAC regime according to tumour response, but pCR rates remain low and mastectomy rates remain high (13.3% and 62.2% respectively in my study cohort). Therefore, manipulation of molecular pathways involved in chemoresistance is likely to have impact on improving patient outcome. My own data suggested that Notch1 inhibition did not synergise with chemotherapy in terms of reducing cell survival/proliferation – however, I also demonstrated poor efficacy of the Notch1 inhibitor used, DAPT. Further research in my supervisor's group shows potential clinical relevance as they showed that Notch1 NICD inhibition does indeed enhance chemotherapy efficacy *in vitro*. A further promising study has been reported by Schott *et al*. They conducted a pre-clinical and a phase I clinical trial evaluating the use of the GSI MK-0752 in combination with docetaxel. For the pre-clinical study, human breast tumour biopsies were transplanted into the mammary gland fat-pad of immune deficient mice. Treatment with MK-0752 achieved reduction in the breast cancer stem cell population in this context. Furthermore, compared to each therapy alone, a combination therapy of docetaxel and MK-0752 resulted in enhanced reduction of tumour size in mice. In the clinical trial, 30 breast cancer patients received MK-0752 with docetaxel and a favourable safety profile was reported (Schott *et al.*, 2013).

In my study, Notch1 NICD expression was significantly up-regulated in breast cancer patients treated with NAC. Whilst I did not establish that MRP1 is an important down-stream target of Notch1 NICD activation, Notch is an oncogene and aberrant Notch signalling plays an important role in breast cancer. Its multiple roles in breast cancer include tumour proliferation, protection of tumour cells from apoptosis, breast cancer stem cell self-renewal, and angiogenesis of the surrounding stroma (Brennan and Clarke, 2013). Therefore, Notch inhibition remains an attractive target, and further pre-

clinical studies are required before Notch inhibitors are to be established for potential clinical use.

Secondly, the hypothesis that oestrogen is involved in defining BCRP levels, potentially via DNA methylation was examined in clinical samples using immunohistochemistry and pyrosequencing assays. While, it was clear that oestrogen activity may impact on BCRP expression, there was no evidence that promoter methylation was involved in this – and indeed I did not find an inverse correlation between protein expression and promoter methylation in the clinical samples. The key clinical implication of these findings relate to the up-regulation of BCRP by NAET and whether this impacts on response to subsequent therapies (see Section 6.3).

BCRP as a drug target

In this study, high BCRP expression post-NAC predicted poor DFS. At present, patients treated with NAC do not receive further systemic treatment unless they are ER positive, and subsequent management is simply clinical surveillance. The group of ER negative patients include patients with triple negative breast cancer who have the worst prognosis due to lack of alternative systemic treatment apart from chemotherapy. Instead of clinical surveillance, patients identified as high risk of recurrence due to high BCRP expression post-NAC may in theory benefit from drugs that target BCRP itself, such as BCRP inhibitors, immediately after surgery. Similarly, patients with high BCRP expression pre-NAET should perhaps be treated with BCRP inhibitors prior to NAET. Such anticipatory approaches are likely to be more successful since the level of potential systemic tumour burden is likely to be significantly lower than at the time of symptomatic disease recurrence where cure becomes unlikely due to the high level of systemic tumour burden (Arteaga, 2013). The following studies highlight the

development of BCRP inhibitors. Kruijtzer *et al* treated patients with range of solid tumour, including ovarian and colon cancer, with chemotherapeutic topotecan and GF120918, a BCRP inhibitor also known as Elacridar, as opposed to topotecan alone. It is worth noting that GF120918 is a dual inhibitor which also inhibits Pgp. The combination treatment resulted in increased bioavailability of topotecan to 97.1%, compared to 40% in topotecan alone group. The addition of GF120918 to topotecan did not result in significant additional toxic side-effects (Kruijtzer *et al*., 2002). However, the study cohort included only 16 patients and did not include patients with breast cancer. Rabindran *et al* transfected the MCF-7 cell line to over-express BCRP. They examined the effect of Fumitremorgin C (FTC) on accumulation of mitoxantrone, doxorubicin, topotecan, and paclitaxel in the BCRP overexpressing MCF-7 cells. Unlike GF120918, FTC is a specific inhibitor of BCRP. Treatment with FTC resulted in potentiation of toxicity of all the aforementioned chemotherapeutic drugs apart from paclitaxel (Rabindran *et al*., 2000). However, FTC is not currently used *in vivo* due to its significant neurotoxic side effects in mice and other animals (Allen *et al*., 2002). Allen *et al* showed that an analogue of FTC, Ko143, achieved potent inhibition of BCRP in human ovarian carcinoma cell line and mouse fibroblast cell line, demonstrated by increased cellular mitoxantrone accumulation. Furthermore, Ko143 was well tolerated in mice with no evidence of tissue pathology in major organs under histological analysis (Allen *et al*., 2002). Therefore, Ko143 could potentially be used in future clinical trials to investigate whether BCRP inhibition results in improved patient outcome. Interestingly, tyrosine kinase inhibitors (TKIs) such as imatinib (Gleevac) and gefitinib (Iressa) have also been implicated in BCRP inhibition. The former is used for treatment of chronic myeloid leukaemia. A study by Ozvegy-Laczka *et al* showed that TKIs caused inhibition of the ATPase activity of BCRP. TKIs also inhibited BCRP-dependent active fluorescent Hoechst dye extrusion in a BCRP-over-expressing HL60 cell line. This shows that functional efflux activity of BCRP protein is attenuated by TKIs without

affecting expression. Furthermore, treatment with TKIs increased the cytotoxicity of mitoxantrone in these cell lines (Ozvegy-Laczka *et al*., 2004).

Prognostic markers: are single markers enough?

Given the complexity of breast cancer biology, information gained from panels of molecular markers is likely to be more informative than individual markers in the clinical setting. Such a panel of molecular markers that provides prognostic information is already in current clinical use in the form of MammaPrint (Agendia) and OncotypeDx (Genomic health) to aid treatment decisions regarding the use of adjuvant chemotherapy (DeMichele *et al*., 2013). The former is a 70-gene signature test in which the genes were identified from a case-control study of young, node negative breast cancer patients with more than 10 years of follow-up. Gene expression profiles of patients who suffered from disease relapse were distinct from patients who remained disease free. The latter examines the expression of 16 outcome-related genes and 5 reference genes, as measured by RT-PCR. These expressions are combined using a mathematical algorithm to calculate the risk of recurrence in patients with ER positive, lymph node negative breast cancer (Dowsett and Dunbier, 2008). These clinical tools provide objectivity in difficult clinical circumstances where the potential survival gain in treating patients with chemotherapy has to be balanced against its potential significant side-effects and costs. As yet, MammaPrint or OncotypeDx have not been validated in the neoadjuvant setting. Future research in breast cancer patients treated with NAC will hopefully identify similar panel of molecular markers, which could be utilised to accurately predict response to NAC and survival in individual patients. No panel of molecular markers exist for patients who are treated with endocrine therapy, perhaps reflecting the fact that patients who are ER positive have an already relatively good prognosis. For patients who are treated with NAET, Ellis *et al* developed pre-operative endocrine prognostic index (PEPI) which consists of pathological tumour size and

nodal status, Ki67 level, and ER status to predict the risk of disease relapse. Those patients who had high PEPI score have higher likelihood of disease relapse and should be recommended adjuvant chemotherapy (Ellis *et al*., 2008). This has particular relevance in our study since BCRP expression was up-regulated by NAET and hence the efficacy of adjuvant chemotherapy has to be in question. My study cohort of 51 patients treated with NAET lacks power to answer this question clearly. However, such question could potentially be answered by the utilisation of clinical samples from trials such as the Perioperative Endocrine Therapy- Individualising Care (POETIC) study (Dowsett *et al*., 2011). In this national multi-centre randomised trial, post-menopausal ER positive breast cancer patients received four weeks of aromatase inhibitors peri-operatively or received no peri-operative treatment with subsets of patients going onto receive adjuvant chemotherapy based on criteria such as PEPI. Examination of BCRP expression in such cohorts has feasibility as change in BCRP expression in the NAET cohort was not influenced by the length of therapy or regime, although unfortunately the samples are currently available only for the trial protocol.

NST has shown to be a valuable treatment modality in aiding identification of predictive markers and in looking at molecular pathways and changes involved in resistance to NST. Its use is currently limited to patients with locally advanced breast cancer who would be obligate candidate for mastectomy at the time of diagnosis. It achieves down-staging of locally advanced breast cancer to enable breast conservation surgery in a subset of patients. In patients where tumour to breast volume ratio is small enough to enable breast conservation surgery at the time of diagnosis, NST is not routinely offered as there is no perceived advantages. In contrast, due to equivalent DFS and OS between NST and adjuvant systemic therapy, international expert consensus by Berruti *et al* argue that NST should be used in routine management of operable breast cancer (Berruti *et al*., 2011). There is a rationale behind this approach, but this has to

be balanced against the likelihood that surgical management of breast cancer will not alter at the end of NST as all patients will receive at least breast conservation surgery at present. More patients will require treatment monitoring in the form of MRI with added costs, and patients will have to live with tumours in situ during therapy, which can result in raised anxiety and psychological morbidity. However, if high proportions of patients treated with NST achieved complete pathological response this would extend the role of NST in a greater number of patients, especially if these patients could be spared surgery. At present, all patients undergo surgery even if MRI monitoring indicates complete radiological response, and this is a pragmatic approach given that MRI is not sensitive enough to determine complete pathological response. Questions about the need for surgery in patients who achieve complete pathological responses have been raised by Rea *et al*, who proposed future trials to investigate the area (Rea *et al*., 2013). However, with the current available regime of NST, the proportion of patients who achieve complete pathological response remains low, especially in the NAET cohort. Hence, the current approach of limiting NST only to patients who are obligate candidates for mastectomy may be appropriate. However, it is hoped that with future molecular research and targeted drug development, the efficacy of NST may improve to such an extent that NST can be extended to a wider cohort of patients.

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9.0 Appendix

9.1 Recipe list

TBS	60ml 2.5M NaCl 20ml 1M Tris Hcl Make up to 1L
TBST	As above with the addition of 1.25ml of 10%v/v Tween-20
10mM Citric acid buffer (pH 6.0)	Add 2.1g citric acid monohydrate to 800ml of distilled water. Add 13ml 2M NaOH solution dropwise until pH of 6.0 is reached. Make up to a final volume of 1L using distilled water.
2M Sodium hydroxide	Add 8g of sodium hydroxide to 100ml of distilled water
0.3% Hydrogen peroxide	Add 1ml of 30% hydrogen peroxide to 99ml of 100% methanol
Scott's tap water	Add 20g of magnesium sulphate and 3.5g of sodium bicarbonate to 1L of distilled water

RIPA buffer	Tris-HCL 10mM EDTA 1mM EGTA 0.5mM NaCl 140mM SDS 0.1% Triton x100 1% Na Deoxycholate 0.1% H2O 1mM PMSF 1mM DTT 1xPIC (P8340; Sigma-Aldrich)

DNA buffer	1M Tris-HCL, 1M. pH 8.0	100ml
	0.5M EDTA	100ml
	dH_20 water	300ml

TAE buffer	Glacial acetic acid Tris base 0.5M EDTA
1% agarose gel	Add 2g agarose to 200ml 1x Tris Acetate EDTA (TAE) buffer (pH 8.0) and heat in 900W microwave for 2 minutes. Once cool, 6ul of ethidium bromide was added.
LB solution	Add a sachet of LB broth EZMix™ powder (L7658; Sigma-Aldrich) to 500ml of distilled water, and autoclave for 1 hour.

LB agar plate	As above with the addition of 7.5g agar and 500ul of ampicillin. 25ml of the resulting solution was poured onto each Petri dish to set.

9.2 Supplementary tables

Total automated histoscore	Total histoscore BK	Total histoscore BW
24.2	37.6	52
47.7	41.6	49.4
0.7	0	0
38.6	106.1	84.6
82.3	43.2	125.1
84.5	45	112.5
65.7	100.8	65.5
84.2	138.2	136.2
129	209.7	173.6
35.2	5.3	40.6
9.6	0	91.9
34.3	12.8	51.3
28.6	36.3	95.2
67.3	180.5	115.1
1.2	0	0
11.1	0	0
15.7	9.3	25.7
27.5	4.8	28.9
0.8	0	0
153.8	220.4	197.5
30.6	8	31.9
6.2	0	0
3.4	0	0
13.3	0	0
10.3	13.3	33.1
0.4	0	0
50.1	15.9	27.2
6.2	0	0
1	0	0
60.4	43.1	90.4
1.8	0	0
1.4	0	0
12.2	0	9.9
61	117.3	104
172.4	263.1	275
0.3	0	0
107	164.4	102.3
30.4	46.1	78.9
170.1	133.7	96.6
8.1	4.7	3.6
31.5	2.5	9.5
20.2	1.8	8.8
20.1	10.9	0
34.2	43.7	18
4	0	0
81.1	134.3	83.3
17.4	0	6
32.3	34.4	45
5.4	4.5	6.3
64	78.1	141.6
1	0	3.1
162.6	170.3	208.2
61.7	33.3	126.9
89.2	115.4	130.5

Table S1: Comparison of manual versus automated histoscores

SLIDE no	Pre-NAC score BK	Pre-NAC score BW	Post-NAC score BK	Post-NAC score BW
1	0	0	3	3
2	3	3	5	5
3	3	2	6	4
4	3	4	0	0
5	0	0	0	0
6	3	3	5	4
7	0	0	2	3
8	0	0	0	0
9	0	0	4	4
10	3	3	7	6
11	2	3	3	3
12	0	0	0	0
13	0	0	8	8
14	4	4	5	5
15	2	2	7	6
16	0	3	8	8
17	0	0	7	7
18	0	0	4	4
19	2	2	7	7
20	2	2	3	4
21	3	3	4	4
22	0	0	3	3
23	0	0	5	5
24	4	3	3	3
25	4	3	0	0
26	0	0	6	6
27	0	0	2	2
28	0	0	7	7
29	0	0	3	3

Table S2: Allred scores for Notch1 expression

Patient number	DNA concentration pre-NAET (ng/ul)	DNA concentration post-NAET (ng/ul)
1	62.2	50.2
2	38.5	295.9
3	22	97
4	53.5	156.1
5	12.7	958.1
6	48.9	60.4
7	81.8	77.8
8	139.1	174.9
9	9.2	454.9
10	6.1	98.8
11	94.8	24.6
12	29	299.2
13		635.2
14	46	122.8
15	8	138.8
16	27.1	161.4
17	47.4	129.3
18	48.5	135.8
19	16.1	124.3
20	45.3	47
21	16.8	907.9
22	65.1	400
23	41.5	68.1
24	108.3	453.5
25	76.1	212.4
26	48.4	452.7
27	9.1	280.2
28	17.4	277.7
29	44.7	25.2
30	10.8	9
31	91.8	17.9
32	12	17.9
33		253.8
34		34.3
35	24.7	259.2
36	13.7	581
37	56.3	127.7
38	63	169.2
39	83.7	60.9
40		338.4
41	15	68.2
42	18.8	711.3
43	40.8	48.9
44	10.2	454.8
45	36.3	68.9
46	114.8	386.1
47	14.7	112.3
48	26.3	143
49	30.3	291
50	43.1	370.3
51		58.1

Table S3: Concentration of DNA extracted from FFPE tissues

Patient	Pre-NAC Pgp	Post-NAC Pgp	Pre-NAC MRP1	Post-NAC MRP1	Pre-NAC BCRP	Post-NAC BCRP
1	0.2	0.3	0.3	64.8	86.3	84.8
2	0.2	45.3	0.03	59.7	1.6	65
3	7.8	26.8	5.5	24.6	64.5	89.7
4	0.6	8.5	0.2	108.5	7.9	88.3
5	44.6	95.1	1.3	28.6	1.8	11.1
6	0.2	7	0.1	1	13.8	65
7	0.2	10.1	0.3	94.4	5.9	72.5
8	0.05	12	0.02	37.1	19.5	30.2
9	56	12.1	0.01	75.3	31	50
10	8.8	100.5	0.02	35.7	58.6	4.3
11	6.5	32.5	0.5	76.6	22.2	13.5
12	0.9	35.4	0.1	80.1	20.5	34
13	8.3	87.8	0.2	84.8	2	39
14	1.7	34	1.3	84.1	66.8	4.8
15	31.2	48.8	35.4	18.2	46.3	24.7
16	79.8	168.7	1.1	39.1	20.8	119.9
17	5.5	13.2	1.6	0.9	15.3	8.4
18	12.7	56	0.5	1.8	85.8	40.6
19	0.4	0.3	0.1	117.5	12.8	29.8
20	3.7	1.4	1	69.2	5.2	55.4
21	8	42.1	0.1	30	98.5	22.3
22	0.1	106.3	0.01	0.5	0.9	161.3
23	0.04	17.2	0.2	82	0.2	82.8
24	8.8	16.3	8.1	14.5	13.8	33
25	9.9	25	6.1	36.7	28.2	25.9
26	16.2	2.9	0.5	161.6	53.8	18.9
27	104	107.9	0.8	9.7	7.8	9.4
28	15.8	58.3	0.4	27.3	2.2	12
29	0.2	1.4	1.5	0.2	1.5	20.4
30	86.7	13.1	0.5	197.5	62.3	11.8
31	48.9	171.4	3.2	55	131.8	133.2
32	0.6	0.2	0.2	202.9	1.6	115.8
33	10.6	117.9	1.8	88.4	9.4	13.9
34	40.1	21.4	0.8	31.7	143.5	73.2
35	61.1	34.5	0.009	28.9	107	44
36	30.8	101.3	0.4	25.7	54.9	5
37	51.2	40.4	0.02	91.5	34.3	23.9
38	1.8	65.3	0.04	57.7	1.6	82.7
39	161.7	32.4	0.2	7.4	13.7	11.6
40	36.8		0.02		5	
41	77.1		0.05		1.7	
42	32		1.3		72.7	
43	34.5		0.7		77.9	
44	97		0.1		7.7	
45	54.5		0.1		0.5	

Table S4: Semi-automated histoscores for Pgp, MRP1, and BCRP

Patient number	BCRP expression pre-NAET (histoscores: 0-300)	BCRP expression post-NAET (histoscores: 0-300)
1	0.6	40.9
2	5.9	105.0
3	6.9	33.8
4	2.0	38.3
5	19.4	6.0
6	0.6	20.7
7	1.9	50.6
8	3.1	75.2
9	0.8	5.5
10	10.4	31.1
11	2.5	23.6
12	7.4	7.5
13	1.7	87.2
14	0.7	7.2
15	2.1	80.0
16	33.7	105.2
17	0.6	13.0
18	7.0	23.6
19	1.9	45.5
20	2.0	59.5
21	1.4	80.7
22	4.5	33.6
23	1.6	16.4
24	1.7	21.9
25	16.3	19.5
26	1.3	7.8
27	1.3	63.5
28	2.9	34.1
29	1.1	31.4
30	33.7	41.2
31	14.0	16.2
32	16.7	33.8
33	80.6	101.5
34	13.3	33.2
35	17.0	50.2
36	1.2	27.1
37	26.7	120.5
38	1.8	31.9
39	4.0	53.4
40	5.1	58.0
41	82.0	139.6
42	27.8	9.0
43	8.7	42.1
44	27.7	19.5
45	71.2	133.4
46	2.8	48.2
47	13.9	25.5
48	31.6	124.5
49	2.4	28.7
50	1.8	15.7
51	0.9	68.8

Table S5: Semi-automated histoscores for BCRP expression in the NAET cohort

	Pre-NAET methylation %					Post-NAET methylation %				
Patient number	pos 165	pos 172	pos 236	pos 250	BCRP expression pre-NAET	pos 165	pos 172	pos 236	pos 250	BCRP expression post-NAET
1	0	0	0	9.51	0.6	5.79	3.31			40.9
2	2.96	0	2.57	15.72	5.9	0	0	0	13.3	105.0
3					6.9	5.59	1.99	7.13	16.35	33.8
4	0	0			2.0	0	0	9.24	14.31	38.3
5					19.4	4.17	2	2.23	7.96	6.0
6	6.54	2.9	0	0	0.6	0	0	0	0	20.7
7	0	0	0	0	1.9	3.41	0	3.54	10.74	50.6
8	0	0	17.84	40.74	3.1	6.23	0	6.84	18.44	75.2
9	2.8	0	0	8.94	0.8			0	23.84	5.5
10					10.4	0	0	0	16.6	31.1
11	0	0			2.5	0	0			23.6
12	1.93	2.18	0	11.41	7.4	0	7	3	14	7.5
13	NA	NA	NA	NA	1.7	0	0	5	20	87.2
14			0	12.85	0.7	0	0	3.82	11.8	7.2
15	0	0			2.1	0	0	0	10.8	80.0
16	0	0			33.7	0	0			105.2
17	2.47	0	3.47	7.35	0.6	0	0	4.85	14.43	13.0
18					7.0	0	0	0	11.36	23.6
19	0	0			1.9	0	7.75	4.1	12.56	45.5
20	0	0			2.0	1.39	0	1.94	8.12	59.5
21	0	0			1.4	0	4	4	13	80.7
22	0	10.06			4.5	0	4.88			33.6
23					1.6	8.25	0			16.4
24	3.31	2.78			1.7	12.34	7.61	0	0	21.9
25	21.97	10.39	10.19	11.39	16.3	6.4	6			19.5
26	6.45	5.92								7.8
27					1.3	2.16	6.39	14.52	33.89	63.5
28					2.9	0	0	5	11	34.1
29					1.1					31.4
30	3.61	0	0	10.73	33.7	0	0			41.2
31	0	0			14.0	4.79	2.56			16.2
32					16.7	0	0	0	0	33.8
33	NA	NA	NA	NA	80.6	0	0			101.5
34	NA	NA	NA	NA	13.3	0	0	2.99	17.03	33.2
35	2.96	0	0	14.39	17.0	0	0	7	11	50.2
36	2.75	0	0	18.83	1.2	0	6.3	8.54	14.99	27.1
37	0	5.02	0	0	26.7	3.77	6.21			120.5
38					1.8	0	0	0	8.45	31.9
39	8.26	3.17	0	12.02	4.0			16.9	18.32	53.4
40	NA	NA	NA	NA	5.1	0	0			58.0
41	2.89	0			82.0	3.68	2.41	2.65	9.67	139.6
42	0	0			27.8	5.84	0	0	0	9.0
43	7.08	4.33			8.7	0	0	3.43	8.53	42.1
44	4.9	0			27.7					19.5
45	3.72	3.22			71.2	3.52	0	2.33	11.07	133.4
46	0	0			2.8	0.00	0	2.79	13.69	48.2
47	6.73	0	0	5.48	13.9	0	0	0	14.02	25.5
48	2.81	0	0	6.47	31.6	0	0	4.17	11.04	124.5
49	5.04	0	0	0	2.4	2.59	2.32			28.7
50	0	0			1.8	2.96	1.55	2.75	10.77	15.7
51	NA	NA	NA	NA	0.9	7.22	0			68.8

Table S6: Pyrosequencing analysis to examine methylation level of BCRP promoter region in the NAET cohort. The table shows degree of methylation for each of the 4 CpG sites pre- and post-NAET. The matching protein expression is also shown in the form of histoscores from immunohistochemistry. The blank columns are where the pyrosequencing assay was unsuccessful despite repeated assays. The 5 NA columns for the pre-NAET methylation analysis are where there were insufficient remaining FFPE tissue to perform macrodissection and subsequent DNA extraction.

9.3 Supplementary figures

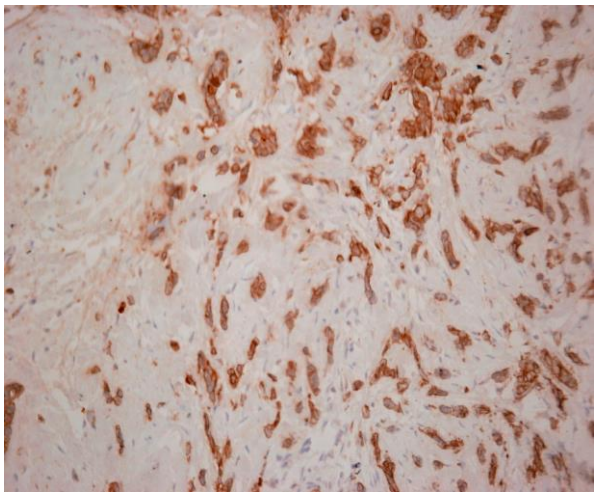


Figure S1: Step-sectioning of pCR blocks resulted in detection of residual tumour cells (anti-cytokeratin AE1/3 antibody; Dako)

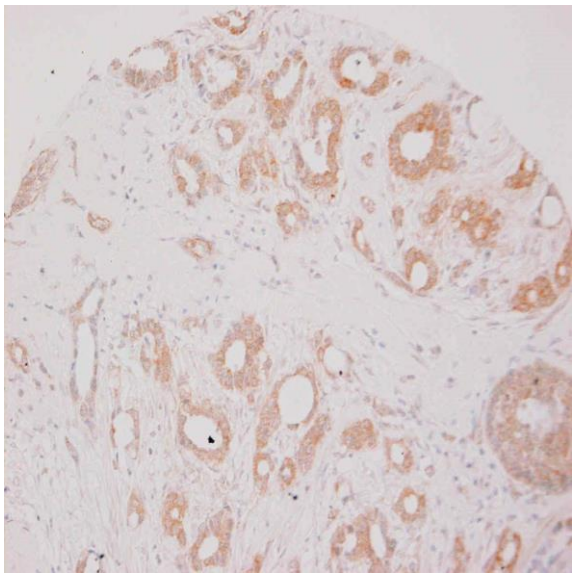


Figure S2a: Optimised TMA staining for Pgp (UIC2), x20 magnification

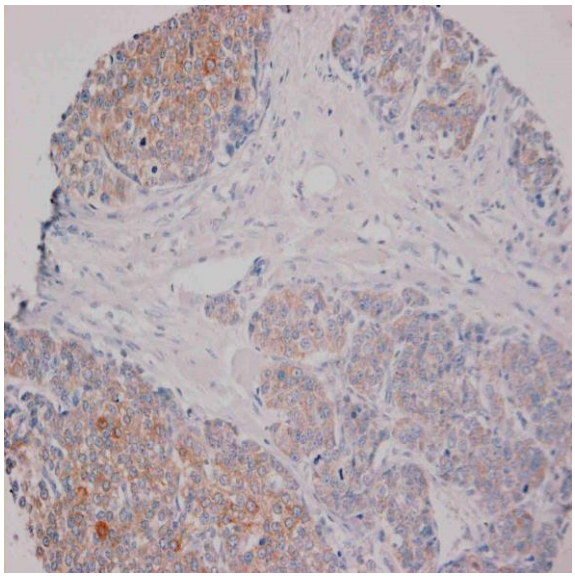


Figure S2b: Optimised TMA staining for MRP1 (QCRL1), x20 magnification

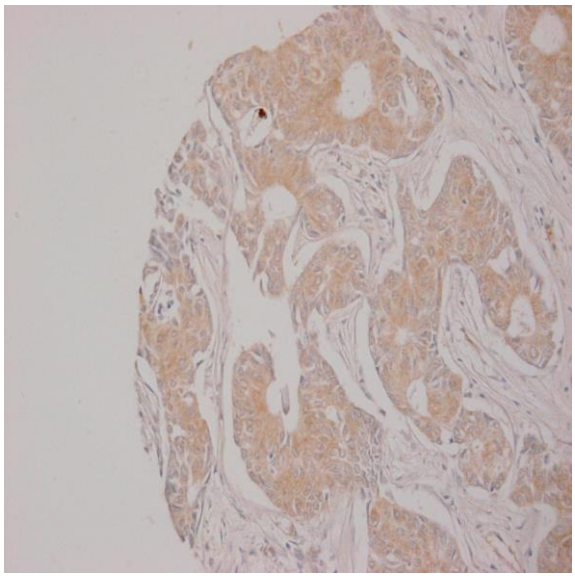


Figure S2c: Optimised TMA staining for BCRP (BXP21), x20 magnification

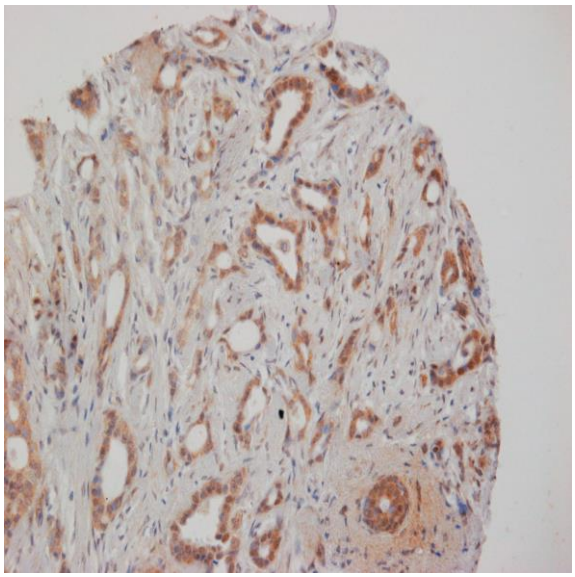


Figure S2d: Optimised TMA staining for Notch1 (anti-activated Notch1), x20 magnification

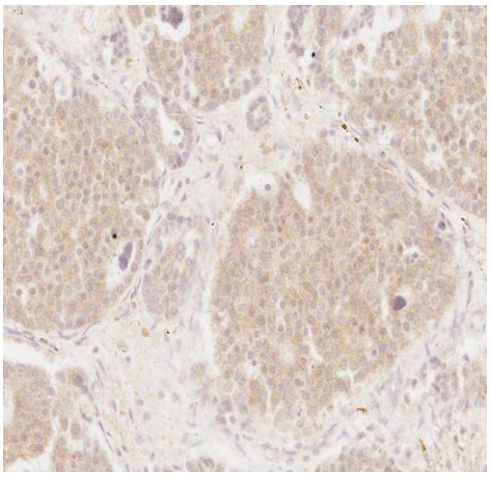


Figure S2e: Pgp expression in axillary lymph nodes

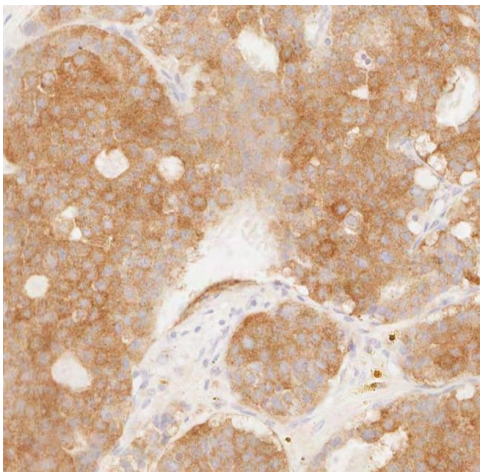


Figure S2f: MRP1 expression in axillary lymph nodes

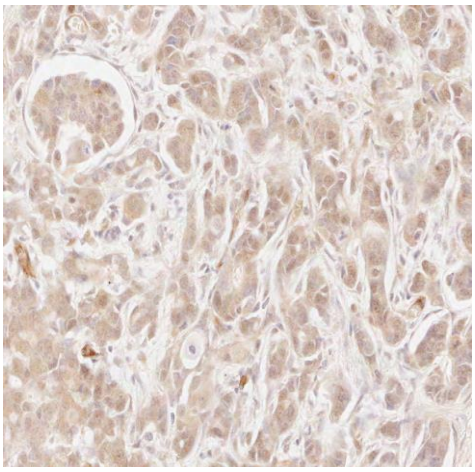


Figure S2g: BCRP expression in axillary lymph nodes

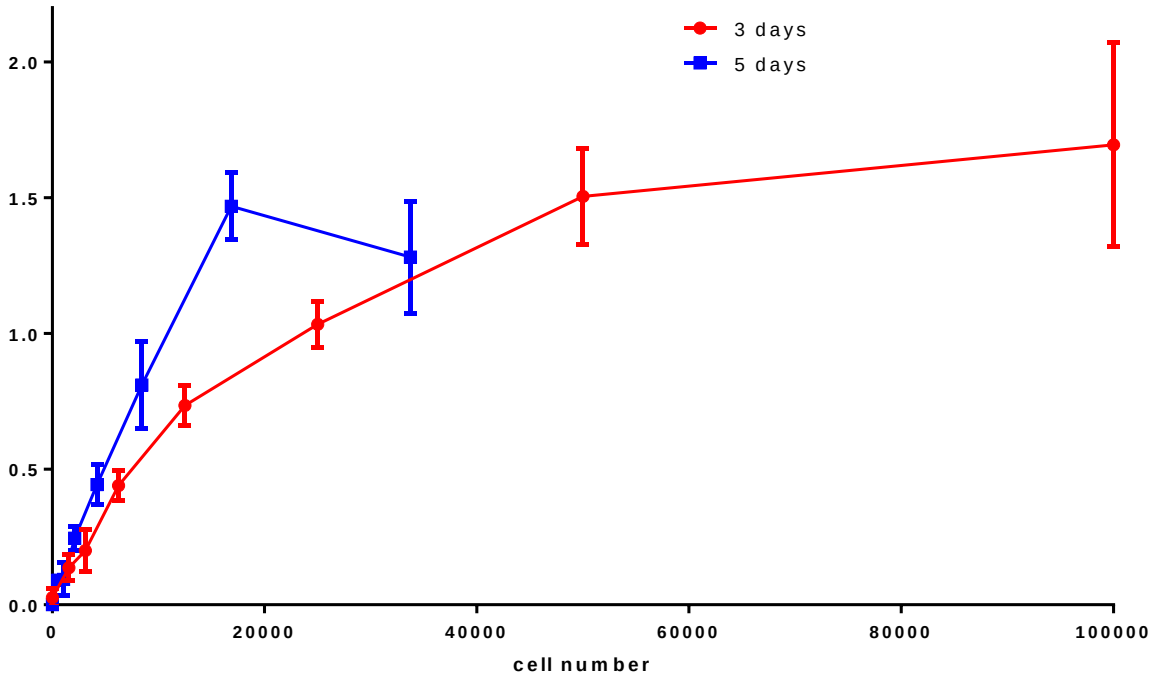


Figure S3: 3 and 5 day MCF7 cell titration curve showing the optical density reading on the y-axis and the initial cell number plated per well on the x-axis. Each point on the graph shows triplicate results. 10,000 cells (3 days) and 7500 cells (5 days) were chosen for the combination assay to allow for log phase growth.

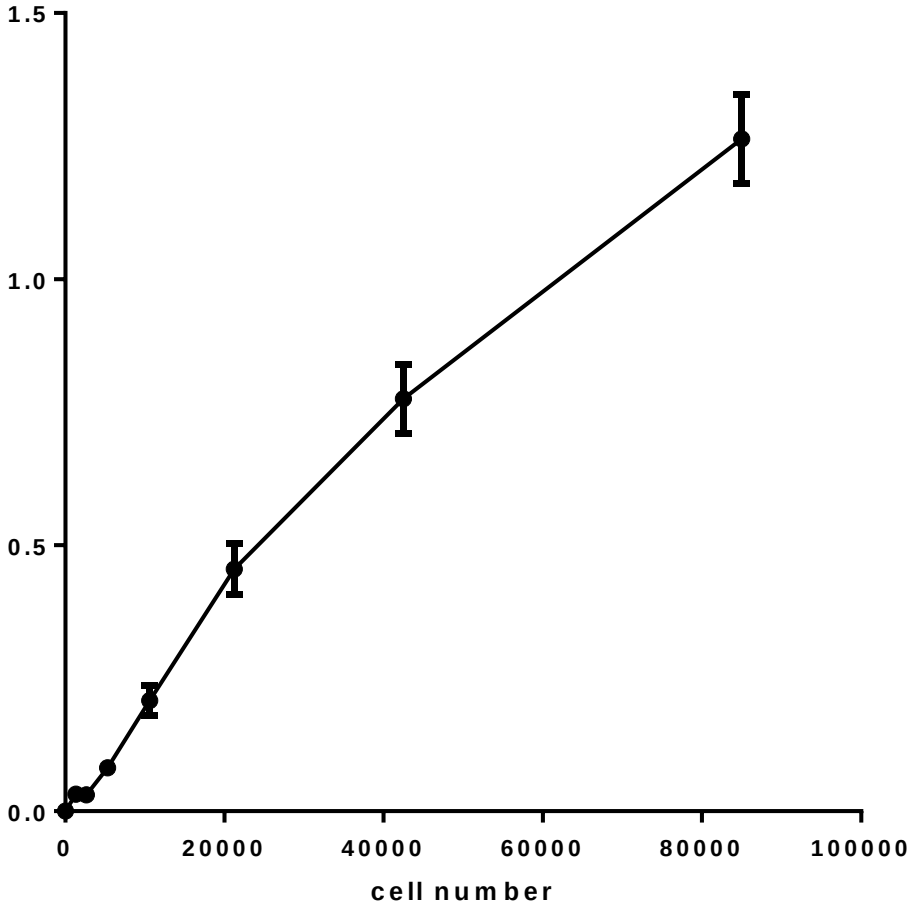


Figure S4: 5 day T47D cell titration curve showing the optical density reading on the y-axis and the initial cell number plated per well on the x-axis. Each point on the graph shows triplicate results. 10,000 cells were chosen for the combination assay to allow for log phase growth.

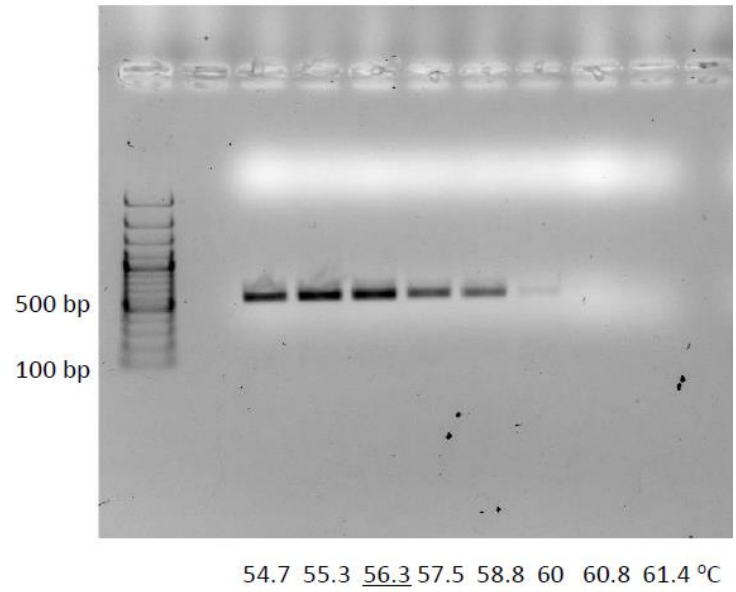


Figure S5: Gradient PCR for bisulphite-modified DNA extracted from H929 cell lines using Turner *et al* primers. This showed an appropriately sized PCR product of around 500 base pairs. The optimal annealing temperature was determined at 56^{o}C.

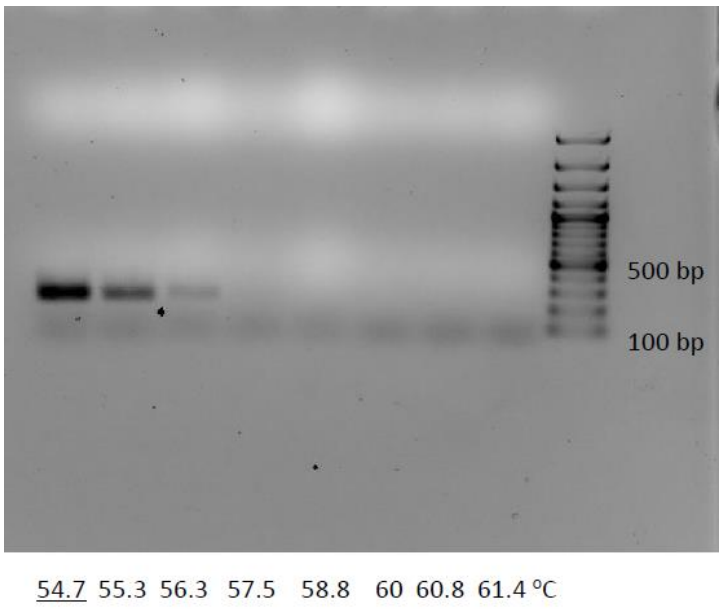


Figure S6: Gradient PCR for bisulphite-modified DNA extracted from H929 cell lines using Chen et al primers. This showed an appropriately sized PCR product of around 250 base pairs. The optimal annealing temperature was determined at 55^{o}C.

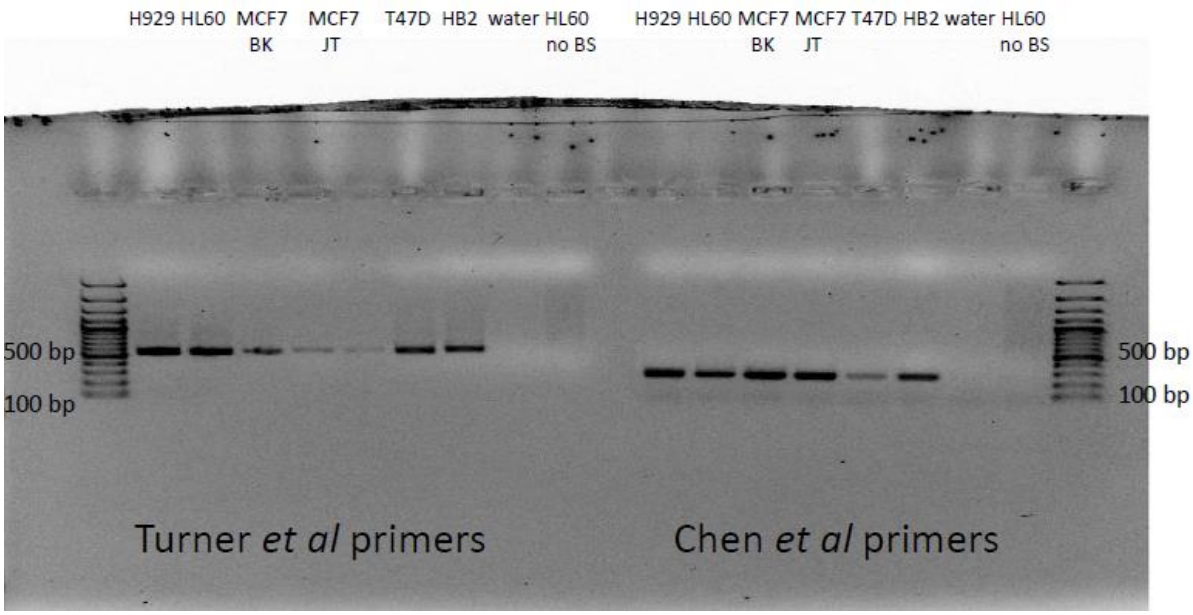


Figure S7: PCR of bisulphite-converted DNA from cell lines using Turner *et al* and Chen *et al* primers. For all cell lines, using Turner *et al* primers resulted in a PCR product of around 500 base pairs, and Chen *et al* primers a product of around 250 base pairs. Negative controls included a lane with nuclease free water instead of bisulphite-converted DNA and DNA from HL60 cell line that was not bisulphite-converted. For MCF7 cell line, an independent bisulphite-converted DNA sample from Dr James Thorne was used.

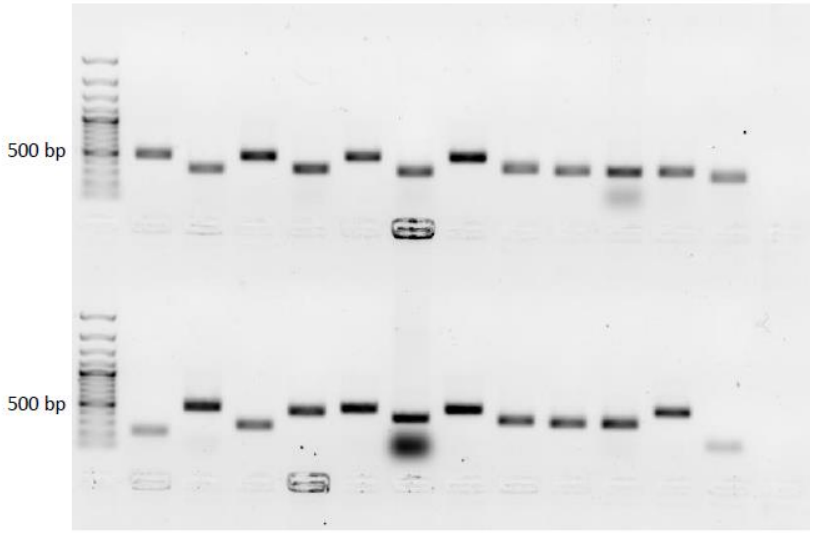


Figure S8: Gel electrophoresis using products from colony PCR of cloned HB2 Chen *et al* amplicon. 23 colonies were randomly selected, and 9 successfully cloned plasmid DNA mini-preps were available for sequencing.

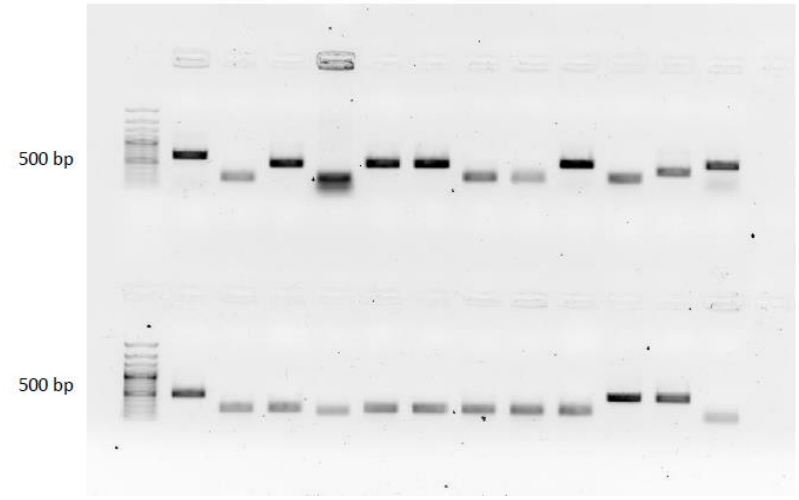


Figure S9: Gel electrophoresis using products from colony PCR of cloned H929 Chen *et al* amplicon. 23 colonies were randomly selected, and 9 successfully cloned plasmid DNA mini-preps were available for sequencing.

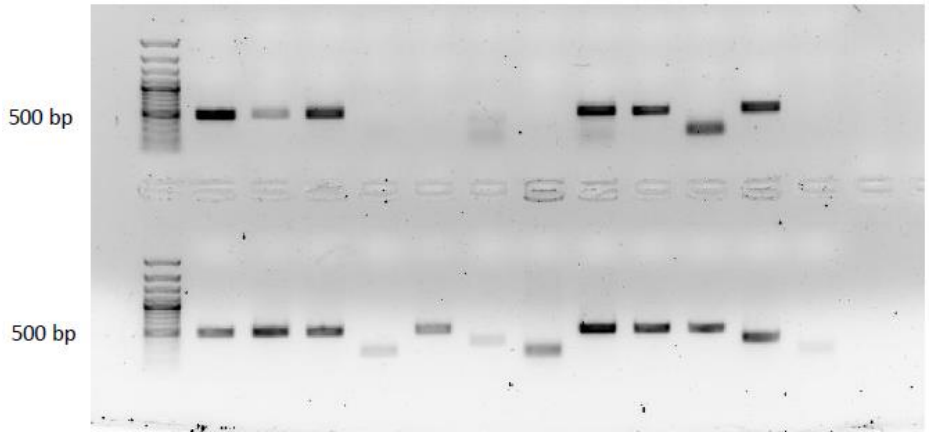


Figure S10: Gel electrophoresis using products from colony PCR of cloned HL60 Chen *et al* amplicon. 23 colonies were randomly selected, and 12 successfully cloned plasmid DNA mini-preps were available for sequencing.

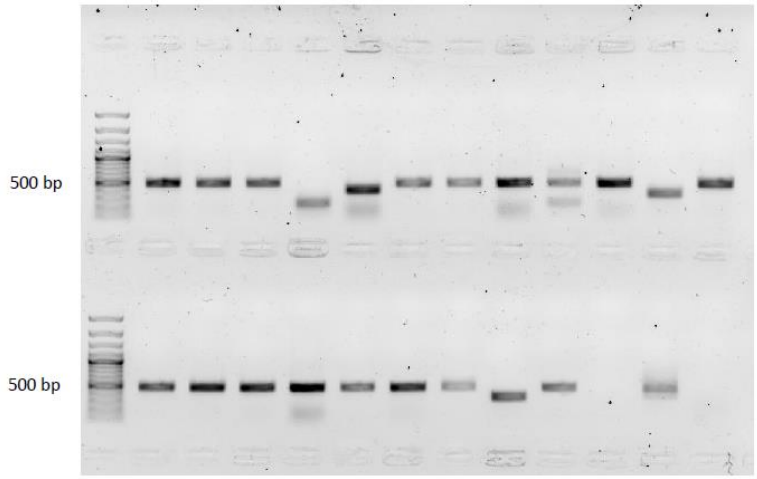


Figure S11: Gel electrophoresis using products from colony PCR of cloned MCF7 Chen *et al* amplicon. 23 colonies were randomly selected, and 12 successfully cloned plasmid DNA mini-preps were available for sequencing.

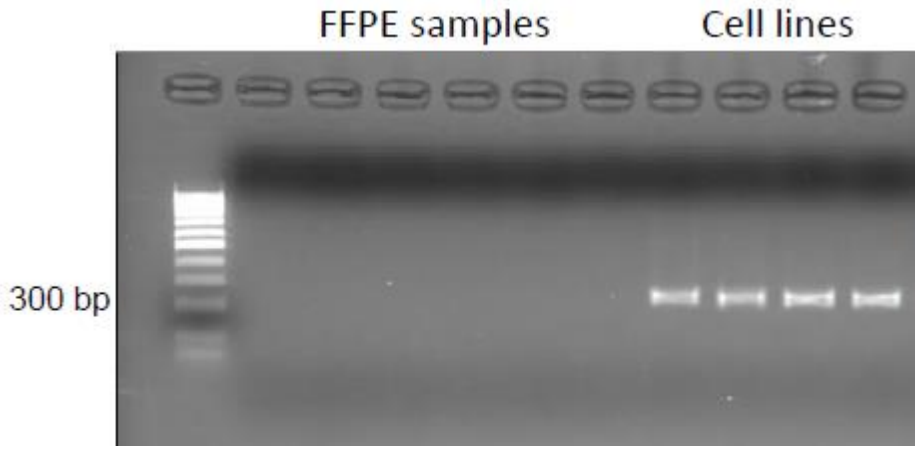
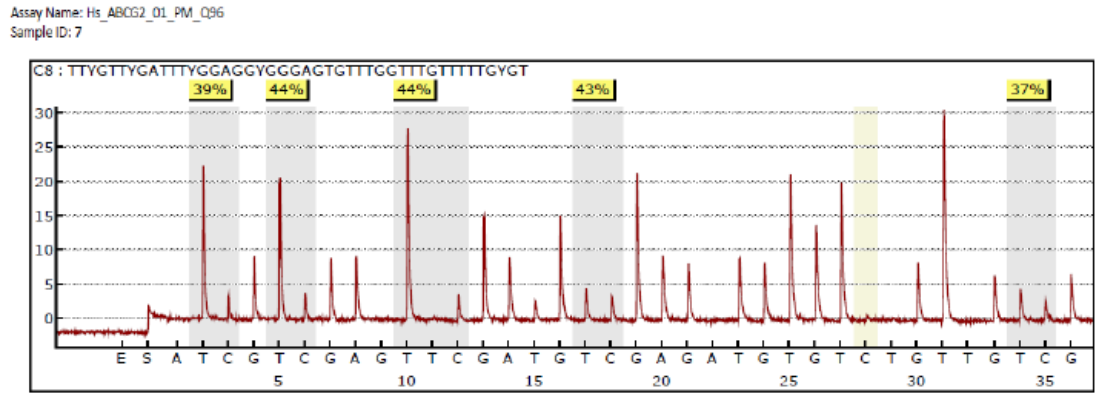
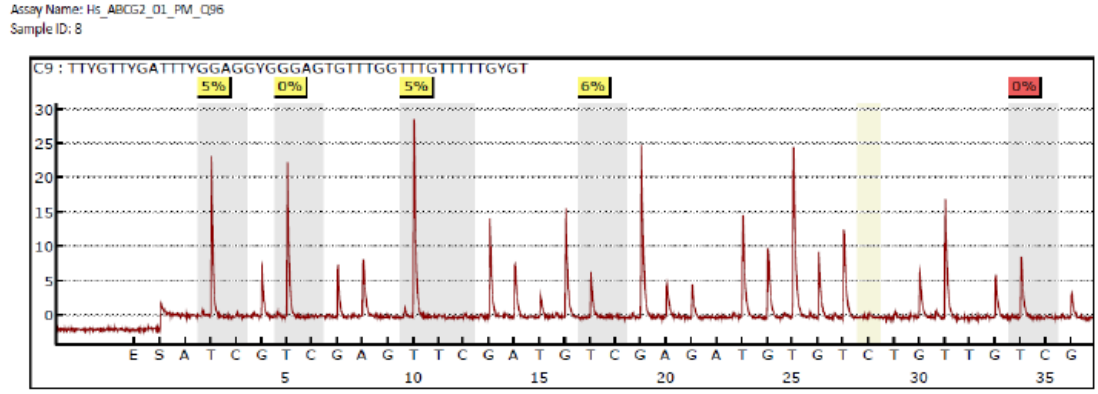


Figure S12: Gel electrophoresis using PCR products of the pyromark assay examining 5 CpG sites. The bands are seen just below the 300 base pair ladder, but were only present for the cell line samples but not for the FFPE samples.

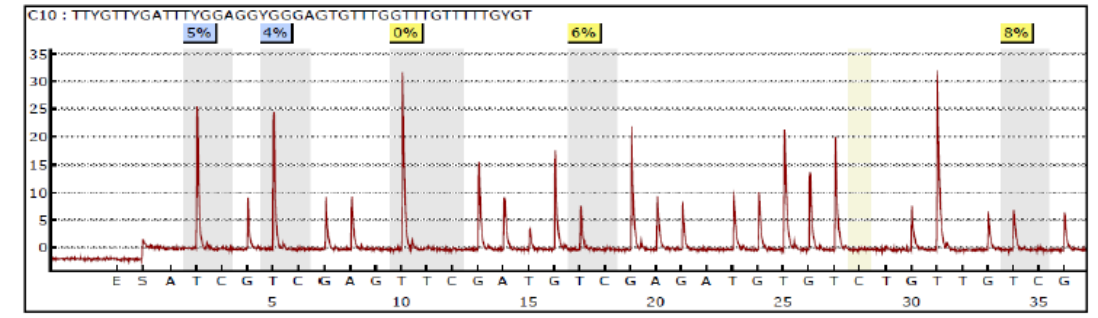
HL60



H929



MCF7



HB2

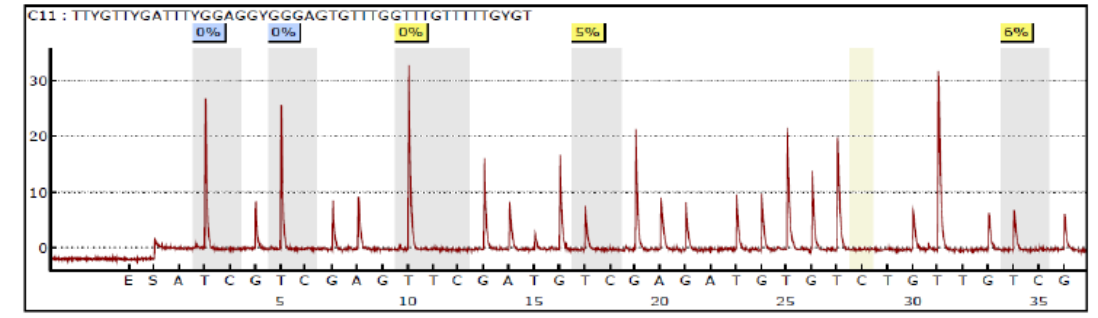


Figure S13: Pyrosequencing analysis for the 4 cell lines examining the 5 CpG sites assigned by the pyromark assay. The 5 CpG sites in H929, MCF7, and HB2 cell lines are unmethylated. However, the equivalent CpG sites in HL60 cell line shows high degree of methylation.

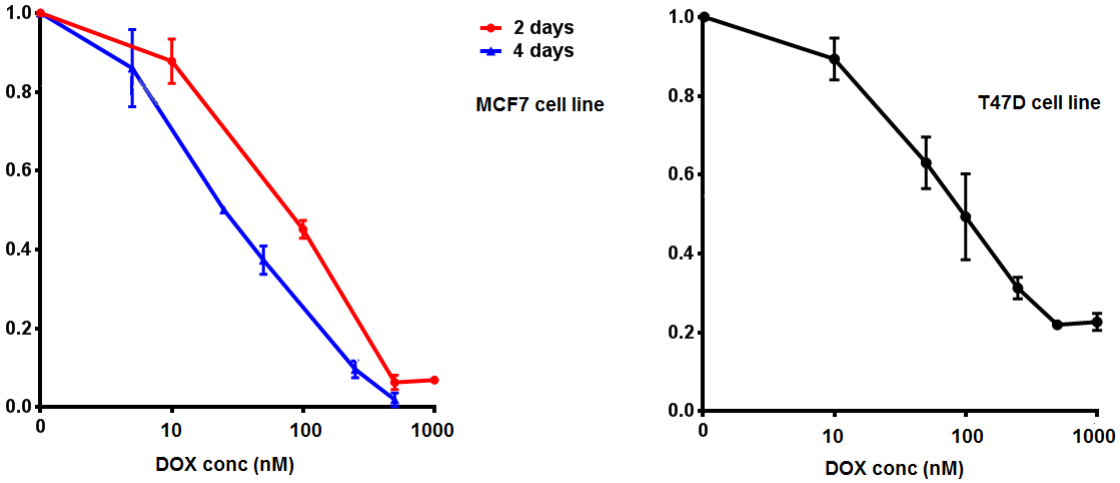


Figure S14: Dose-response curve for doxorubicin (MCF7 and T47D cell lines) showing optical density reading on the y-axis (normalised to untreated) and log 10 of doxorubicin concentration added to the wells at 24 hours on the x-axis (0 to 1μM). This allowed determination of approximate range of IC values after 2 and 4 days of treatment with doxorubicin for MCF7 cells, and after 4 days of treatment for T47D cells. Each co-ordinates represent triplicate values and optical density readings from wells containing medium only was subtracted.

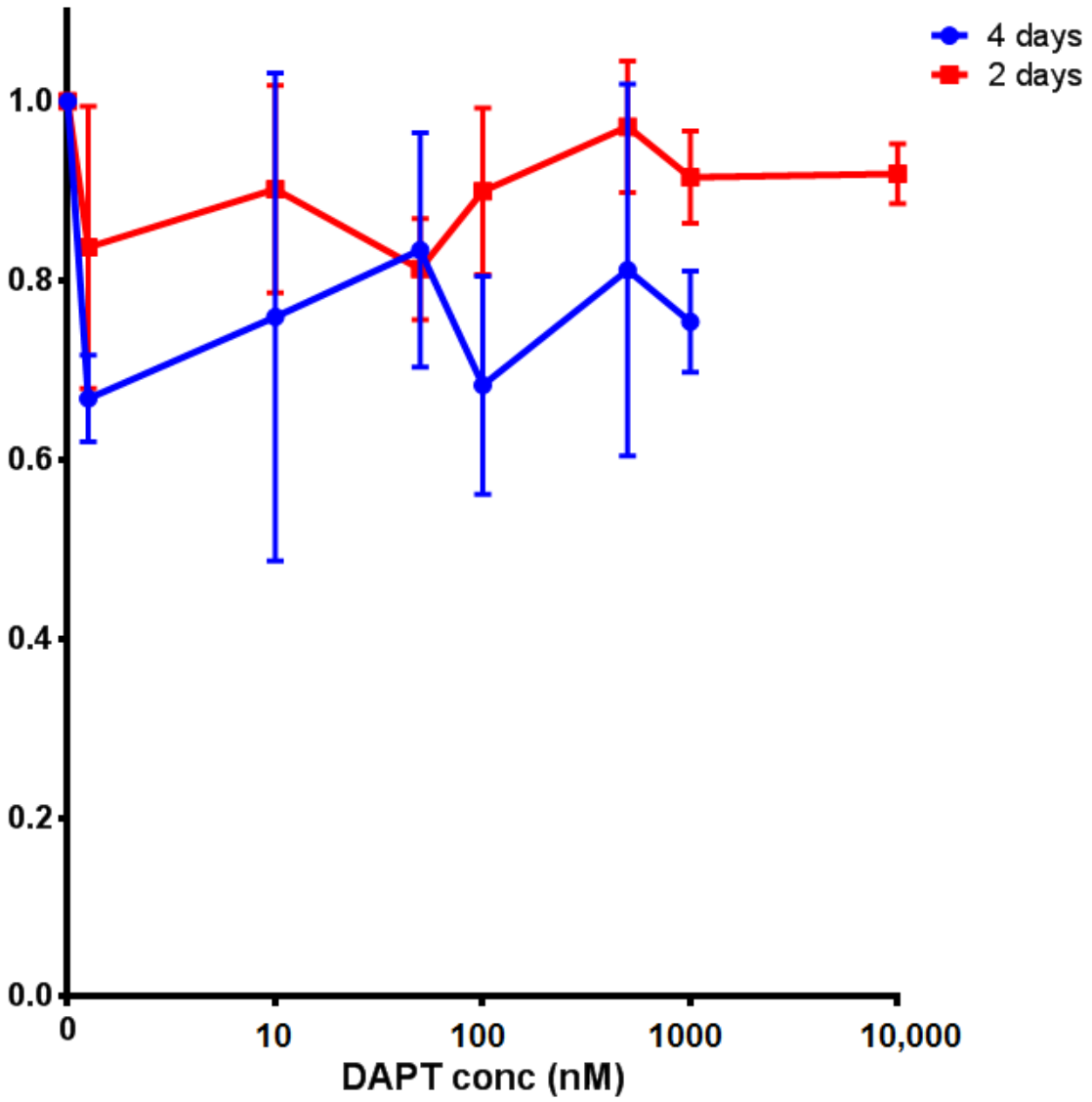


Figure S15: DAPT 2 and 4 days titration curve (MCF7 cell line) showing optical density reading on the y-axis (normalised to untreated) and Log 10 of DAPT concentration added to the wells at 24 hours on the x-axis (0 to 10μM). Increasing concentration of DAPT on its own did not have any effect on cell growth/survival. Each co-ordinates represent triplicate values and optical density readings from wells containing medium only was subtracted.

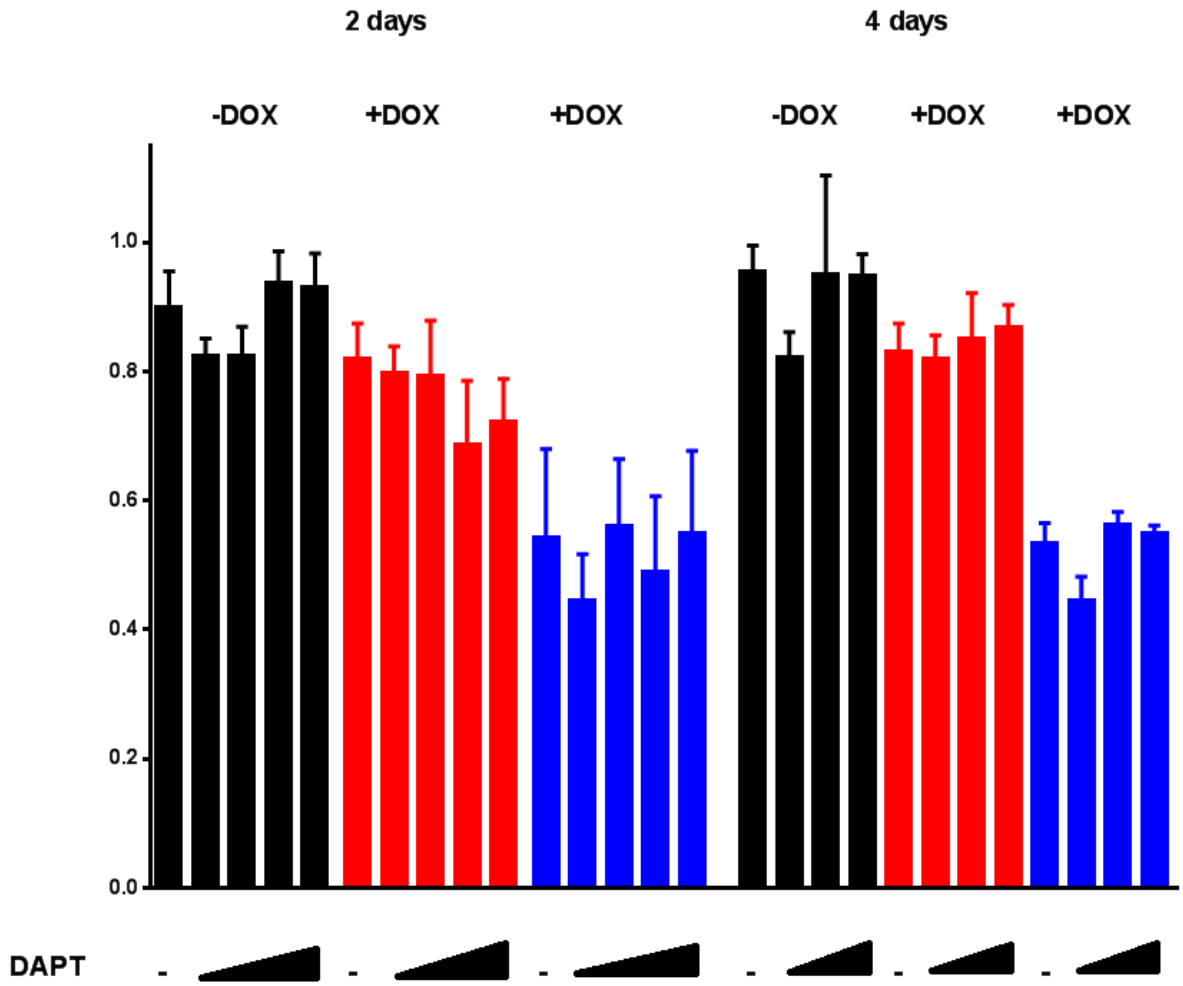


Figure S16a: 2 and 4 day combination assay in MCF7 cell lines for the lower DAPT concentrations (1nM to 1uM). Y-axis shows optical density reading. The first black bar denotes the controls (-), with increasing DAPT. The first red bar denotes the IC10 values without DAPT (-), with the remainder showing IC10 doxorubicin with increasing DAPT. The first blue bar denotes the IC50 values without DAPT (-), with the remainder showing IC50 doxorubicin with increasing DAPT.

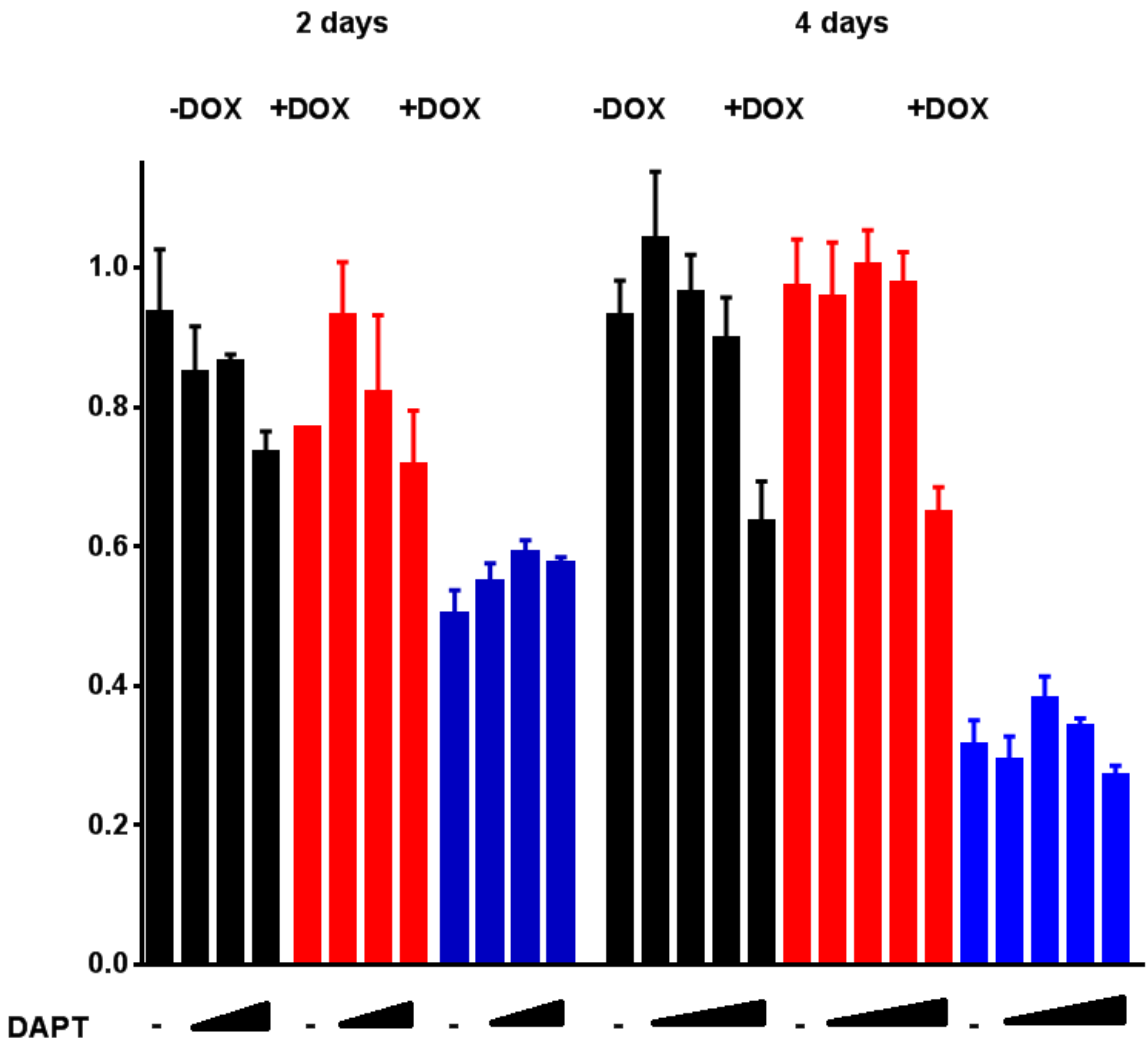


Figure S16b: 2 and 4 day combination assay for the MCF7 cell lines for the higher DAPT concentrations (1uM to 100μM). Y-axis shows optical density reading. The first black bar denotes the controls (-), with increasing DAPT. The first red bar denotes the IC10 values without DAPT (-), with the remainder showing IC10 doxorubicin with increasing DAPT. The first blue bar denotes the IC50 values without DAPT (-), with the remainder showing IC50 doxorubicin with increasing DAPT.

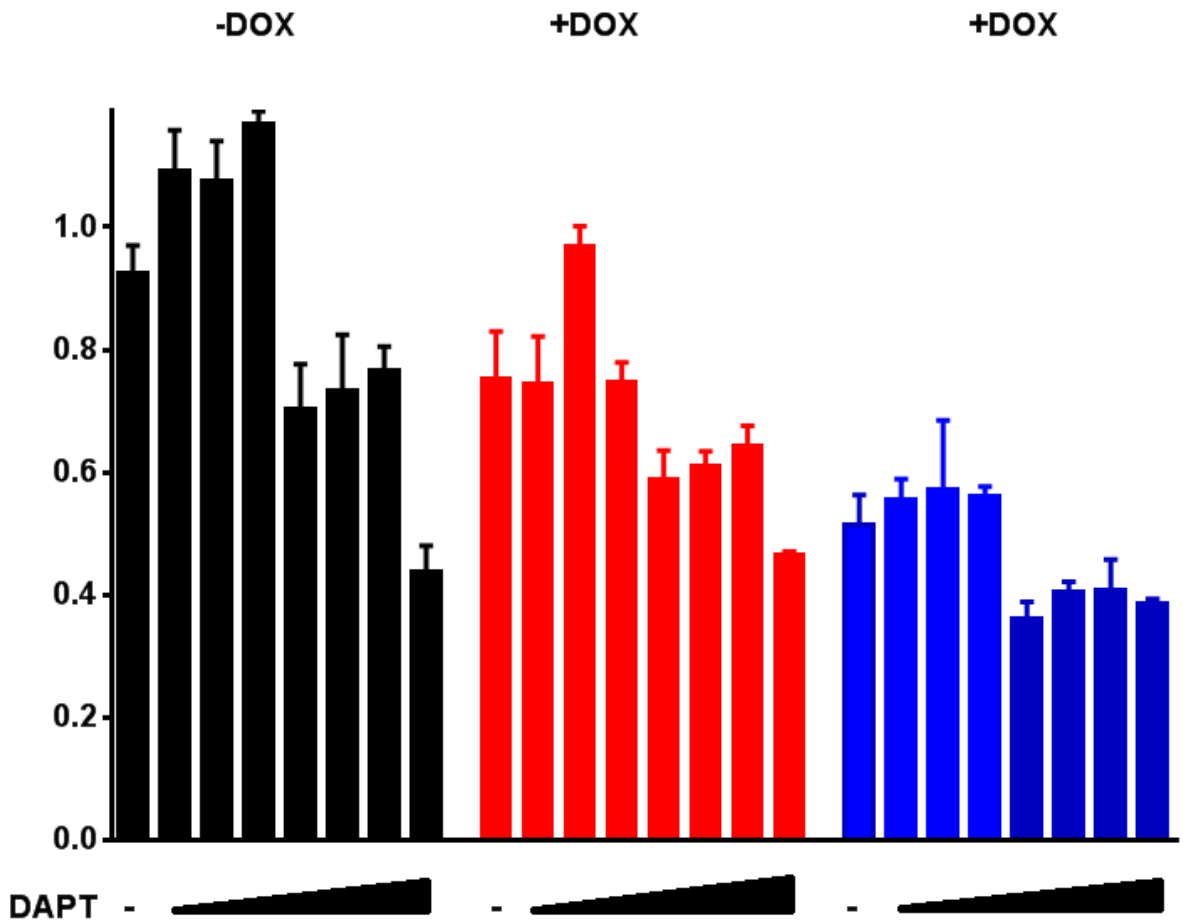


Figure S17: 4 day combination assay using T47D cell lines (DAPT concentrations of 1nM to 100uM). Y-axis shows optical density reading. The first black bar denotes the controls (-), with increasing DAPT. The first red bar denotes the IC10 values without DAPT (-), with the remainder showing IC10 doxorubicin with increasing DAPT. The first blue bar denotes the IC50 values without DAPT (-), with the remainder showing IC50 doxorubicin with increasing DAPT.

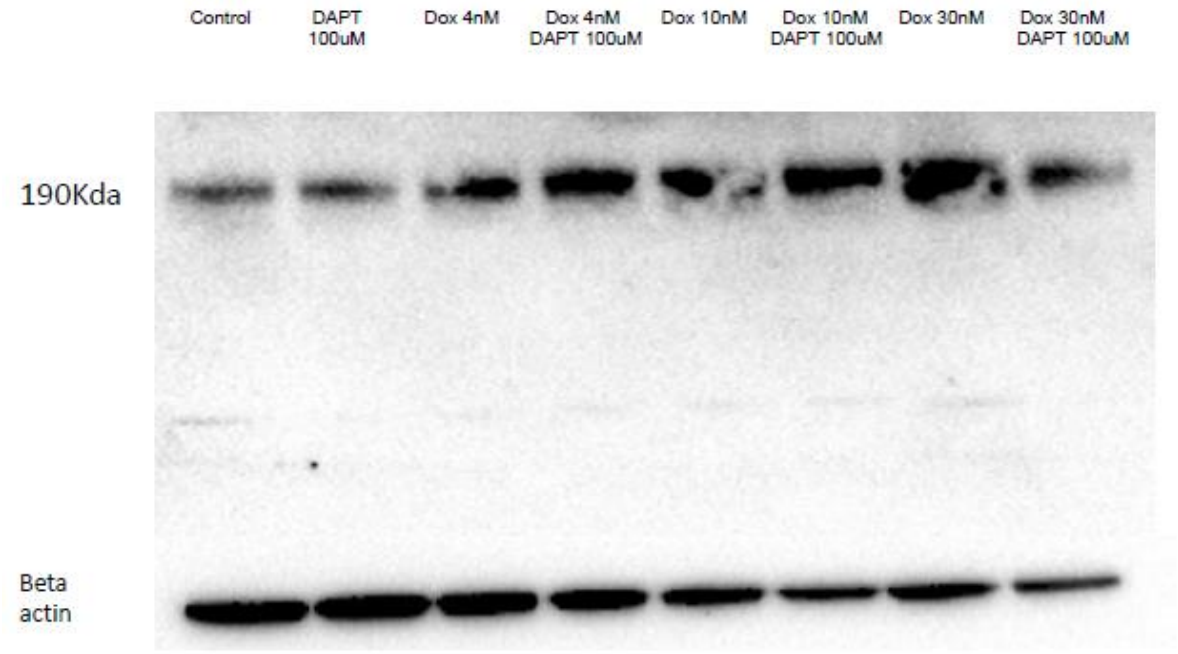


Figure S18: Western blot to examine MRP1 expression using MCF7 another independent cell lysates treated with doxorubicin +/- DAPT. Increasing doses of doxorubicin treatment led to a dose-dependent up-regulation of MRP1 expression. DAPT treatment did not result in down-regulation of MRP1 expression however. Combination therapy of doxorubicin and DAPT did not cause significant down-regulation of MRP1 expression in general.

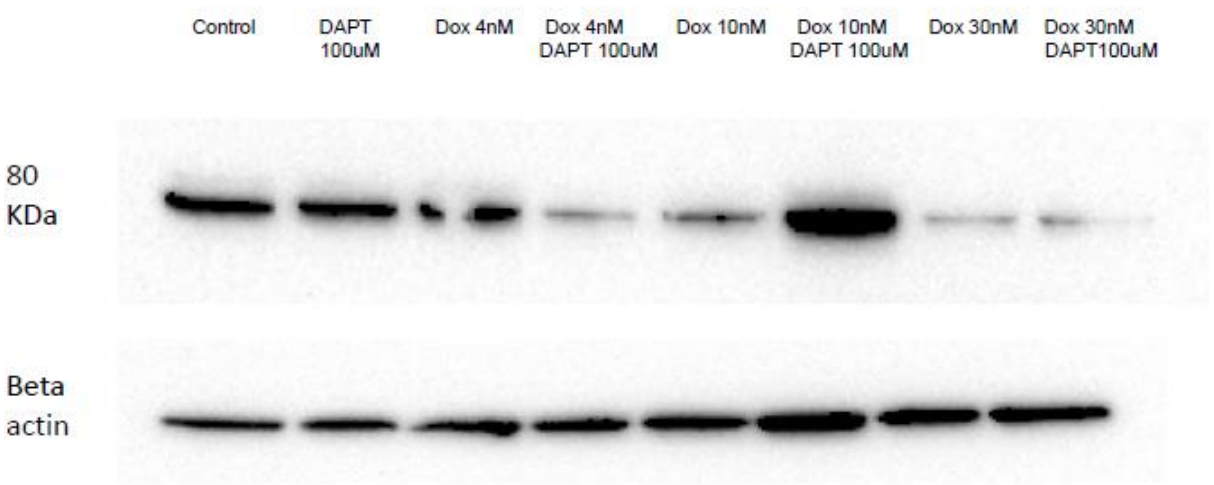


Figure S19: Western blot to examine Notch1 NICD expression using an independent MCF7 cell lysates treated with doxorubicin +/- DAPT. Increasing doses of doxorubicin resulted in the down-regulation of Notch1 NICD expression. Treating the cells with DAPT did not result in down-regulation of Notch1 NICD expression. However, the combination of doxorubicin and DAPT led to down-regulation of Notch1 NICD expression.

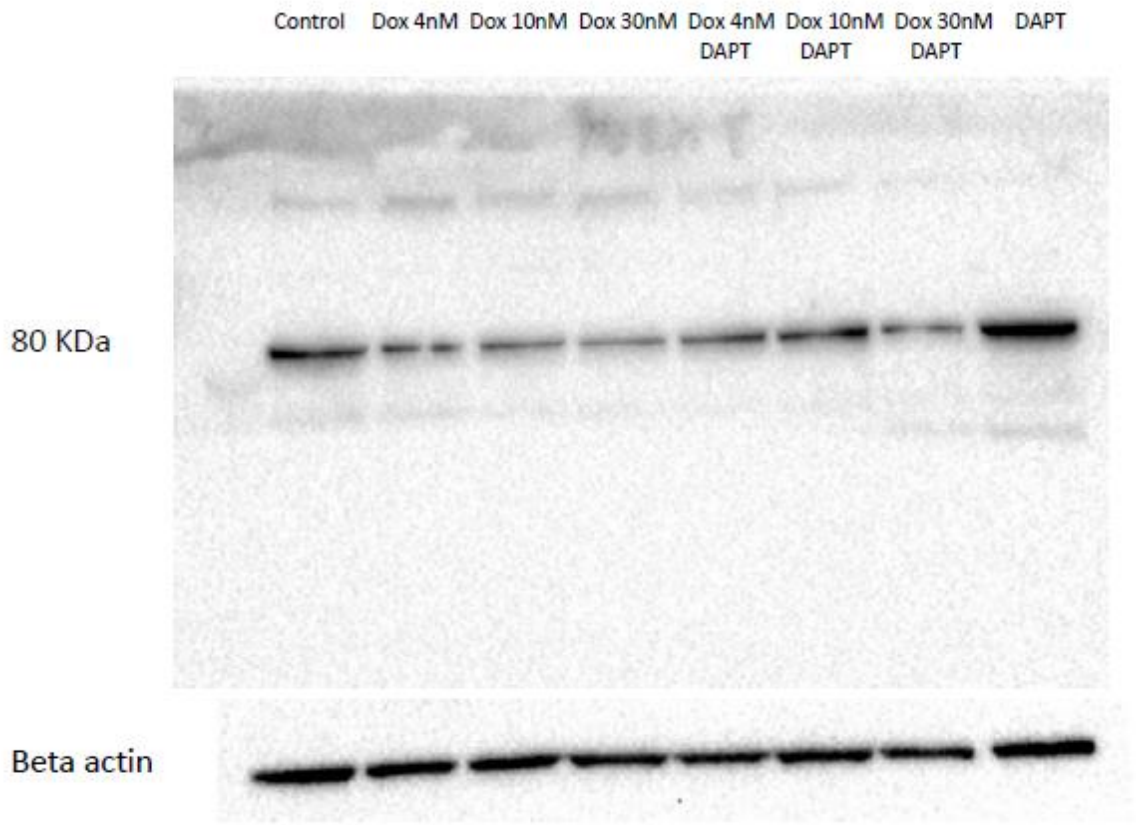


Figure S20: Western blot to examine Notch1 NICD expression using another independent MCF7 cell lysates treated with doxorubicin +/- DAPT. Increasing doses of doxorubicin resulted again resulted in the down-regulation of Notch1 NICD expression. DAPT treatment did not result in down-regulation of Notch1 NICD expression. However, the combination of doxorubicin and DAPT led to down-regulation of Notch1 NICD expression.