

Investigation of Association between Natural Variants in Sub-Cellular Pathways and Breast Cancer Chemotherapy Response.

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Abstract

Invasive breast cancer is the commonest cancer in the UK and adjuvant chemotherapy is often used to reduce the risk of local and distant recurrence in early stage disease. However, it is estimated that 70-80% of women receive no benefit from chemotherapy. In the current climate of personalised medicine the aim of the treating physician is to maximise individual benefit of treatment whilst minimising exposure to harmful side effects.

Here we discuss the current tools available to stratify patients' treatment including clinicopathological and molecular features, clinical guidelines, computer based risk calculators, gene expression profiles and minimal invasive disease markers. We also review the major chemotherapy drugs in breast cancer, both historically and current regimes. This leads us to the notion that hypothesis generated biomarkers are required to help determine chemo-responsiveness of cancers.

We describe why single nucleotide polymorphisms (SNPs) are the ideal candidate for a biomarker given that they are constant between cancers, easy to detect and reproducibility is reliable. Although many pathways have been implicated in cancer and response to treatment we have focused on predictive SNPs for hypothesis testing of specific candidate examples; apoptosis, cell cycle, metastor genes, drug metabolism and. We discuss the pathways in detail including their known relevance to breast cancer and current chemotherapy agents.

It was our hypothesis that specific variants would be enriched in chemo responsive groups of breast cancers and we aimed to test such associations by creating a panel of candidate SNPs for a range of subcellular processes.

Methods

Full ethical approval was obtained to use breast cancer samples stored in the Liverpool Candis Cancer Research Tissue Bank and obtain clinical information from patient case notes held in both Royal Liverpool and Broadgreen hospital and Clatterbridge Centre for Oncology. Patients who had undergone primary breast cancer surgery and received chemotherapy between 1993 and 2005 were identified.

SNP assays were developed using 96 well DNA panels from healthy volunteers using commercially Taq man real time polymerase chain reactions (PCR) and custom gel based assays as required. Tumour DNA was either available as a macromolecule from the tissue bank or extracted from frozen or paraffin embedded tissue using commercially available kits. Once probes had been optimised they were used in duplicate on DNA samples with a number of positive and negative controls. Overall survival and disease free survival were used as end points

Results

A cohort of 303 patients was analysed a number calculated to achieve statistical significance. All patients received chemotherapy but the regimes changed over the study period with anthracyclines and taxanes becoming more prevalent as time progressed. The presence of adverse clinical features was still associated with poorer outcome despite chemotherapy being given.

Two of our apoptotic pathway SNPs (Rs 1042522, Rs 2279225) had some association with survival and sensitivity to taxanes. While our drug metabolism pathway variants weren't expected to be associated with clinical features we found some trends linking variation with possible cause.

A polymorphism associated with alternative splicing of cyclin D1 appears to be associated with response to anthracycline based chemotherapy. We hypothesise that if this SNP is associated with increased expression of the alternative cyclin D1b protein in our population, as suggested by other published studies, then the associated difference in proliferation and subsequent effects on the DNA damage response pathways can explain our findings. We have also found a possible link between SPP1 variants and overall survival ($p=0.02$) and chemotherapy agent sensitivity.

Discussion.

This work supports the hypothesis that pathway associated genetic variants can be associated with outcome in a chemotherapy treated cohort of early breast cancers and that the nature of chemotherapy is important in specific instances. It has provided a foundation for further work to be performed both on validation of these results and for testing in larger cohorts from previous clinical trials. It provides continued support for the use of SNPs in point of care testing towards truly personalised cancer treatment.

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1. Chapter 1 - Introduction

1.1 Adjuvant Chemotherapy for Breast Cancer

Invasive Breast cancer is the most common cancer in the UK, affecting 46,000 women per year. 5-year survival rates exceed 80%, but over 1000 women still die each month because of the disease. The prevalence is over 296,000 individuals still alive having contracted the disease. Local control of early breast cancer can be achieved using surgery and radiotherapy in combination. Adjuvant, systemic hormone and cytotoxic therapy reduce the risk of local and distant relapse. However, 70-80% of treated patients are likely to have survived without having received chemotherapy but it is difficult to ascertain which patients specifically will benefit [1]. Current NICE guidelines suggest that intermediate and high risk patients should be considered for chemotherapy, yet 30% of node negative, oestrogen receptor positive patients will relapse whilst 40% of node positive women will survive for at least 10 years without recurrence [1].

Chemotherapy regimes can cause many side effects that are unpleasant, potentially harmful to patients and can have long-term consequences. Ideally clinicians would direct chemotherapy only to those who are likely to benefit, thereby reducing recurrence rates whilst optimising patient well being and efficiently using health care resources. Current practice uses known prognostic indicators whilst evaluating the potential benefit from adjuvant treatment. Clinicians have routinely used clinicopathological features to determine risk and evolving molecular methods such as biological classification and gene arrays (all discussed here) have added layers of prognostic information. However, the expense of molecular prognostication has meant that it is not routinely available to all in the UK. The goal should therefore be to use clinicopathological and molecular features together to compliment each other in a cost effective manner. The aim of this study

therefore is to investigate molecular approaches to stratifying patients for treatment, in particular the use of natural variants.

1.2 Clinicopathological features of Breast Cancers and Prognostic

Significance

Standard practice uses clinicopathological features of patients and tumours to stratify patients into prognostic groups, guiding treatment selection. Prognostic scoring tools such as the Nottingham Prognostic Index use these features in an attempt to quantify patients' risk. - summarised below.

1.2.1 Tumour Grade

In the UK, breast cancer is graded according to the Nottingham combined histological grade, which is the Elston-Ellis modification of the Scarff-Bloom-Richardson system[2]. It is an objective grading system encompassing three features; tubule formation, nuclear pleomorphism and mitotic counts. Each group can be scored 1-3 giving an overall score of between 3 and 9. Grade 1 breast cancer is well differentiated and has a score of 3-5; grade 2, moderately differentiated, a score of 6-7 and grade 3, poorly differentiated a score of 8-9. It is a valuable score irrespective of tumour type.

It has been demonstrated that this system is reproducible and has low inter-observer bias [3-5]. Numerous studies have demonstrated that grade shows independent significance in overall survival (OS) and disease free survival (DFS) [6, 7] including a large study (2219 patients) with a median follow up of 111 months (**figure 1.1**) [2]. The study also showed that grade was significant even in sub groups based on size (pT1a, pT1b, pT1c and pT2) and lymph node size. This is important as the Breast Task Force of the American Joint Committee on Cancer lacked clear evidence for the role of grade in small tumours and therefore did not include histological tumour grade when setting its staging criteria [8].

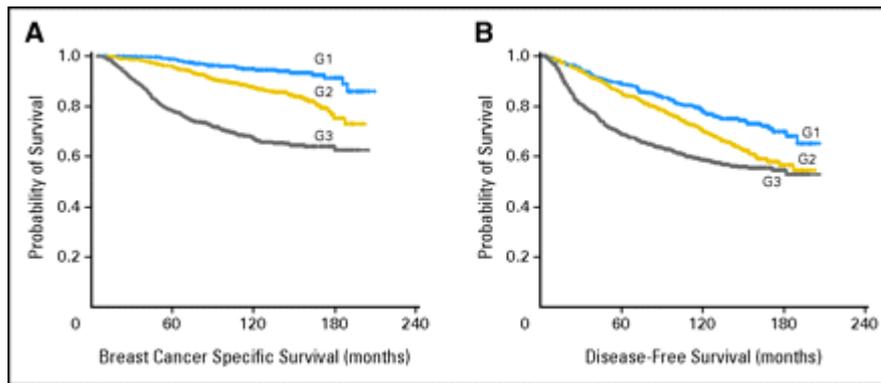


Figure 1.1 Relation between histological grade and (A) breast cancer-specific survival (B) disease-free survival in months.

Taken from Rakha et al, Journal of Clinical Oncology, 2008 [2]

Grade forms part of numerous prognostic indexes including the Nottingham Prognostic Index (see later) where it has equal weighting with lymph node status [9] and the Kalmar prognostic index, where it has a higher value (1.57) than lymph node stage (0.79) or size (0.31)[7].

1.2.2 Tumour Size

Tumour size is considered to be a powerful predictor of outcome, **figure 1.2**[10, 11]. It is a time-dependent prognostic factor reliant on both the time between tumour development and detection and the balance between tumour cell proliferation and apoptosis. The positive correlation between size and axillary lymph node stage is documented well [11-13]; with increasing tumour size and increasing lymph node stage being highly significantly associated with poorer outcome, although the two factors are independent of each other [11, 14].

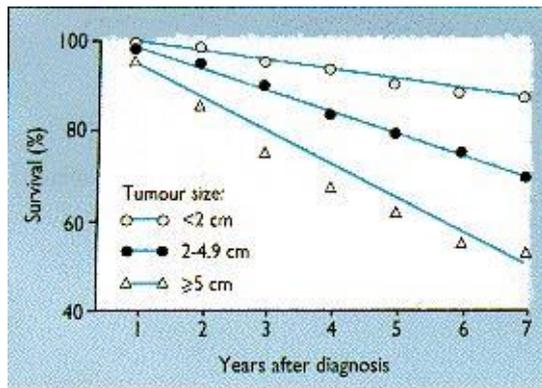


Figure 1.2 Breast cancer patient survival in relationship to size of the original tumour.

Taken from Miller et al., *ABC of Breast Diseases: Prognostic Factors*. BMJ, 1994. [15]

However, the importance of size as a prognostic indicator has recently been questioned as some small tumours appear to behave more aggressively [16]. Standard practice considers the largest diameter of the tumour as size but other values may be significant. As metastatic cells are typically dislodged from the tumour surface, the surface area may also be important. This also takes into account multi-centric tumours having larger surface areas and the convoluted nature of some tumours. To date such measurements of size have not been studied in relation to outcome.

A recent review has questioned whether size correlates with survival in all subtypes particularly the triple negative and basal like breast cancers[16]. The hypotheses proposed include the presence of a proportionally large, but fixed, population of cancer stem cells or that clonal evolution driven by genomic instability leads to a metastatic phenotype.

1.2.3 Axillary Lymph Node Status

Axillary lymph node status (discussed here as simply lymph node status) of metastatic deposits is one of the strongest predictors of outcome in breast cancer. The absolute number of lymph nodes involved is one of the most important prognostic factors, reflected in the revised TNM staging of breast cancer[8]. There is a common consensus that positive lymph node status and outcome are inversely related. In a study involving 24,740 patients

in the surveillance, epidemiology and end results (SEER) programme[11] and a study involving 20,547 cases from the American College of Surgeons [17] survival declined linearly with increasing numbers of histologically positive nodes (up to 21 positive nodes).

Lymph node status can be described by anatomical level of dissection or absolute number. Level one nodes lie lateral to the lateral border of the pectoralis minor muscle, level two behind it and level three lie medial to the medial border of pectoralis minor. The 5-year survival if level one nodes are positive is 65%, 45% if level two nodes are positive and 28 % if there is level 3 nodal involvement. The number of positive nodes is more closely related to survival than the level of involvement[9].

It is therefore mandatory that the axilla be staged as part of breast cancer treatment.

Clinical assessment is unreliable [18, 19] and ultrasound although fairly specific is only moderately sensitive [20]. It is recommended that all patients should undergo histological staging. Traditionally this has been thorough axillary lymph node dissection or by 4-node lymph node sampling, however the ALMANAC (Axillary lymphatic mapping against nodal axillary clearance) has provided level 1 evidence for the use of sentinel lymph node biopsy in early breast cancer [19]. A sentinel lymph node is any lymph node that receives direct lymphatic drainage from a primary tumour site. In breast cancer there is a mean of 3 sentinel lymph nodes and the false negative rate is 0-11%.

At present, a metastatic deposit of 2mm or greater is classified as positive, those less than 2mm but greater than 0.2mm a micrometastasis and those less than 0.2mm isolated tumour cells. Standard techniques for assessing the sentinel lymph node are lacking and there is no evidence to date on the relevance of positivity detected by molecular methods. In the UK, most pathologists follow the ALMANAC method of processing nodes in 3mm thick slices perpendicular to the long axis of the node and one node per cassette. The relevance of micrometastasis and isolated tumour cells in guiding adjuvant therapy is unknown, although

a recent study of Dutch patients suggests that the presence of these is associated with a reduced 5 year DFS in women with favourable early stage breast cancer who did not receive adjuvant therapy and that the survival was improved if adjuvant therapy was given[21].

1.2.4 Lymphovascular Invasion

The presence of tumour in vascular or lymphatic spaces, lymphovascular invasion (LVI), correlates well with local and regional lymph node involvement[22, 23] and appears to be reproducible among pathologists [24]. However, even in patients who are histologically lymph node negative, LVI correlates with early recurrence, distant metastasis and OS [25-27]. In a large study of lymph node negative women the presence of LVI was prognostically significant and independent of grade, size and tumour type for OS (**figure 1.3**)[28] . LVI should therefore be considered in addition to other factors in adjuvant therapy planning in lymph node negative women.

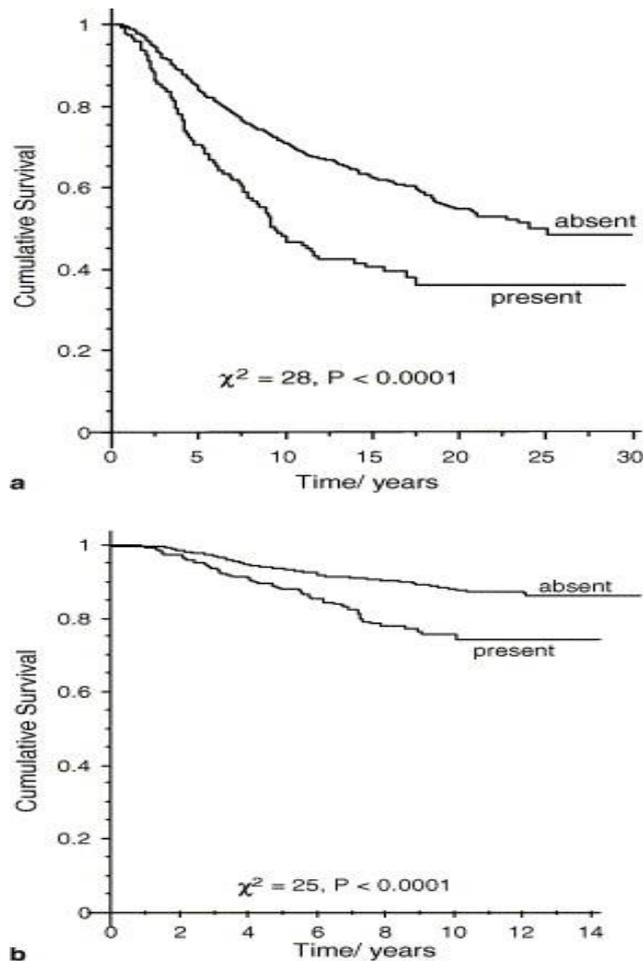


Figure 1.3 Lymphovascular invasion and breast cancer specific survival of women with node-negative operable invasive carcinoma of the breast: (a) no adjuvant therapy group; (b) selective adjuvant therapy group.

Taken from Lee et al, Eur J Cancer. 2006 [28]

1.2.5 Stage

Breast cancer stage is a classification scheme describing the primary tumour, regional lymph node status and metastasis. Based on clinical and pathological staging it is used to guide management of patient care.

1.2.6 Nottingham Prognostic Index

The Nottingham Prognostic Index (NPI) attempts to describe the inherent aggressiveness of a tumour using the time dependent and biological factors described above. The score is derived from tumour size (pathological size in cm multiplied by 0.2), lymph node status (1

for negative nodes, 2 if 1-3 nodes positive and 3 if 4 or more nodes are positive), and grade (scored 1,2 or 3)[24]. Arbitrary cut off points are used to risk stratify patients into significant prognostic groups, **table 1.1**.

Prognostic Group	Score	5 year survival
Excellent	2-2.4	93%
Good	2.4-3.4	85%
Moderate	3.4-5.4	70%
Poor	>5.4	50%

Table 1.1 Summary of Nottingham Prognostic Index (NPI) scores and prognostic groups

The score has been validated by many studies including prospectively [9, 29, 30].

1.3 Molecular Features Of Breast Cancers In Relation To Treatment

1.3.1 Oestrogen Receptor Status

Oestrogen receptors (ER) are steroid receptors located in the cell nucleus. The degree of expression of ER on the breast cancer cells can predict a patient's response to hormone therapy. In unselected patients 30% will respond to endocrine therapy; while in those patients that are ER positive 78% have a chance of responding to therapy. If there is no ER expression then response to hormone therapy will not occur [31-33]. However, ER is not of independent prognostic significance, probably due to its close relationship with grade [31].

1.3.2 HER2-*neu* status

It is estimated that 15-25% of breast cancers overexpress HER2/*neu* protein or amplify the gene and this is associated with a more aggressive clinical course [34-36]. Single copy overexpression of the protein (that is protein overexpression in the absence of gene amplification) is rare [37] and both amplification and overexpression are correlated with a

poor prognosis[38]. The poor prognosis associated with HER2 overexpression is due to the clinical manifestation of its many biological functions – increased proliferation, cell survival, invasion and metastasis and increased angiogenic activity[39, 40]. HER-2 is an important target for monoclonal antibodies based treatments such as Trastuzumab (Herceptin). The status is commonly determined by a combination of immunohistochemistry assessing the expression of the HER2 oncoprotein and fluorescence *in-situ* hybridisation (FISH) assessing the number of gene copies per chromosome 17.

HER2 gene amplification has been shown to be independently predictive of OS and DFS in node positive patients [34] and over expression/amplification is associated with a poor prognosis in this sub group. However, the significance in node negative patients is less clear [41-43]. HER2 over expression has been associated with resistance to hormonal therapy [44-46].

1.4 Decision Aids for Adjuvant Therapy.

Several attempts have been made to summarise the prognostic features described above into reliable, reproducible tools for breast cancer physicians. A recent review article has summarised the tools currently available to aid clinicians in making the decision in whether or not to proceed with systemic chemotherapy [47]. They can be grouped into clinical guidelines, computer based risk calculators, gene expression profiles and minimally invasive disease (**table 1.2**)

Clinical Guidelines	St Gallen International Expert Consensus
	NCCN Clinical Practice in Oncology
Computer based risk calculators	Adjuvant! Online
	Neo adjuvant!
Gene Expression Profiles	Molecular classification of breast cancer
	Oncotype DX
	Mammaprint
Minimal Invasive Disease	Bone marrow micrometastasis
	Circulating Tumour Cells

Table 1.2 Decision aids for management of early breast cancer

1.4.1 St Gallen International Expert Consensus Panel

The St Gallen International Expert Consensus Panel have met since 1978 and aim to bring into focus contemporary insights and produce general principles based upon available evidence and expert opinion[48]. The March 2007 meeting looked specifically at a target orientated approach to adjuvant systemic therapy in early breast cancer. Risk stratification is based on histopathological features -**Table1.3** although it should be noted that there is some disagreement amongst the panel members.

Low Risk	Intermediate Risk	High Risk
Node Negative AND ALL of pT<2cm Grade 1 Absence of peritumoral vascular invasion ER and/or PR over expressed HER2/<i>neu</i> gene neither overexpressed nor amplified Age ≥ 35	Node Negative And at least 1 of pT>2cm Grade 2-3 Presence of peritumoral vascular invasion ER and PR absent HER2/ <i>neu</i> gene either overexpressed or amplified Age >35 OR Node Positive (1-3 nodes) and ER expressed HER2/ <i>neu</i> gene neither overexpressed nor amplified	Node Positive (1-3 nodes) And ER/PR absent or HER2/ <i>neu</i> gene either overexpressed or amplified OR Node positive ≥ 4 nodes

Table 1.3 St Gallen Risk Stratification

1.4.2 NCCN Clinical Practice in Oncology

The National Comprehensive Cancer Network (NCCN) began to develop guidelines in 1995. They are defined as “systematically developed statements to assist practitioner and patient decisions about appropriate health care for specific clinical circumstances” [47]. They use algorithms to define recommendations in an orderly fashion using categories of evidence and levels of consensus. Level 1 is equivalent to high quality evidence with a uniform level of consensus, level 2A a lower quality of evidence but a uniform level of consensus, level 2B a lower quality of evidence and a non-uniform level of consensus and level 3 no empirical evidence and surrounding controversy. There are annual meetings of individual specialists from a wide range of specialties who evaluate and expertly judge the scientific data. The first breast cancer treatment guidelines were published in 1996 and there have been subsequent updates [49-51]. These cover treatment of all stages of breast cancer including

methods of risk stratification for recurrence. They take into account age, menopausal and endocrine status on the benefits achieved by chemotherapy and endocrine therapy.

1.4.3 Adjuvant! Online

Adjuvant! Online (www.adjuvantonline.com) is a computer based risk stratification tool that provides a quantitative estimate of benefit of adjuvant systemic therapy for women with early stage breast cancer [52]. It provides visual, both numerical and graphical, printouts to share with patients if wanted. Factors including age, co-morbidity, grade and size of tumour, lymph node burden and ER status are used to predict 10 year OS, breast cancer specific survival and event free survival. The software is based on information gained from the American SEER (Surveillance Epidemiology and End Results) database and on Early Breast Cancer Trialist's Collaborative Group meta-analysis [1, 53, 54]. While it has been validated in a population based study [55] and physicians report that it is easy to understand and helps them understand their patients' treatment preferences [56] there have been concerns raised over its applicability in UK patients as the programme tended to overestimate overall and breast cancer specific survival in an Oxfordshire population[57].

1.5. Biomarker Mediated Molecular Classification of Breast Cancer

Using complementary DNA (cDNA) microarrays representing over 8000 genes, a subset of 496 mRNAs has been demonstrated to have variation in expression between a set of 65 different breast cancers [58]. Cluster analysis further allowed the cancers to be grouped into ER positive, (luminal) and ER negative (basal like, HER2 overexpressing and normal like) subtypes. These subtypes were found to be prognostic markers for OS and recurrence free survival. It is perhaps not surprising that the basal like and the HER2 overexpressing tumours had the worst prognosis as these cancers show a high frequency of HER2 oncoprotein or TP53 mutations. It has been demonstrated that the basal like cancers are

likely to represent the ER-ve, PR-ve, HER2 –ve cancers, the HER2 overexpressing the ER-ve, PR-ve, HER2 +ve, the luminal A the ER+ve, PR+ve, HER2 –ve and the luminal B ER+ve, PR+ve, HER2 +ve [59]. Interestingly the subtypes have also been found to have distinct sensitivities to neo-adjuvant chemotherapy [60].

1.5.1 Oncotype DX

Oncotype DX is a clinically validated 21-gene assay, which, determines a recurrence score (RS), for grouping lymph node negative, ER positive patients into recurrence risk categories [61]. 250 candidate genes selected from published literature, genomic databases, and experiments based on DNA arrays performed on fresh frozen tissue were tested in 3 independent clinical studies of breast cancer patients. From these studies 16 known cancer genes and 5 reference genes were selected to form an algorithm based on gene expression level. The 16 cancer genes can be grouped according to pathway; proliferation (Ki-67, STK15, Survivin, Cyclin B1, MYBL2), invasion (stromelysin, cathepsin) HER2 genes (HER2 GRB7) and ER genes (ER, PR, Bcl2, SCUBE2).

Both large and smaller population based case-control studies have validated that Oncotype DX can predict 10-year distant recurrence in this group of patients [62, 63]. RNA is extracted from formalin fixed paraffin –embedded tumours and after purification analysis is performed by reverse-transcription polymerase chain reaction. A RS is generated placing patients in a low, intermediate or high-risk group. It also appears to predict the magnitude of chemotherapy benefit with tumours with a high RS having absolute benefit from chemotherapy and those with a low RS having minimal or no benefit [64]. It was less obvious for those with an intermediate score and for this reason a multicentre trial, trial assigning individualised options for treatment (TAILORx), is currently on-going, aiming to recruit over 10,000 American and Canadian women[65]. The RS has been shown to be more

accurate than the NCCN guidelines as an indicator of prognosis for node negative, ER positive women with early breast cancer and to be cost effective [66]. The utility and adoption of the Oncotype Dx assay provides credence in support of further investigations of pathway-based responses to treatment.

1.5.2 MammaPrint

MammaPrint is a 70-gene, breast cancer mRNA expression profiling microarray that predicts risk of metastasis in women with node negative early breast cancer, regardless of ER status. It groups patients into low or high risk[67] and has been shown to be a more powerful predictor of the outcome of disease than standard systems based on clinical and histological criteria including Adjuvant! [68]. Prospective validation of MammaPrint is ongoing through the EORTC 10041/BIG 03-04 MINDACT (Microarray In Node-negative and 1–3 node positive Disease may Avoid ChemoTherapy) trial, which is the first prospective trial to evaluate the clinical utility of a molecular-based signature for the adjuvant treatment of early breast cancer [69]. There is early evidence to suggest that MammaPrint may be useful as a predictor of the benefit of adjuvant chemotherapy [70].

1.6 Cancer Biology Classification of Breast Cancers

1.6.1 Bone Marrow Micrometastasis

A large meta-analysis has shown that the presence of micrometastasis in the bone marrow at the time of breast cancer was diagnosed is associated with poorer outcome as defined by all cause mortality, breast cancer related mortality, local disease recurrence and distant metastasis [71]. This was true even for those groups classified as low risk by histopathological features. Bone marrow micrometastasis (BMM) is also independent of lymph node metastasis and may therefore represent a different mechanism of spread [72]. It may be that metastasis genes play a role in the development of future models of

treatment using BMM (discussed later), for example the use of adjuvant bisphosphonates may lead to long-term improvement in survival in patients with BMM[73]. However, bone marrow can be difficult to obtain, and before treatment stratification based on the presence of BMM, further clinical trials are essential. This is particularly true in women with node negative tumours less than 2cm, as the potential benefit derived from treatment needs to outweigh the morbidity of a bone marrow biopsy.

1.6.2 Circulating Tumour Cells

The presence of circulating tumour cells (CTC) in the peripheral blood of breast cancer patients is increasingly being used, either for staging or predicting disease progression[47]. In metastatic breast cancer increased levels of CTCs are associated with shorter progression free survival and worse OS [74-76]. However, to date no information is available on whether CTC detected at diagnosis can be used for treatment stratification and prospective randomised controlled trials are needed to look at the potential for prediction of response to adjuvant chemotherapy.

1.7 Use of Major Chemotherapy Drugs in Breast Cancer and Predictors of Treatment Response.

Current practice is for patients with moderate to high risk breast cancers based on histological, endocrine and HER2 markers to be offered adjuvant chemotherapy if clinically appropriate. The classification systems described above are not based except incidentally on the mechanisms affecting treatment response and although they assess risk, the majority do not predict response to treatment and mechanism based biomarkers that make this possible are needed.

Since the 1970s, a regime of cyclophosphamide, methotrexate and fluorouracil (5-FU) (CMF) has predominantly been used for adjuvant chemotherapy treatment of breast cancers. Randomised trials emerging in the mid to late 1990s supported the use of an anthracycline containing regime rather than CMF. Over the time scale of our cohort, CMF alone was steadily replaced with an anthracycline-containing regime (see chemotherapy cohort results figures 3.1 and 3.2). Exceptions to this occurred when patients were involved in any clinical trials or whose fitness status contraindicated anthracyclines. More recently the use of Taxanes has been advocated in the adjuvant treatment of node positive patients, but taxanes were not routinely used during our study period.

1.7.1 Chemotherapy Drugs

1.7.1.1 Cyclophosphamide

Cyclophosphamide is an alkylating agent, a member of one of the original classes of anti-cancer drugs. It causes specific sites of alkylation on purine bases leading to crosslinking of DNA strands and induction of apoptosis[77]. Cyclophosphamide is a pro-drug and requires biotransformation by a group of P450 cytochrome enzymes[78].

1.7.1.2 Methotrexate

Methotrexate is a member of the antimetabolite group of anti-cancer agents and specifically is a dihydrofolate reductase (DHFR) inhibitor. [77]. The active form acts as a co-enzyme for methylation in various metabolic processes including the synthesis of purine nucleotides and thymine. Thymidylate synthase (TS) catalyses the transfer of a carbon atom from tetrahydrofolate to target molecules by oxidising the folate ring of the tetrahydrofolate back to dihydrofolate. As DHFR is used repeatedly to allow this process to recur, continuous DHFR activity is required.

Methotrexate enters cells via specific folate receptors or by folate carriers. It then binds to the active site of DHFR preventing synthesis of tetrahydrofolate[79]. Thereby purine and thymine nucleotides cannot be created and DNA/RNA cannot be synthesised. This inhibits cell repair and replication.

1.7.1.3 Fluorouracil (5-FU)

The commonest used pyrimidine antagonist in anti-cancer therapy is 5-FU. It blocks the formation of normal pyrimidine nucleotides via enzyme inhibition with multiple consequences. It also interferes with DNA synthesis after incorporation into a replicated DNA molecule.

Thymine differs from uracil (a normal component of RNA) by the presence of a methyl group on the 5th carbon in the pyrimidine ring. This methyl group is added by TS. If, instead, a 5-FU molecule is present in this position instead of a uracil then TS cannot add a methyl group due to the presence of a fluoride atom at the 5th carbon. Therefore thymine can't be made and subsequently DNA cannot be synthesised[80].

5-FU is converted to its main active metabolite 5-fluorouridine monophosphate (F-UMP) that competes with uracil to be incorporated into RNA. This leads to inhibition of cell growth. Another active metabolite 5-fluoro-2'-deoxyuridine-5'-O-monophosphate (F-dUMP) inhibits TS[81].

Folate metabolism is a good example of a biological pathway with relevance to chemotherapy because folate has a key role in normal cell growth and replication and is also a target for chemotherapy agents[82]. There are 3 key proteins within the folate metabolism pathway; the drug transporter protein Reduced Folate Carrier (RFC); the regulatory enzyme 5, 10 methylenetetrahydrofolate reductase (MTHFR) and the drug target enzyme Thymidylate synthase (TS). Methotrexate, a folate analogue and 5-fU are folate

pathway inhibitors. Folate analogues are structurally similar to folate but are able to inhibit the action of various enzymes in folate metabolism (in the case of methotrexate, primarily dihydrofolate reductase) [83-85]. 5-fU is a fluoropyrimide that develops a stable complex with TS that inhibits enzyme activity and leads to reduced levels of proliferation[86]. There are many studies demonstrating how polymorphisms in the genes for the 3 key proteins of the folate pathway affect the response to methotrexate or 5-fU; RFC [87]; MTHFR [88-94] and TS [95-100].

1.7.1.4 Anthracyclines

The first two anthracyclines, doxorubicin and daunorubicin were originally isolated from *streptomyces peucetius*. Epirubicin is a semi-synthetic derivative of doxorubicin [101].

Anthracyclines have a wide range of biological activity including anti-bacterial, immunosuppressive, anti-parasitical, and anti-tumour. The limiting factors in their use as a chemotherapy agent are the development of tumour resistance and cardio-toxicity. Patients can develop dilative cardiomyopathy and congestive cardiac failure with cumulative use.

The exact mechanism of action of anthracyclines is elusive. It is probable that they are a topoisomerase poison, leading to the initiation of DNA damage [102]. Topoisomerases modify the topology of DNA without altering structure or sequence. Anthracyclines act by stabilising a reaction intermediate in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II leading to the inhibition of DNA resealing.

Doxorubicin has also been reported to inhibit topoisomerase I [103].

Other theories concerning mode of action have included intercalation into the DNA resulting in inhibited synthesis of macromolecules; the generation of free radicals leading to DNA damage or lipid peroxidation; DNA binding and alkylation; DNA cross linking;

interference with DNA unwinding or DNA strand separation and helicase activity and direct membrane effect[101].

1.7.1.5 Taxanes

Taxanes are naturally occurring compounds derived from the Taxoidaceae tree family. Paclitaxel was developed first but due to solubility and availability issues a semi-synthetic drug, docetaxel was developed. They are anti-mitotic drugs, stabilising GDP-bound β -tubulin dimer in the microtubule, thus leading to cell-cycle arrest in G2/M. [77] [104]. This prolonged mitotic arrest induces phosphorylation, and therefore inactivation, of the anti-apoptotic protein Bcl-2 in a seemingly TP53 independent process [105].

1.7.2 Breast Cancer Adjuvant Chemotherapy Regimes.

It has been demonstrated that both sequence and timing of chemotherapy regimes are important, and therefore block sequential regimes have become standard [106]. There are many variations in specific regimes used in breast cancer and a single recommendation has not been given. CMF has been used for breast cancer chemotherapy since the 1960s and multiple trials have confirmed the efficacy of anthracycline containing regimes. In 1998 the world overview of trials performed by the Early Breast Cancer Trial Collaborative Group (EBCTCG) reviewed 11 trials of anthracycline-containing regimes verses CMF. They demonstrated that anthracycline regimes gave an additional 12% proportional decrease in risk of recurrence and an absolute reduction of 3.2%. In addition, there was an additional 11% proportional decrease in the risk of death with an absolute reduction of 2.7%[54]. This was updated in 2005 and with the additional follow up anthracyclines continued to give significant improvements in DFS and OS across all age groups[107].

The EBCTCG report however did not consider taxanes. The most recent NICE guidelines (2006) have considered a number of RCT looking at the use of taxanes. Although the regimes used have been variable there have been modest improvements in patient

outcome with the addition of taxanes to an adjuvant regime. The only current guidelines in the UK at present are that docetaxel should be given concurrently with doxorubicin and cyclophosphamide for lymph node positive early breast cancer [108].

A number of the patients in our cohort were involved in chemotherapy trials that include tAnGo, TACT, and NEAT.

1.7.2.1 tAnGo

tAnGo was an international randomised phase III trial evaluating gemcitabine in paclitaxel containing epirubicin/cyclophosphamide based adjuvant chemotherapy. It commenced in 2000 and was based on pre-clinical evidence of a potentially favourable interaction between paclitaxel and gemcitabine and encouraging results with gemcitabine in advanced breast cancer [109]. 4 cycles of epirubicin 90mg/m² and cyclophosphamide 600mg/m² followed by 4 cycles paclitaxel 175mg/m² and gemcitabine 1250mg/m² were compared with the same regime without the gemcitabine.

3152 patients were randomised and stratified according to country, age, radiotherapy, nodal status, ER and HER2/*neu* status. There has been no difference in DFS or OS between the groups indicating no therapeutic advantage to the addition of gemcitabine [110].

1.7.2.2 TACT

The Taxotere as adjuvant chemotherapy trial (TACT) was a randomised phase III trial comparing 8 cycles of fluorouracil 600 mg/m², epirubicin 60 mg/m², cyclophosphamide 600 mg/m² (FEC) or epirubicin 100 mg/m² for four cycles followed by CMF (cyclophosphamide 600 mg/m², methotrexate 40 mg/m², and fluorouracil 600 mg/m²) with 4 cycles of FEC followed by 4 cycles of docetaxel 100 mg/m². As well as looking for differences in DFS and

OS the trial sought to identify specific subgroups that may benefit from taxanes, which had proven challenging.

The inclusion criteria included women over 18 years of age within 8 weeks of definitive surgery for invasive early breast cancer (stage pT1-3, pN0-1, M0), which was either node positive disease or high-risk node negative (grade 3, ER receptor negative or lymphovascular invasion present). 4162 women were randomised and after a median follow up of 51.8 months there was no significant difference in DFS or OS. Subgroup analysis on either pathological or clinical features also failed to show any significant differences [111]. Further analysis based on biological features is planned.

1.7.2.4 NEAT

The National Epirubicin Adjuvant Trial was a phase III randomised treatment trial commencing in 1996 comparing anthracycline based epirubicin plus CMF with standard CMF treatment at a time when evidence for adjuvant chemotherapy regimes in breast cancer was lacking. 2391 patients had a median follow up period of 48 months. Highly significant findings of a 28% advantage in DFS and a 30% advantage in OS in those receiving an anthracycline were reported[112]. The addition of an anthracycline was also found to be tolerable and acceptable in all patient groups [113]. The conclusion was that Epirubicin plus CMF is superior to CMF alone as adjuvant treatment for early breast cancer.

1.7.3 Molecular and Cell Biological Factors in Relation to Chemotherapy Response

Whilst biomarker mediated methods are proving useful for breast classification with regard to prognosis, the markers used are selected on the basis of their statistical power rather than any mechanistic significance and where mechanism is implied this is incidental to the process. By exploring signal transduction pathways it is also possible to construct hypotheses concerning genes that ought to have mechanistic and therefore diagnostic

significance. Instances of acquired and natural genetic variants having an influence on the relative effectiveness of signal transduction are known and candidates for explaining differences between the relative chemoresponsiveness of cancers. Natural genetic variants are of particular interest here because they are constant between cancers, unlike acquired variants, which may be specific to a given cancer sample.

Chemotherapy agents exert their effect through many actions; consequently there are diverse ways that the behaviour of individual breast cancers could be influenced. Apoptosis has been considered as the primary means by which chemotherapy agents exert their effects [114-116], Other non-apoptotic modes of action include through angiogenesis, proliferation and invasion.

1.7.3.1 Single nucleotide polymorphisms

Single Nucleotide Polymorphisms (SNPs) are variations in the human genome where the sequence varies by one nucleotide between any two individuals. They account for 90% of all polymorphisms in humans, occurring on average every 1 in 300 nucleotides. The 1000 Genomes project, which is surveying such variation in 3 member, family triads (2 parents, one child) estimates that over 15 million exist in the population overall [117]. SNPs within coding areas can be synonymous resulting in the same polypeptide sequence or non-synonymous (missense variants) resulting in an alternative polypeptide. The latter can also affect structure and therefore function. Functional consequences in non-coding sequences are also possible if polymorphisms affect regulatory sequences. Loss of protein sequences can also result if missense variations result in a premature stop codon and therefore a truncated polypeptide or involve small insertions or deletions, which result in non-sense translation beyond the point of the variant. Correct splicing can also be affected by variants at the splice junctions or within the sequences that regulate splicing.

SNPs that alter function have great potential as biomarkers for personalised medicine. They are stable, widespread and relatively easy to detect. Naturally occurring genetic variants in the population could therefore influence the pathways regulating key processes required for the development and progression of cancer and thus influence chemotherapy response. Moreover they would then serve as mechanistic biomarkers of treatment response. Stoehlmacher et al [118] for example studied the prediction of clinical outcome to 5-fU combination chemotherapy in refractory colorectal cancer by assessing 10 polymorphisms within 8 genes involved in the metabolism and detoxification of 5-fU. This comprehensive pathway evaluation revealed that a combination of favourable genotypes could identify those patients that would gain the greatest benefit from treatment.

1.7.3.2 Apoptosis

Apoptosis is programmed cell death and requires the synthesis of mRNA and proteins. It is a normal end process of the cell cycle and the most common mechanism by which the body eliminates damaged or unneeded cells without local inflammation from leakage of cell contents [119, 120]. TP53 can direct damaged cells into an apoptotic pathway. Genetic mutations resulting in decreased apoptosis can allow a tumour cells proliferative uncontrollably[121] and virtually all cancer cells contain genetic alterations that enable evasion of apoptosis[122]. The apoptotic response of cells is mediated by the activation of a caspase cascade, which can be initiated via 3 pathways, intrinsic, extrinsic and extracellular **(Figure 1.4)**.

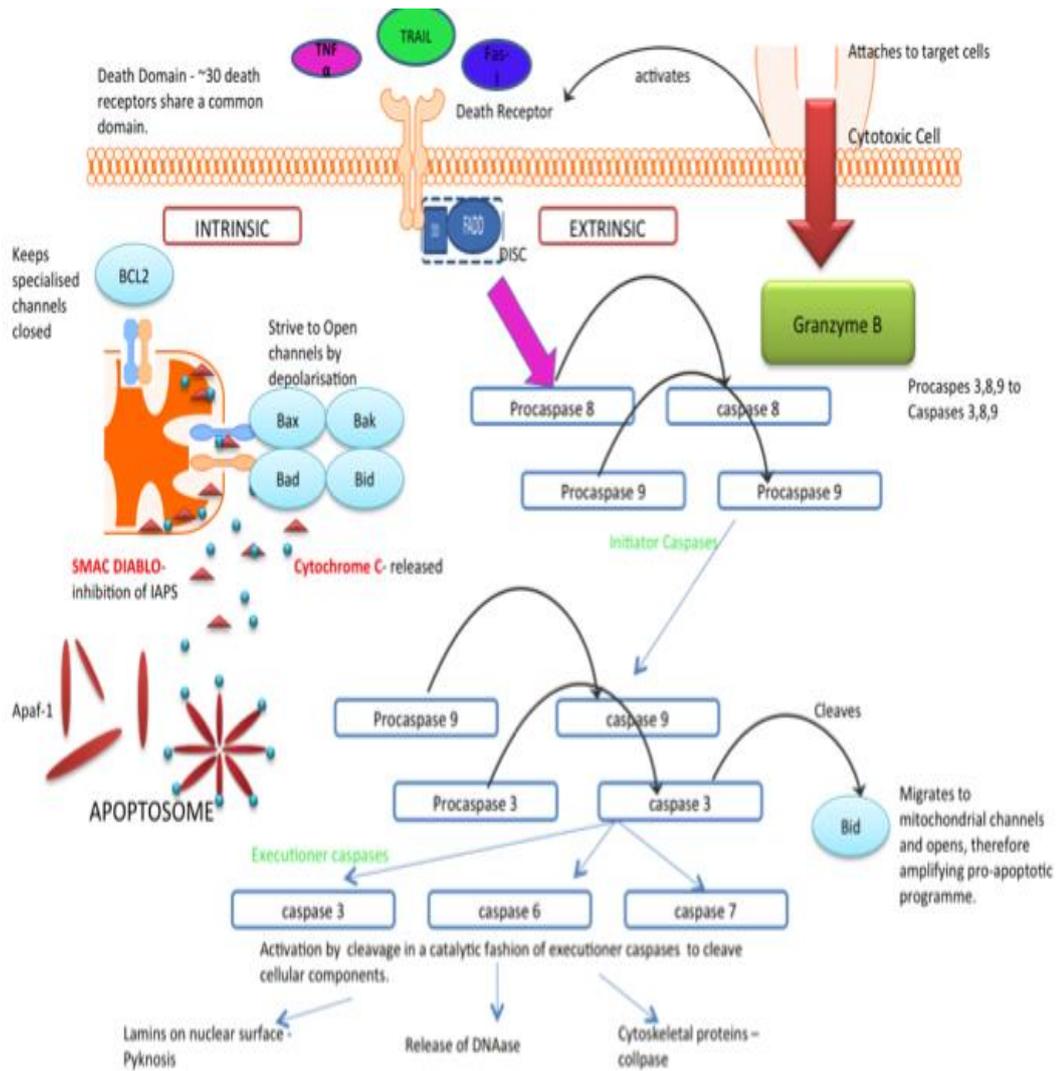


Figure 1.4 The 3 pathways leading to apoptosis

The intrinsic pathway is mediated via the BCL2 family proteins which act to regulate the permeability of the mitochondrial membrane [123]. There are 24 family members, 6 anti-apoptotic and 18 pro-apoptotic. BCL2 itself has an anti-apoptotic effect and operates on the outer membrane of mitochondria to keep specialised channels closed along with BCL-XI [124-126]. Antagonistically, Bax, Bad, Bak and Bid all act to open the mitochondrial channels allowing cytochrome C to be released from in between the inner and outer mitochondrial membranes [127, 128]. The transcription of Bax is activated by p53 [129, 130] and Bad can be phosphorylated by Akt/PKB [131], decreasing its ability to open the channels [132]. As well as the release of cytochrome C, the opening of the channels allows

fragmentation to take place and the collapse of the ATP generating system that is required for signal transduction.

Once released into the cytosol, cytochrome C associates with Apaf-1 protein and forms an Apoptosome [133, 134] that acts to cleave the latent cytoplasmic protease from procaspase 9 to caspase 9 [135]. This initiator caspase commences a sequence of cleavages in which one protease cleaves the next one to achieve activation, each time acting catalytically so that a minor signal at the top of the cascade can be amplified to a large number at the bottom where they ultimately cleave death substrates, proteins whose degradation creates the cellular changes associated with apoptotic death [136, 137].

The executioner caspases, which are activated by the initiator caspases, caspases 3, 6 and 7 cleave the cellular components such as the lamins on the inner nucleus surface leading to pyknosis [138], cell collapse and also allow the release of DNAase [139]. Caspase 3 also cleaves the BCL2 related protein Bid, activating it and allowing it to migrate to the mitochondrial channels [140]. This allows more channels to open and amplifies the pro-apoptotic response.

The opening of the mitochondrial channels also allows the release of other intramembrane proteins such as Smac/DIABLO [134]. This inactivates the Inhibitors of Apoptosis (IAPs) that normally block caspase activity by either binding directly with caspases and inhibiting proteolytic activity or by marking caspases for ubiquitylation and degradation [135, 141, 142]. The release of Smac/DIABLO results in the caspases being free to initiate the proteolytic cleavages that lead to apoptosis.

The extrinsic pathway is initiated when ligands from the Tumour Necrotic Factor (TNF) family bind with death receptors on the cell membrane. These death receptors are a trans membrane protein and approximately 30 share a common cytoplasmic death domain [133,

143-145]. Once activated by TNF- α , TRAIL or Fas-ligand for example, a Fas associated death domain (FADD) protein bind to the death domain forming a death inducing signalling complex (DISC)[146-149]. This activate the cleavage of procaspases 8 and 10 into active caspases 8 and 10 which also act as initiator caspases for the executioner caspases 3,6,7, thus linking in with the intrinsic pathway[136, 143, 150].

The third pathway is mediated by cytotoxic T lymphocytes and natural killer cells. Killer cells can either activate death receptors such as Fas thereby initiating the extrinsic pathway[151], or they can attach to the cell membrane of target cells and release a protease granzyme B[152, 153]. Once internalised, granzyme B can cleave and activate the procaspases 3, 8 and 9 and converge with the cascade[154, 155].

Cells can utilise all of the 3 possible pathways to apoptosis and therefore variation in their susceptibility to apoptosis is expected depending upon prevailing condition. Cells using primarily the extrinsic pathway are less able to avoid apoptosis by BCL2 overexpression for example because death receptors communicate directly with the caspase cascade.

TP53 can control the apoptotic pathway by directly promoting transcription of Bax and Fas receptors[156-158]. It also transcribes for IGFBP-3 protein which is released from cells and binds to Insulin like growth factor-1 (IGF1)[159]. IGF1 operates through its own cell surface receptors to produce trophic (survival) signals in the cell. On binding to IGFBP-2, IGF1 is sequestered and the reduced levels lead to the inhibition of survival signals and places the cell in danger of succumbing to apoptosis[160].

TP53 status is proven as a prognostic factor in breast cancer, however its role as a predictive factor is more controversial and trials generally don't support the use of p53 for patient selection to treatment[161]. There are a number of studies that both support [162-169] and refute relationship between TP53/p53 and response [168, 170-191]. There are a

number of possible explanations for this apparently conflicting evidence; given that the multiple activities of p53 and its capacity to induce apoptosis may depend on criteria such as type of drug used, drug dose, tumour type and the mutation spectrum of the tumour, it is possible that the apoptotic pathways induced by the drugs tested were independent of p53 [192, 193]. All the studies above used immunohistochemical techniques of differing methodologies and antibodies as well as scoring TP53 as either a protein or mutation. It has been shown that around 30% of tumours harbouring TP53 mutations are recorded negative by immunohistochemistry and that not all gene mutations lead to an increase in protein levels[174]. Therefore assessment of TP53/p53 status should include gene-sequencing techniques. Studies evaluating TP53 by sequencing have reported mutations to predict for chemoresistance in haematological malignancies [194-197].

1.7.3.2.1 TP53/p53 and CMF responsiveness.

Askmalm et al [198] reviewed a clinical trial where adjuvant therapy was randomised to CMF chemotherapy or radiotherapy with or without tamoxifen in women with lymph node positive disease or tumour size over 30mm after mastectomy. Using immunohistochemistry, DNA extraction and PCR with single strand conformational polymorphism techniques, TP53 mutations were identified in 20% of 266 patients with a median follow up of 11.5 years. Those with a TP53 mutation had a trend towards CMF being better than radiotherapy for distant recurrence (RR=0.72, 95% CI 0.32-1.6) and those with no TP53 alterations had a non-significant trend towards a decreased benefit from CMF (RR1.4, 95% CI 0.78-2.4).

In a sequence-based analysis of TP53 status Andersson et al [199] extracted RNA from frozen tumour samples of 376 lymph node positive patients. 174 had received adjuvant CMF chemotherapy.28% were found to have a mutation and although TP53 mutations predicted for a worse recurrence free survival and OS in patients receiving CMF, this was

not significant on multiple regression analysis. The mutation type and location were not shown to be of any significant importance. The authors concluded that their study was lacking in numbers to make any powerful conclusions.

1.7.3.2.2 TP53 and anthracycline responsiveness

Geisler et al [200] looked at a series of 94 patients with locally advanced breast cancer receiving doxorubicin monotherapy. 91 samples were available for snap freezing and analysis of TP53 mutations. In keeping with reported literature 26 patients (28%) had TP53 mutations in their tumour samples (16 missense, 6 deletions/splices and 4 nonsense). Taken as a homogenous group, TP53 mutation did not predict response to therapy. However mutations affecting the L2/L3 domain (n=19) predicted for resistance to doxorubicin (p=0.008), as did non-missense mutations (p=0.025).

In a series of 108 patients receiving chemotherapy for metastatic breast cancer, Rahko et al[201] assessed a sub group of 30 patients receiving anthracycline based therapy. They found that 73% of those with mutant TP53 progressed on therapy compared with only 33% with wild type p53.

In the TAX 303 clinical trial single agent doxorubicin was compared with single agent docetaxel as first or second line therapy in advanced breast cancer [202]. Using genomic DNA extracted from paraffin embedded tumour denaturing high performance liquid chromatography was performed to identify p53 mutations in 78 tissue samples. 34 p53 mutations were detected with 22 confirmed with sequencing. There was a trend for p53 mutation to decrease the probability of response to doxorubicin and increase the probability of response to docetaxel but these were not statistically significant. When examined in detail there were 12 patients with mutation who had been treated with doxorubicin. 6 mutations were located at exon 5 and 3 mutations at exon 7, all of which

had no response to therapy. 2 mutations at exon 6 or 8 responded well. In the 10 patients with p53 mutations treated with docetaxel there were 3, 4, 2 and 1 mutations at exons 5, 6, 7, and 8 respectively. Responses to docetaxel were reported in all exons with exception of exon 7.

1.7.3.2.3 p53 and Taxanes

Several studies have assessed the association of TP53 mutation and response to docetaxel and found no significant association with immunohistochemical staining [203-205]. In one genomic DNA analysis study of 50 breast tumours and the response to docetaxel there was no significant association found leading the authors to conclude that the mutational loss of TP53 does not confer to resistance against docetaxel[206]. This is in keeping with *in vitro* studies showing no association between TP53 mutation and resistance to paclitaxel[207]

Although it is generally thought that taxanes induce apoptosis via a p53 independent pathway, TP53 status may influence cell-cycle progression following mitotic arrest. Ooe et al [208] therefore assessed TP53 mutation and response to neoadjuvant docetaxel. They showed that TP53 mutations had a trend towards a lower response to treatment but not significantly and also that L2/L3 region domains tended towards lower response, but again not significantly.

They went on to assess which genes were expressed differently between mutated TP53 tumours and wild type TP53 tumours. 13 genes were differently expressed with statistical significance, and 3 of these were differentially expressed between responders and non-responders (chaperonin containing TCP1 subunit 5 (CCT5), regulator of G-protein signalling 3 (RGS3) and SNARE protein YKT6). These were all upregulated in TP53-mutated tumours and were associated with a resistance to docetaxel, leading to a possible new insight into the molecular mechanism of resistance to docetaxel.

1.7.3.2.4. TP53 SNP – codon 72 (rs1042522)

A SNP in codon 72 of TP53 gene was identified in 1986[209]. A C to G base change results in a proline (Pro) to arginine (Arg) residue. It resides in the Pro rich region of TP53 that has been shown to be important in mediating the apoptotic response[210]. In a European population the minor allele (C) has a frequency of 23%. It has been demonstrated that wild type TP53codon 72 Arg has greater apoptotic potential both in the presence [211] and absence of chemotherapies[212-215].

This variant has been studied in breast cancer. It may represent a potential risk factor for breast tumorigenesis. TP53 codon 72 Arg homozygosity has been linked with increased risk in Brazilian[216], Turkish[217] and Greek [218] populations but a further Turkish study found that the increased frequency of the Pro allele and the presence of a Pro allele was associated with breast cancer[219].

TP53 codon 72 may also have some prognostic significance. In a population of 414 breast cancer patients, genotype was significantly associated with DFS [220]. In those with wild type TP53 (346) Pro/Pro variant had worse DFS than those with Pro/Arg or Arg/Arg. A Finnish breast study also demonstrated that patients homozygous for Pro had a poorer survival [221].

However, in a metastatic cohort those patients homozygous for Arg had a significantly shorter time to progression and OS than those heterozygous [222]. Patients with LOH of Arg allele were diagnosed at an earlier age than those with loss of a Pro allele and were also associated with worse survival [223]. No correlation has been found between genotype and clinicopathological parameters [224, 225].

In 557 Japanese patients with breast cancer, the Pro/Pro genotype was again associated with poorer DFS. This was especially significant in patients who had received adjuvant

chemotherapy[226]. In patients who received adjuvant hormone or no adjuvant therapy, genotype was not associated with DFS. In a pathway based analysis of SNPs with relevance to 5 fU therapy in breast cancer it has been suggested that TP53 codon 72 may have a direct, allele specific role in 5fU mediated response [227]. Pro/Pro homozygosity also predicted for poor response to anthracycline based neo-adjuvant chemotherapy in a study of 110 patients [225].

A randomised treatment trial of 220 patients found that in ER positive breast cancer having at least one Pro allele had better distant recurrence free survival when randomised to tamoxifen leading the authors to suggest that those ER positive cancers lacking a Pro allele may be a candidate for treatment other than tamoxifen[228].

1.7.3.3 MDM2

MDM2 has the ability to inactivate the function of p53 through ubiquitination and degradation by direct binding to the p53 protein [229, 230]. MDM2 overexpression has been associated with many types of cancer where it has been shown to be involved in the inactivation of wild type TP53 thereby obliterating cell cycle checkpoint control [231]. Intracellular MDM2 expression is controlled at the levels of protein stability, gene transcription and transcript translation[232]. Upon stress or hormonal signalling various transcription factors, among them p53, bind to response elements in the MDM2 gene promoter in the first intron [233]. A [G/T] SNP in this intron at position 309 generates a novel binding site for the ubiquitous transcriptional activator SP1 and causes higher MDM2 levels and consequently, attenuated p53 response in stressed or oestrogen exposed cells [234-236]. This is a hallmark of some tumour types in humans [237-239].

It is the G allele of SNP 309 that increases the DNA binding affinity of the transcriptional activator, SP1, which results in high levels of MDM2 mRNA and protein in human cells

[240]. The increased MDM2 levels are associated with the attenuation of the p53 pathway and the acceleration of tumour formation in both hereditary and sporadic cancers [234, 241, 242].

Whilst MDM2 SNP 309 has been associated with increased risk of some cancers [243], [244], [242], [245], [234], [246] [247], most studies find no increased risk for breast cancer [248-252]. SNP 309 has been implicated in earlier age of onset of Li-Fraumeni syndrome and sporadic cancers [234]. The GG genotype has been associated with both the risk of breast cancer and earlier age of onset in Taiwanese women [253] and poor prognostic features in a Scottish population [254]. In 557 Japanese primary breast cancer patients MDM2 SNP 309 was not associated with DFS [226]. In a study to find interaction between SNP 309 and tumour p53 expression for breast cancer survival it has been shown that TT genotype was associated with increased risk ratio for death for both mutant and aberrant p53. Tumour TP53 status was not associated with breast cancer survival among carriers of GT or GG which is consistent with a dominant effect of the variant allele [250]. A strong interaction between 309 status and tumour TP53 status appears to modify the association between TP53 status and breast cancer survival.

There are a lack of studies on MDM2 variants and chemotherapy response in breast, but Nayak *et al* [255] have demonstrated that cancer cell lines harbouring MDM2 SNP309 are resistant to certain topoisomerase II targeting drugs. Increased expression of MDM2 resulted in lower topoisomerase II, the target of drugs, following drug exposure. Decreased expression of topoisomerase II α has been shown previously to be a mechanism of resistance to topoisomerase II targeting drugs [256, 257].

Also amplification and overexpression of MDM2 in human sarcomas can stimulate cell proliferation, enhance cell survival and induce resistance to conventional chemotherapy [238, 258, 259]. However, the SNP 309 genotype does not predict survival, progression free

interval or sensitivity to platinum based chemotherapy in a study of ovarian, tubal and peritoneal carcinomas [260].

1.7.3.4 BCL2 family genes

B-cell CLL/ lymphoma 2 (BCL2) is located on chromosome 18q21 and encodes an integral inner mitochondrial membrane protein of 26kDa that involved mainly in inhibiting apoptosis [261]. Several experimental models have shown the BCL2 gene to protect cells against death induced by a myriad of insults including most chemotherapeutic agents [262-264] implying that Bcl-2 over expression may play a role in the resistance to chemotherapy via the inhibition of apoptosis.

A number of studies have assessed the role Bcl-2 in the prediction of response to chemotherapy in breast cancer. Several authors have failed to find any association either in the neo-adjuvant [115, 167-169, 172, 179, 188-190, 265] or metastatic settings [191].

Takamura *et al* [189] demonstrated a weak association with increased Bcl-2 expression and increased response to neo-adjuvant cyclophosphamide/epirubicin treatment but no association with docetaxel therapy in a study of 70 patients. This trend of Bcl-2 overexpression being associated with better response was also seen in a neo-adjuvant study using mitomycin C, mitozontrano, methotrexate and tamoxifen[190].

In a study of adjuvant CMF chemotherapy verses adjuvant tamoxifen in node positive women, Bcl-2 expression correlated with improved recurrence free survival in both groups and improved OS in the group receiving chemotherapy[170]. However in a study of 55 patients in a metastatic setting receiving either CMF or CAF chemotherapy a higher response rate was seen in patients with Bcl-2 negative tumours although this did not equate to improved survival[171].The absence of Bcl-2 correlating with improved response has also been demonstrated in neo-adjuvant studies[186, 266].

Changes in Bcl-2 expression with treatment have also been evaluated. Whilst some studies show no change over treatment [167, 190] others have demonstrated an increase in post-treatment Bcl-2 levels in all tumours ($p < 0.03$) [115]. A trend towards increased expression in responders to CMF or anthracycline therapy ($p = 0.063$) is contradictory to a finding that a decrease in Bcl-2 expression relative to pre-treatment level correlated with a complete response [267].

BAX is a gene that functions closely with BCL2 as a pro-apoptotic protein [268]. It has been demonstrated that all Bcl-2 positive tumours are also BAX positive [266]. The absence of BAX protein has been demonstrated to be significantly associated with both improved [266] and poorer [269] response to chemotherapy (FAC and CEF respectively). In a study of 231 patients receiving one of 5 chemotherapy regimes no association between BAX and treatment outcome was demonstrated [167].

These variable findings suggest that it is possible that the determination of BCL2 status alone is not sufficient in assessing the competency of the Bcl-2 apoptosis pathway.

Buchholz *et al* [266] hypothesised that Bcl-2 (anti-apoptotic) over expression would be associated with a poor response to treatment, while BAX (pro-apoptotic) over expression would be associated with a good response. In fact, they found that both were associated with a poor response. This led to theory that a transcription factor involved in the regulation of both protein products may be significant in controlling apoptotic response.

We have therefore considered variations in both these genes.

1.7.3.4.1 BCL2 -938 C>A (Rs 2279115)

BCL2 gene consists of 3 exons and 2 promoters [270]. The second promoter, P2, lays 1400 base pairs upstream of the translation initiation site and decreases the activity of the P1 promoter and therefore functions as a negative regulatory element [271, 272]. In 2004 Park

et al identified 6 SNPs in Bcl-2 from direct sequencing of a Caucasian population [273]. Haplotype analysis yielded a significant linkage disequilibrium between a SNP in the inhibitory P2 promoter (-938 C>A) and a silent SNP in exon 1 (+21 A>G). Nüchel et al [274] demonstrated that the -938 SNP had a statistically significant impact on transcriptional activity. The -938 C allele contains a putative binding site for the important transcription factor SP-1 which was abolished by in the A allele. The C allele has thereby been associated with increased P2 promoter activity and binding of nuclear proteins leading to overall decrease in Bcl-2 promoter transcriptional activity and Bcl-2 expression.

The -938 SNP was also demonstrated to be relatively common, in the control population 36% were AA, 63% AC and 21% CC, proportions subsequently confirmed by further authors [275-278]. The high prevalence of this SNP and its proven influence over Bcl-2 expression [274, 279] has led to studies in various tumour types to assess its role as a prognostic marker. The CC genotype is associated with poor outcome in various cancers [279], [277] [278] confirming its important role in solid tumours. [280].

Bachmann et al [276] hypothesised that breast cancer patients with a genotype of AA (associated with increased levels of Bcl-2) would have a survival benefit. There was no association with clinic-pathological features, including ER and HER2/*neu* status but the SNP was demonstrated to be associated with Bcl-2 levels in breast cancer.

Increased levels of Bcl-2 were associated with increased survival overall. On subgroup analysis this only remained significant in the lymph node positive population. However, in lymph node negative patients the CC genotype was independently predictive of a worse survival. This gives the SNP the potential to be a candidate to identify those lymph node negative high-risk patients who may benefit from chemotherapy.

There are no further published studies to date of this interesting SNP in breast cancer. Also, given the tendency of the CC genotype to have a significantly higher apoptotic index [275], there is a lack of studies associating this SNP with chemotherapy response.

1.7.3.4.2 Bax -248 G>A (Rs 4645878)

Bax is located on chromosome 19 and contains 6 exons [281]. Drug-induced apoptosis *in vitro* correlates with the Bcl-2/Bax ratio [282, 283] and chemosensitive cells show rapid induction in Bax[283]. Transcription factors binding sites found in the Bax gene promoter include p53 response elements and NFκB binding sites which are involved in the regulation of gene expression [130, 284, 285]. Mutations found in the promoter region containing p53 response elements and in the open reading frame (ORF), which encodes for dimerization domains [130], have been shown to abolish its pro-apoptotic function and promote tumour formation [286, 287]. A frame shift mutation within a homodimeric stretch of 8 consecutive guanosines has been observed in more than 50% of colon cancers [287]. Although there is no evidence yet of a Bax frame shift mutation in breast cancer, loss of Bax expression is reported in some breast malignancies. In a series of 119 metastatic breast cancers loss of Bax was seen in a third of cases and was associated with a shorter OS, faster time to progression and a failure to respond to chemotherapy [269]. In contrast to this cohort of patients treated with chemotherapy in whom loss of Bax is a prognostic indicator, significant correlations of Bax expression and outcomes have not been observed for patients with localised disease[288]. This suggests that the prognostic significance of Bax in breast cancer may be limited to chemotherapy treated cohorts. Reduction in the expression of Bax is known to contribute to the unresponsiveness to chemotherapy drugs [289]. Bax G (-248) variant is associated with protein expression, stage progression and failure to achieve complete response to chemotherapy in CLL[290] [291].

Although decreased Bax expression is associated with prognosis in solid cancers [292] to date there has only been one published study associating Bax SNPs and solid tumours. In a study of 814 patients with head and neck squamous cell carcinoma (SCCHN) and 934 controls there was no association between risk and Bax G (-248) A SNP[280].

The population frequency of the GA genotype and the AA genotype in healthy populations has been found to be 14-21% and 0.8-1.2% respectively [280, 291]. Given the relatively high prevalence of this SNP in a normal population and the central role of Bax in mediating chemotherapy induced cell death in many tumours, including breast, it indicates a need for genotypic studies of this SNP.

1.7.3.5 CDKN1A; Codon 31 (rs1801270) and 3'UTR (rs1059234)

CDKN1A, is a tumour suppressor gene located on chromosome 6p21.2 and encodes a 21KDa protein [293] that belongs to the CIP/KIP family. The protein is a cyclin dependent kinase (CDK) inhibitor that is essential for cell growth, differentiation and apoptosis. The expression of CDKN1A is upregulated by p53 in response to DNA damage leading to either cell cycle arrest at the G1 checkpoint or apoptosis[294]. Somatic mutations in the CDKN1A gene are rare in human malignancies [295] but decreased expression in tumours (including breast [296]) has been associated with poor prognosis [297, 298]. It is therefore possible that genetic variation may modulate the expression of CDKN1A.

One hundred and six SNPs have been reported in CDKN1A in dbSNP including 6 non-synonymous. One common SNP is found in exon 2 which causes a non-synonymous Ser-Arg substitution at codon 31 (rs1801270)[299]. Fifteen SNPs are found in the 3' untranslated region (UTR), only two of which have an allele frequency of over 10%. RS1059234, CDKN1A C70T causes a single C-T substitution 20 base pairs downstream of the stop codon at exon 3.

As the 2 commonest SNPs [300], both this SNP at 3'UTR and at codon 31 are thought to alter CDKN1A function.

Because of the important role of CDKN1A in the p53 pathway, many studies have tried to associate cancer risk with polymorphisms in the many cancers including breast with variable results [301-303], [301, 304], [305-308], [305, 309-311], [312] | [313], [314], [315], [316, 317] [306].

The underlying mechanism by which CDKN1A might affect cancer risk is unclear; however the codon 31 SNP causes a serine to arginine substitution in the zinc finger motif which could alter the protein's function [306]. Or, as 3'UTRs have been shown to be important for RNA stability and gene regulation [318, 319], it is possible that the polymorphism in 3'UTR may increase the risk of carcinoma by altering mRNA stability thereby affecting intracellular levels of CDKN1A protein; although this hypothesis remains to be tested [299].

No breast cancer treatment trials have been published however, CDKN1A 3'UTR SNP has been interrogated for an association with outcome in ovarian cancer treated with platinum based chemotherapy in the form of cisplatin and paclitaxel [320]. Although the SNP on its own was not associated with outcome, p53 codon 72 AA genotype simultaneously with a CDKN1A 3'UTR CC genotype did demonstrate a statistically significant longer progression free survival.

1.7.4 Cell Cycle.

In order to proliferate a cell must progress through the cell cycle. Immediately after mitosis, mitogenic growth factors will encourage a new cell to remain in the cell cycle whilst growth inhibitory factors such as transforming growth factor β (TGF- β) encourage cells to enter G0[321]. The time between the birth of a daughter cell and the subsequent onset of DNA synthesis is known as G1. The restriction point (R point) late in G1 is a critical determinant

of whether cells will progress to the S phase of DNA synthesis, and therefore grow. It is the point of growth factor independence. The deregulation of R point decision making machinery is a mechanism for uncontrolled cell proliferation and therefore cancer[322].

The cell cycle clock that controls progression through the cell cycle does so by deploying cyclin-dependent kinases (CDKs) (**Figure1.5**). These are serine/threonine kinases, which share approximately 40% amino acid sequence identity with one another[323]. Another protein class, cyclins, then associate with CDK, forming active cyclin-CDK complexes. Early and mid G1 is guided by CDK 4 and CDK6 which associate with the D type cyclins (D1, D2, D3)[324]. At the R point E type cyclins associate with CDK2 that enables the phosphorylation of appropriate substrates required for entry into S phase[325]. As the cell enters S phase A type cyclins replace E types as partners of CDK2 allowing S phase to progress[326, 327]. Later on in S phase, A type cyclins switch partners from CDK2 instead associating with CDC2. As the cell enters G2, A type cyclins are replaced by B type and the B-CDC2 complex triggers the start of S phase to metaphase [328, 329].

Whilst the levels of available CDKs vary only minimally the availability of cyclins is variable. E, A and B type cyclin levels are cell cycle dependent with gradual accumulation followed by rapid degradation meaning that the cell cycle clock moves in one direction[330]. The rapid degradation is triggered by the actions of ubiquitin ligases that attach polyubiquitin chains to the cyclins[331, 332]. D type cyclins however vary according to levels of extracellular mitogens, serving to convey signals from the extracellular environment to the cell nucleus[333].

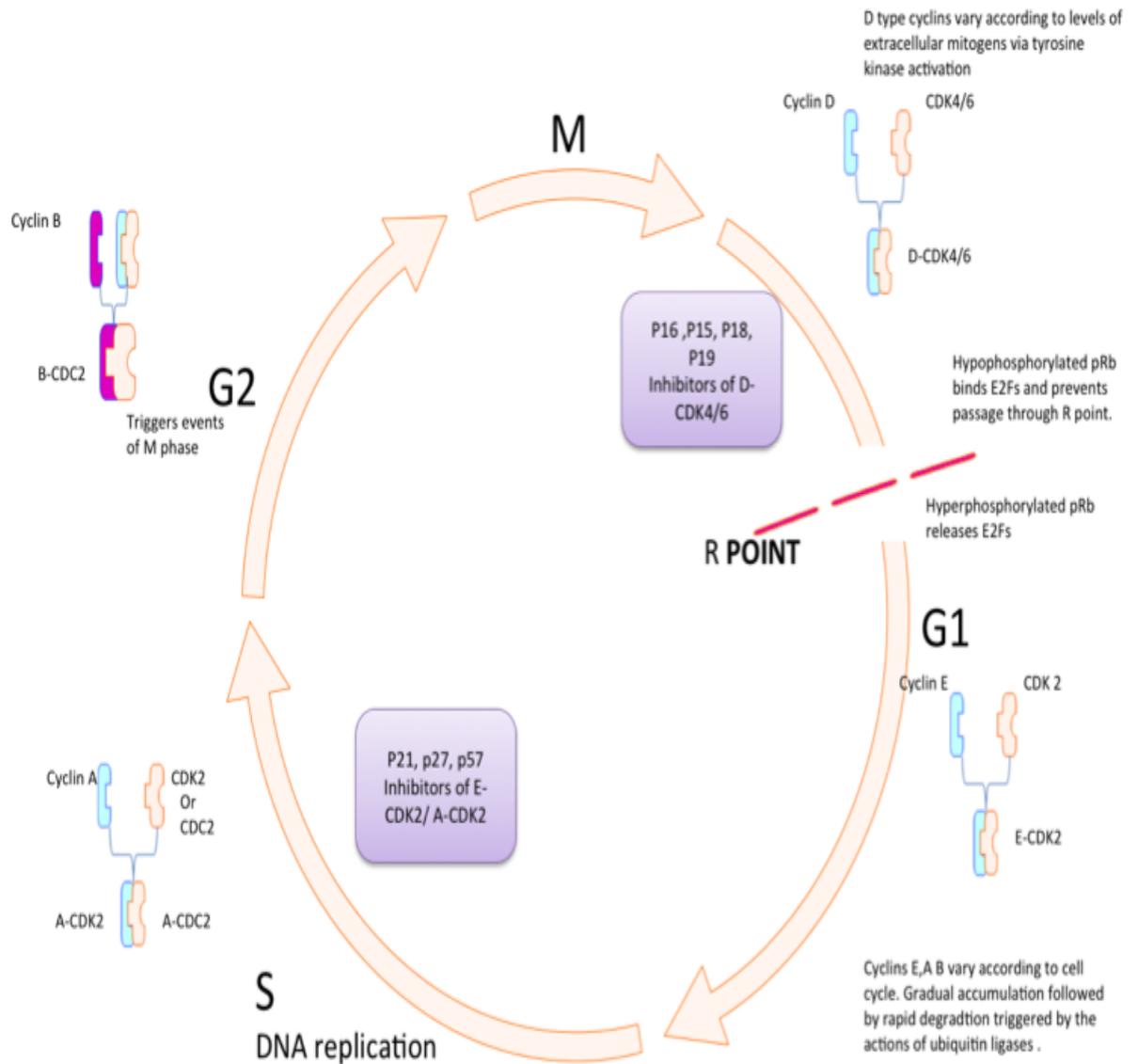


Figure 1.5 Regulation of G1/S phase transition

It is possible for the cell to block the action of cyclin-CDK complexes with CDK inhibitors (CKI). These can be grouped into 2 classes; the (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}), and the cip/kip proteins which inhibit the remaining cyclin-CDK complexes (p21^{Cip1}, p27^{Kip1}, p57^{Kip2})[334, 335]. TGF- β when applied to epithelial cells antagonises cell proliferation[336]. It does this by increasing levels of p15 which blocks cyclin D-CDK4/6 complex formation and inhibits those already formed preventing progression of the cell to the R point[337].

However, if the cell has already passed the R point the cyclin D-CDK4/6 is unnecessary explaining why TGF- β is only growth inhibitory during early and mid G1.

Although TGF- β has a weak stimulatory effect on p21, levels of this protein mainly increase due to physiological stresses such as damage to the cell genome. If a damaged genome is detected in G1 then the resulting increased p21 blocks advance to the R point by inhibiting E-CDK2 thereby preventing progression to S phase and preventing inadvertent copying of still damaged DNA sequences[338]. P21 also inhibits PCNA halting already initiated DNA synthesis until repair is complete[339].

The D-CDK4/6 and E-CDK2 complexes dictate the cell progression past the R point by controlling the degree to which the retinoblastoma protein (pRb) is phosphorylated[340].

The Rb tumour suppressor gene is growth inhibitory and codes for a nuclear phosphoprotein, pRb, which is the molecular governor of R point transition [341]. pRb is unphosphorylated in G0. In early G1 D-CDK4/6 complexes initiate the weak phosphorylation which blocks passage through the R point. Hyperphosphorylated pRb however then permits passage through the R point, where it remains hyperphosphorylated through the remaining cell cycle. It is stripped of its phosphate by protein phosphatase type 1 (PP1) as the cell exits mitosis. A decrease in cyclin D levels resulting from a decrease in mitogens causes the pRb to lose its phosphate group preventing transgression to the R point[342-344].

The Myc oncoprotein located in the nucleus, functions as a growth promoting transcription factor by targeting the D2 gene and increasing CDK4 activity[345]. Increased expression of D2 and D-CDK4 complexes leads to hypophosphorylation of pRb and subsequent E2F release. In the same way, decreased expression of p16 (which usually functions to inhibit D-CDK4/6) leads to excess D-CDK4/6 complexes and inappropriate phosphorylation of pRb allowing more cells to move freely into S phase.

Deregulation of the Rb pathway, either by mutation, viral oncoproteins, excess mitogenic signals or the actions of oncoproteins, leads to unconstrained proliferation and therefore it is likely to be disrupted in most, if not all, types of tumour cells.

1.7.4.1 Cyclin D1 G/A870 (rs 9344)

Cyclin D1 (CCND1) expression is induced as a delayed early response to many mitogenic signals and is associated with the cell's transition into the proliferative cycle. Although most widely known as a cell cycle gene it has been shown that it also has an activity outside the cell cycle. Breast cancers over expressing CCND1 failed to show enhanced proliferation rates and control of the retinoblastoma (RB) pathway was retained [346, 347]. It has been demonstrated that CCND1 is a modifier of gene transcription with the largest class of transcription factors being regulated belonging to the nuclear receptor super family including the oestrogen receptor (ER) [348, 349] and the androgen receptor (AR)[350, 351]. Given its role in both the cell cycle and gene transcription it is therefore an ideal suspect to contribute to carcinogenesis. CCND1 alterations are tumour specific [352] and 40-50% of breast cancer specimens have been shown to have gene amplification or overexpression [353]. Forced over expression of CCND1 in breast mouse models leads to cancer development and gene knock down can protect from breast cancer showing the importance of CCND1 in breast cancer [354, 355].

As a result of alternative splicing 2 isoforms have been recognised, CCND1a and CCND1b. It is the presence of a stop codon within intron 4 which gives rise to the truncated CCND1, which harbours a unique COOH terminus that is devoid of exon-5 encoded sequences [356, 357]. The alternatively spliced transcript has been detected in many cancers including breast [358], [359, 360] [361]. CCND1b has unique activities distinct from CCND1a and has been shown to enhance oncogenic activity compared with CCND1a [352, 362, 363]. In

mouse fibroblast models CCND1b, but not CCND1a, induced *in vivo* tumour formation and anchorage independence [362-364]. Interestingly in breast cancer cells CCND1b exclusively induced resistance to oestrogen antagonists and could therefore be contributing to therapeutic failure in ER +ve breast cancers. In breast cancer both CCND1 isoforms are regulated at the transcriptional level through the actions of the ER [365, 366].

The CCND1b protein is defective in several motifs that typically regulate CCND1 turnover including the PEST motif and threonine 286. PEST motif is critical for the degradation of CCND1 [367]. Threonine 286 phosphorylation modulates protein localisation and stability [368-370], and is present in exon 5 encoded sequences and thereby is absent in CCND1b. As a consequence CCND1b has been shown to be a nuclear protein [358, 362, 363]. This is relevant, as T286 mutation resulting in nuclear localisation is known to promote oncogenic transformation[369]. CCND1b, but not CCND1a, has been independently associated with adverse patient outcome for recurrence, distant metastasis and breast cancer specific death [358].

The presence of a SNP at the splice donor site, G>A870, is reported to influence alternative splicing. The A allele is thought to be associated with transcript b expression [371-375] but as GG genotypes can also produce CCND1b, and AA can produce CCND1a, additional events may co-operate to influence splicing. It is thought that the A allele hinders the splicing event allowing for read through into intron 4 and production of the variant splice product[352]. There are discrepancies in the data with regard to influence of G>A870 polymorphism and overall cancer risk. A meta-analysis in 2008[367] reviewed 60 papers covering 9 tumour types including breast cancer. 4718 cases of breast cancer in total were assessed with 5183 controls and the AA genotype was weakly associated with breast cancer risk. The G allele of this SNP has also been associated with HER2/*neu* expression, perhaps

because decreased levels of CCND1 decrease levels of factors down regulating HER2/*neu* [376].

1.7.5 Metastatic transformation

Normal cells require a surface to proliferate and are said to proliferate in an anchorage dependent manner. Cancer cells, however, have the ability to proliferate in an anchorage independent manner and this is partly due to deregulation of the integrin-signalling pathway[377-380]. Integrins serve to sense an attachment of cells to the extracellular matrix and identify the specific molecular components (collagens, laminins, proteoglycans, fibronectins)[381, 382]. They have 3 functions; to physically link cells to the ECM (via the α and β subunits); to inform cells whether or not tethering to specific ECM components has been achieved and to facilitate motility by making and breaking contacts with the ECM. They are unusual in that as well as transmitting extracellular signals inside the cell they also use cytoplasmic signalling to control binding to the ECM[383-385].

Integrins belong to a large family of heterodimeric transmembrane cell surface receptors made up of α and β subunits[386]. It is the different combinations of these subunits that determine ligand specificity. The various glycoproteins of the ECM act as ligands for the integrin receptors. Calcium and magnesium ions act as a bridge between the ligands and the α subunit[387]. Upon binding, the ligand-integrin complexes cluster to form focal adhesions[388, 389]. As well as affecting the organisation of the cytoskeleton by the cytoplasmic domains linked to actin, vinalin and paxillin, the formation of focal adhesions activates the cytoplasmic signalling pathway leading to migration, proliferation and cell survival [390, 391].

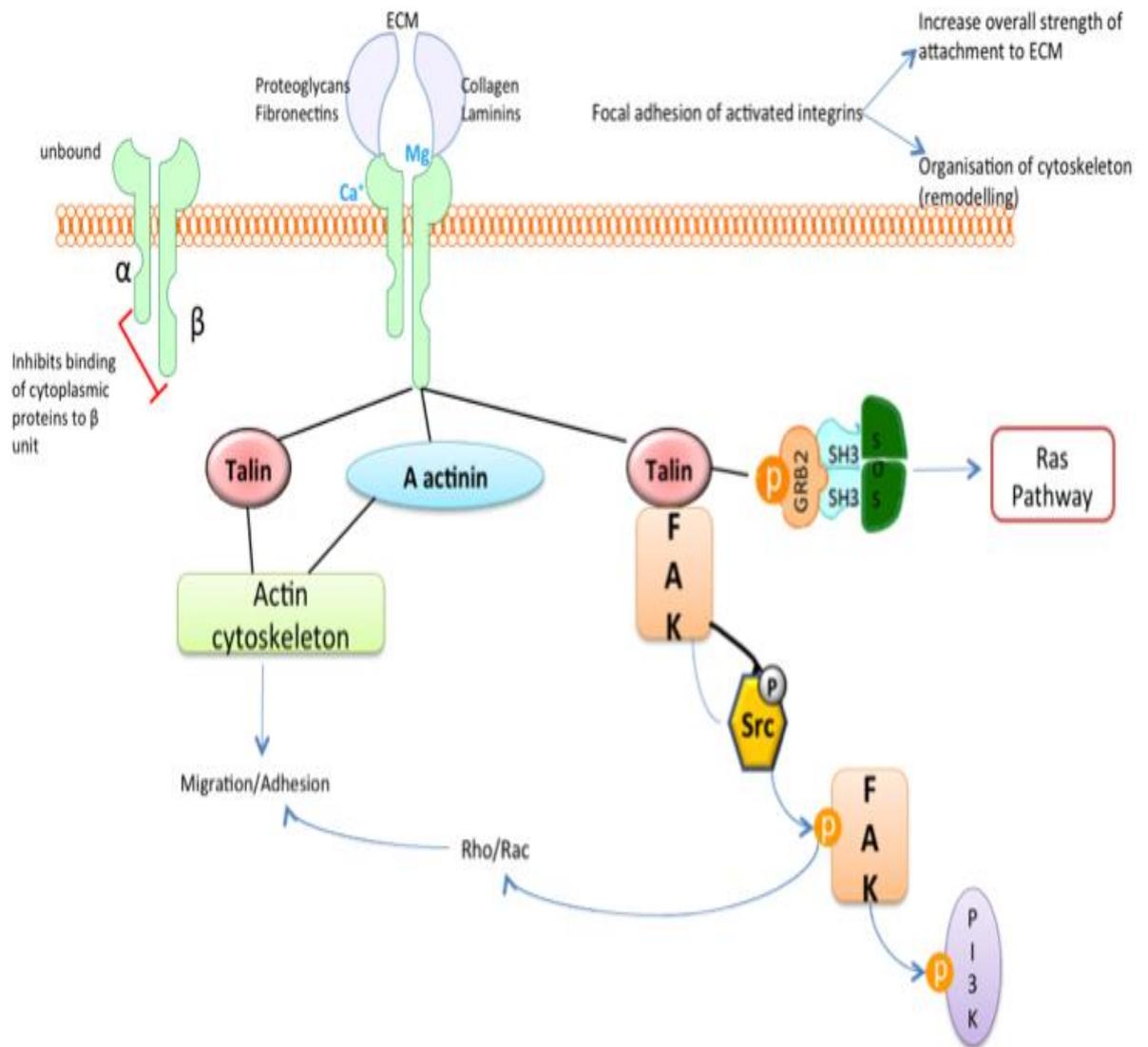


Figure 1.6 Integrin signalling

Deregulation of the integrin signalling pathway (**figure 1.6**) allows cancer cells to proliferate in an anchorage independent manner. Integrins are heterodimers consisting of an α and β subunit. Unbound the heterodimer is unable to activate the cytoplasmic signalling pathways but once Ca and Mg facilitates the binding of various ECM glycoproteins the α subunit can form complexes and local adhesions, activating various pathways via talin, α -actinin and FAK. Phosphorylation of FAK can subsequently activate the Ras pathway while talin and α -actinin control the cytoskeleton

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is capable of phosphorylating its own and other tyrosine residues associated with the cytoplasmic tails of the β -subunits of integrins[392, 393]. The phosphotyrosine residues on FAK provide a docking site for Src which can then proceed to further phosphorylate FAK providing additional docking sites for SH2 containing molecules such as Grb2, Shc, PI3K and PLC γ [394, 395]. Grb2 can then proceed to recruit Son of Sevenless (Sos) by forming physical bridges, which can activate the Ras pathway.

Integrins therefore work in conjunction with growth factor receptors such as EGF and PDGF (which sense soluble growth factors) by sensing an attachment to the insoluble scaffolding of ECM providing the mechanisms of detection for the 2 preconditions necessary for cell proliferation (that is, mitogenic growth factors and adequate anchorage to the ECM)[396].

In order to be classified as malignant, neoplastic cells must have breached the basement membrane; classed as invasive. The first step of acquiring invasiveness is by undergoing EMT [397, 398]. This is controlled via the E-cadherin pathway (discussed above) where E-cadherin and cytokeratins levels decrease and vimentin, an intermediate filament component of the mesenchymal cell cytoskeleton, levels increase[399]. This is accompanied by the replacement of E-cadherin with N-cadherin. The TGF- β pathway enhances the EMT pathway (discussed later).

In the TNF- α pathway, **figure 1.7**, inflammatory cells, notably macrophages, produce TNF α which bind to the TNF α receptor, forming trimers. The conformational change involved leads to the dissociation of SODD from the intracellular death domain allowing the adaptor protein TRADD to bind to the death domain. TRADD then recruits TRAF2 and RIP (a serine-threonine kinase) allowing TRAF2 to recruit the multi-component protein IKKK enabling RIP

to activate it. The NF- κ B family members are usually inhibited by IK- β whose phosphorylation by activated IKKK leads to its ubiquitylation and degradation leaving NF- κ B free to migrate to the nucleus where it acts as a co-activator to transcribe anti-apoptotic and mitogenic genes. The TNF- α pathway induces EMT and leads to activation of the nuclear factor - κ B (NF- κ B) pathway[400, 401]. This pathway leads to the induction and maintenance of EMT and blockage of NF- κ B signalling prevents expression of EMT[402]. NF- κ B family members are transcription factors that can form homo or heterodimers in the cytoplasm[403]. The commonest form is a heterodimer of p65 and p50 subunits. This is sequestered in the cytoplasm by a third polypeptide Inhibitor of κ B (I κ B) leaving the complex inactive[402]. I κ B phosphorylation leads to its rapid destruction leaving NF- κ B free to move to the nucleus where it acts as a transcription factor for over 150 genes. TGF- α , interleukin-1B and reactive oxygen species (ROS) all stimulate the kinase which leads to the phosphorylation of I κ B[404]. NF- κ B activates anti-apoptotic genes such as Bcl-2 and IAP-1/-2 and also mitogenic genes such as Myc and cyclin D1[405-411]. Although it is rare to find NF- κ B pathway components in mutant form, the pathway is often hyperactivated in cancer cells[412].

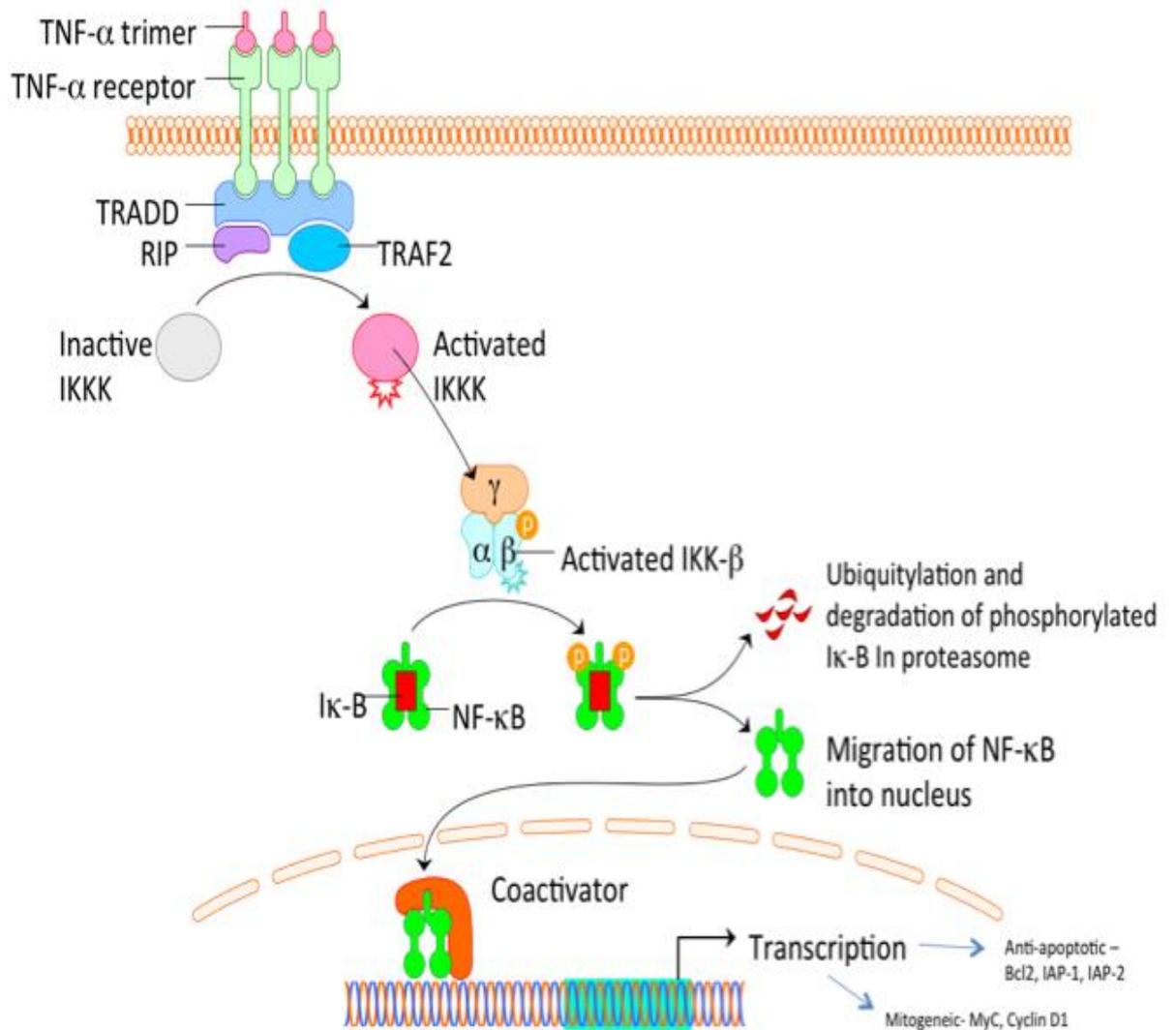


Figure 1.7 TNF-α pathway

As EMT is the complex biological process that enables cancer cells to undergo phenotypic changes in order to become invasive, it also needs to allow the cells to burrow into the basement membrane, excavating passages through the ECM. To do this matrix metalloproteins (MMPs) are required. These are usually secreted by recruited stromal cells such as macrophages, mast cells and fibroblasts and dissolve the ECM to create space for more cells[413]. They do this by cleaving the ECM components such as fibronectin, tenascin, laminin, collagens and proteoglycans. This is part of a normal cellular response as MMPs creates space for newly divided daughter cells and allows remodelling.

There are two categories of proteases; those which are Zn^{+}/Ca^{+} dependent, MMPs and those which require a serine residue at the active site classed as serine proteases, for example plasminogen. Both groups are secreted as inactive pro-enzymes, and it is the balance of pro-enzymes, protease and protease inhibitors that maintain normal function. In cancer, the balance favours proteolysis.

The MMPs can be grouped into their specific substrates, collagenases, gelatinases and stromelysins and can therefore digest all individual components of ECM, removing physical barriers to invasion and metastasis[414]. While the majority of MMPs function as soluble enzymes in the spaces between cells, 6 function are membrane anchored and cleave cell surface adhesion molecules such as cadherins and integrins[415]. The active site of MMPs contains a histidine.glutaminate.any amino acid.glycine.histidine motif[416]. The two histidines co-ordinate the Zn^{+} that is essential for their activity.

Tissue inhibitors of metalloproteinases (TIMPs) bind MMP and put them in inactive configuration. They are broad-spectrum inhibitor proteins that contain cysteine residues arranged so that six intra-chain –S-S- bonds are formed. Increased levels of TIMPs are associated with decreased metastatic potential[417].

Urokinase plasminogen activator (uPA) is an extracellular protease secreted by stromal cells as an inactive pro-enzyme. It has direct and indirect ECM cleaving ability[418, 419]. It binds to the uPA receptor becoming tethered and active and can cleave and activate the pro-MMPs directly[420]. It can also cleave plasminogen to its active plasmin, which both cleaves pro-MMPs and activated TGF- β 1, which encourages invasion in progressing cells[421, 422]. It makes sense that in animal models, uPA and uPA receptor complex inhibitors block tumour growth and metastasis[423]. An increased level of uPA receptor in the serum is associated with a poor prognosis in cancer patients [424-428].

In cancer the disruption of the integrin system is complex but it is clear that cancers have different types and membrane distribution of integrins compared to their normal counterparts. In general the integrins required for tissue expression tend to be underexpressed whilst those needed for migration are normal or increased. Migration is determined by the ECM on which the cells move and therefore on the cell receptors which recognise ECM. For example Secreted Phosphoprotein 1 (SPP1), more commonly known as osteopontin, is a phosphorylated glycoprotein found in all body fluids, extracellular matrix (ECM) components, and the proteinaceous matrices of mineralized tissues [429]. It contains an RGD (arginine-glycine-aspartate) motif[430] and similar to other ECM proteins assists both as an immobilised ECM molecule in mineralized tissue and as a cytokine in body fluid containing the RGD sequence, allowing RGD-dependent interactions with integrin receptors and subsequent cell attachment and signaling[431, 432]. SPP1 has been shown to interact with a number of different integrins via the RGD sequence[431]. It also increases uPA expression possibly secondary to integrin binding and subsequent uPA receptor binding and subsequent signalling[433]. There is also evidence to suggest that SPP1 induces the expression and activity of members of the metalloproteinase family [434, 435].

Variants discussed in this pathway group will be classified as metastor genes

1.7.5.1 SPP1 -443 (Rs 11730582), -66 (Rs28357094)

Many solid cancers have been shown to overexpress SPP1, including breast [436, 437], and SPP1 expression is particularly high in metastatic tumours [438, 439]. It been demonstrated that as well as having SPP1 delivered to them by circulating cells, breast cancer cells can synthesize their own protein [440]. Increased levels of intracellular SPP1 in breast cancer are independently associated with prognosis [441] and increased serum SPP1, associated with breast cancer [442], can be prognostic in metastatic cancer [443, 444]. It has been

suggested that SPP1 may be a mediator to bone specific metastasis in breast cancer and different SPP1 expression levels may impact on tissue specific metastasis [445, 446]. The metastatic ability of breast cancer cells can be significantly reduced by SPP1 gene knockdown [447, 448].

Giacopelli *et al* [449] analysed the promoter region 500bp upstream of the transcription start site of SPP1 and identified 3 common variants, -66, 156 and -443. SNP -66 was at a site shown to modify the binding affinity for SP1/SP3 transcription factors. Allele-specific binding of SP1 transcription factor to the -66 polymorphism has been demonstrated in a leukemic cell line [450]. This study also showed that the specific haplotype -443C/-156G/-66T was associated with significantly enhanced promoter activity compared with 5 other allelic variants tested.

SNP -156 is within as yet uncharacterised RUNX2 (a transcription factor associated with osteoblast differentiation) binding site. Giacopelli *et al* [449] also demonstrated that one haplotype conferred a significant decreased level of SPP1 expression in all cell lines.

SNP -443 is reported as a Tag SNP, and has been shown to exert influence on SPP1 gene regulation in melanoma cells [451]. It was demonstrated that the CC genotype was associated with significantly increased levels of mRNA expression in metastatic melanoma tissue and melanoma cell lines. The sequence immediately preceding the -443 position, AAGTTC/T is very similar to the c-Myb core binding motif CAGTT and the authors managed to demonstrate that c-Myb binds to this region in an allele specific way and induces the enhanced activity of the -443C allele compared with the -443T allele.

Given that haplotypes in SPP1 affect promoter activity and the relevance of SPP1 levels to breast cancer metastasis as well as the suggestion that SPP1 levels may be related to patient response and survival in chemotherapy treated cancer patients [452], it is of

interest to assess the haplotype of breast cancer patients and any association with outcome.

1.7.6 XBP-1 -116C>G (Rs 2269577) and the unfolded protein response pathway

XBP-1 is a transcription factor that is upregulated as part of the unfolded protein response (UPR) [453]. The UPR is triggered by the accumulation of misfolded proteins with the endoplasmic reticulum leading to endoplasmic reticulum stress. This halts further protein translation, allowing the cell a chance to restore normal function. Endoplasmic reticulum stress also allows the signalling pathways that lead to the increase of molecular chaperones involved in protein folding to be activated. If these responses do not occur within a given time frame then the UPR initiates apoptosis.

X-box binding protein -1 (XBP-1) has been found to be over-expressed in breast cancers [454, 455] and has been shown to be oestrogen responsive in both normal breast tissue [456] and breast cancer cell lines [457]. Gomez et al [458] demonstrated that XBP-1 over-expression is associated with oestrogen independence and resistance to anti-oestrogens *in vitro* and that it altered the expression of several apoptotic and cell cycle genes. This led to the promotion of cell survival via alteration of the intrinsic apoptotic pathway. Davies et al have demonstrated that XBP-1 isoforms affect prognosis of endocrine therapy for breast cancer patients, perhaps explained by the favouring of apoptosis over cell survival of particular variants [459].

XBP-1 also regulates the expression of the tissue inhibitor of metalloproteinases, SPP1 and osteocalcin [460]. A SNP at -116C>G is significantly associated with protein expression [461].

To date there are no published studies assessing this SNP in the promoter region of XBP-1, proven to modulate expression, and cancer risk, prognosis or response to therapy. It will

therefore be of interest to assess the association of XBP-1 genotype and long-term outcome of breast cancer patients.

1.7.7 Drug Metabolism

Differences in drug response can be dependent on patient age, sex, disease and drug interactions as well as genetic factors. All stages from absorption, distribution, interaction with targets (receptors and targets), metabolism and excretion will have potential genetic variation affecting outcome. It is perhaps drug metabolism that has been studied most with genes affecting Phase I reactions (oxidation, reduction and hydrolysis) and Phase II conjugations reactions (acetylation, glucuronidation, sulfation, and methylation) looked at specifically.

1.7.7.1 *Xenobiotic metabolising genes*

Xenobiotic metabolising genes actively participate in the metabolism of drugs and toxicants. They can be grouped in phase I enzymes which can activate or inactivate administered drugs (e.g. cytochrome p450) or phase II enzymes which usually inactivate the active form of the drug or its metabolites by conjugation (e.g. Glutathione S-transferases). While the genetic profile of the tumour may affect the effect of drugs used, it is the genetic background of a patient with respect to metabolising enzymes and drug transporters that determines the relation between drug dose and plasma concentration and thus thereby the therapeutic effect[462].

1.7.7.2 *Cytochrome p450*

Cytochrome p450 (CYP450) is a large subfamily of enzymes involved in the oxidative metabolism of drugs, playing a major role in their activation and/or elimination. The enzymes are expressed mainly in the liver and CYP3A, CYP2D6 and CYP2C families account

for the metabolism of over 75% of prescribed drugs and the activity of these can be affected by diet, smoking behaviour and co-medication. Various SNPs in family members have been associated with decreased protein levels [463], activity levels [464], drug metabolism and drug clearance[465, 466].

It has been demonstrated that CYP2D6 genotype is related to DFS and time to progression in tamoxifen treated patients [467] and that CYP2C19 genotype can predict those that are likely to have the best outcomes with tamoxifen[468]

1.7.7.2.1 Cytochromes and taxanes

Docetaxel is extensively metabolised to yield inactive oxidation products by the CYP450 system, mainly CYP3A subfamily [469]. A number of genotypes of CYP3A have been shown to affect docetaxel clearance [470-474] although the clinical impact of this still needs to be clarified. The metabolism of paclitaxel is primarily through oxidative metabolism and biliary excretion with only 5-10% being renally eliminated [475]. CYP2C8 enzyme is responsible for conversion of paclitaxel to its main metabolite 6 α -OH paclitaxel. CYP3A4 is responsible for a minor pathway conversion to 3'-p-OH[476]. Various SNPs of these genes have demonstrated alteration in paclitaxel metabolism [464, 477] although once again, there are no published studies assessing clinical response.

1.7.7.2.2 Cytochromes and cyclophosphamide

CYP3A4 and CYP3A5 are responsible for the majority of cyclophosphamide metabolism [478]. SNPs in these genes as well as CYP2C19 and CYP2B6 have been implicated in altered pharmacokinetics and/or treatment outcome. Not all studies have found the same correlation indicating that the influence of these polymorphisms may not be very high and might be missed in underpowered studies[462].

1.7.7.2.3 CYP2B6, Nr Gene 5 (Rs4802101), Intron 3 (Rs4803419), Intron 5 (Rs2279345)

CYP2B6 is a highly polymorphic gene that plays a key role in the biotransformation of up to 25% of drugs and many chemical compounds including cyclophosphamide and tamoxifen [479, 480]. Cyclophosphamide is a pro drug that requires activation by CYP450 to 4-hydroxycyclophosphamide and CYP2B6 has the highest activity in this step [481]. In a study involving breast cancer patients receiving chemotherapy the Nr Gene 5 SNP was found to have a frequency of 68.4%, Intron 3 SNP 81.6% and Intron 5 SNP 70.9% [482]. These SNPs were found to be good predictors of toxicity and were associated with decreased 4-hydroxycyclophosphamide.

The Nr Gene 5 SNP lies within a putative binding site for HNF-1 and has a modest effect on protein levels; The CT and CC genotypes are associated with a 1.42 and 1.81 fold decrease in protein when compared with TT. The Intron 3 SNP is thought to be associated with a splicing variant in which the protein lacks exons 4 to 6 [482].

No studies have to date been published on the survival of breast cancer patients, historically receiving cyclophosphamide, and associated CYP2B6 polymorphisms. Given its importance in the pharmacological pathway it is important to assess this further.

1.7.7.3 Glutathione S-transferases

Glutathione S-transferases (GSTs) are involved in the detoxification of various chemotherapeutic drugs by catalysing the conjugation of glutathione (GSH) [483, 484]. 8 GST iso-enzymes have been identified with α , μ and π (GSTA, GSTM, GSTP) representing the major classes.

Both enzyme expression and the effect of polymorphisms have been assessed in regard to chemotherapy response.

GSTP is expressed in normal tissues at varying levels in different cell types and abnormal GSTP activity and expression have been reported in a wide range of tumours including breast [485-489]. Studies of GSTP expression in breast cancer have not uniformly demonstrated a difference in prognosis according to GSTP expression levels [486-488]. However, increased expression of GSTP has been documented to contribute to drug resistance of ovarian carcinoma [490], head and neck cancer [491] and lung squamous cell carcinoma [492].

1.7.7.3.1 GST expression and anthracyclines

DNA reactive metabolites of doxorubicin are substrates for GST mediated GSH conjugation. Transfection of a cDNA encoding GSTP into drug sensitive cells resulted in an increased resistance to doxorubicin[485]. An increase in GSTP1 expression has been associated with resistance to doxorubicin[493] and doxorubicin has been reported to induce the expression of GSTP1 through the AP1 transcription factor in leukaemia cells[494].

In a study of 116 breast tumours, GSTP1 positive tumours showed poorer prognosis than those with no GSTP1 expression when all patients were treated with adjuvant chemotherapy consisting of 49% anthracycline containing regimen [495]. In a neo-adjuvant study of 42 patients with primary breast cancer over 5cms given FAM chemotherapy (5-fU, adriamycin, mitomycin), GSTP1 negative tumours had a significantly greater reduction in tumour size and a significantly increased apoptotic rate[496]. GSTP1 positive tumours seemed to be resistant to FAM chemotherapy suggesting an important role for GSTP1 in detoxifying one or more of the drugs in the protocol.

Conversely, in 171 patients receiving adjuvant chemotherapy (163 receiving an anthracycline containing regime) for breast cancer, increased GSTP1 expression was an independent predictor of increased 5-year DFS [497]. The authors hypothesise that as GSTP1 is one of the major isoenzymes, low levels will reduce the global activity of GST and consequently reduce glutathione (GSH) consumption in GST catalysed reactions. The increased levels of GSH would block apoptosis and promote proliferation of tumour cells. This was initially suggested when increased GSH levels were found to be associated with relapse in childhood acute lymphoblastic leukaemia [498] and confirmed in cell studies showing intracellular GSH levels determining cell sensitivity to drug induced apoptosis [499]. The authors also suggest that the role of GSTP1 on cell proliferation may be the reason behind their results. However, GSTP1 is an inhibitor of c Jun N terminal kinase (JNK) [500, 501], which is implicated in the control of cell proliferation [502, 503]. Inhibition of JNK blocks the signal transduction of MAP kinase pathway resulting in cancer cell survival [504, 505], meaning that increased GSTP1 should lead to increased cancer cell survival and poorer 5 year survival.

1.7.7.3.2 GSTs expression and taxanes

Few studies have directly assessed the effect of GSTs expression and response to chemotherapy. Cell line studies have shown GSTP1 expression to be associated with resistance to docetaxel in breast cancer [506] and non-small cell lung carcinoma [507]. In one neo-adjuvant study of 62 breast cancer patients receiving docetaxel or paclitaxel monotherapy the mean reduction rate in GSTP1 negative tumours was significantly higher than in GSTP1 positive tumours[508]. The authors also assessed the genomic DNA from 48 tumours and found no association with GSTP1 methylation and response to taxanes. However, no association was found between GSTP1 expression and response to paclitaxel in metastatic breast cancer [509].

1.7.7.3.3 Glutathione S-Transferases, Codon 105 (Rs 1695) and Codon 114 (Rs 1138272)

Numerous polymorphisms have been demonstrated in GSTP1 including a G to A resulting in isoleucine (Ile) or valine (Val) at codon 105 in exon 5 [510, 511] and a C to T resulting in alanine (Ala) or Val at codon 114 in exon 6 [512]. Codon 105 substitution is located near the substrate-binding site of the enzyme and the variant is fairly common in a Caucasian population [513]. The codon 114 Val variant is infrequent occurring in less than 15% of Caucasians [512]. These two SNPs lead to 4 haplotypes, the common GSTP1*A with Ile at codon 105 and Ala at 114, GSTP1*B with Val at 105 and Ala at 114, GSTP1*C with Val at both positions and the rare GSTP1*D with Ile at 105 and Val 114 [512]. These polymorphisms result in both structural and functional differences [512, 514-516].

The frequency of the codon 105 Val allele has been shown to be significantly higher than controls in bladder, testes, prostate, lung and breast cancer [517-519]. It has recently been demonstrated that women who are homozygous for the Val allele at codon 105 are at a significantly decreased risk of breast cancer [520]. This is in contrast to earlier work where the presence of the Val allele was associated with a non-significant trend towards increased risk [519]. Both studies only contained small numbers of patients and so these findings need to be validated on a larger scale. Maugard *et al* [521] showed that there is a significant 2.18 fold increase in breast cancer risk with non- GSTP1*C genotypes, demonstrating that the codon 114 Val allele may have a protective effect against carcinogenesis.

Thiotepa is a polyfunctional alkylating agent. It has been demonstrated that GSTP-1 content is an important determinant of tumour cell sensitivity to thiotepa [522]. Haplotype analysis has shown that GSTP1*A variant is the most efficient in conjugating thiotepa suggesting that these polymorphisms may be relevant in tumour cell resistance to alkylating class

drugs [523]. Although thiotepa is not routinely used in breast cancer chemotherapy regimes, cyclophosphamide acts through related alkylating intermediates and it is therefore likely that polymorphisms will have a similar effect as GSTP1 has also been shown to catalyse GSH conjugation of reactive cyclophosphamide metabolites in *in vitro* studies [524]. Sweeny *et al* [513] reviewed the codon 105 status of a cohort of 189 breast cancer patients, for which 95 % had received cyclophosphamide chemotherapy. They demonstrated that the hazard of death among Val/Val carriers was 30% of that of Ile/Ile women. They also found that the time to recurrence was significantly less for those with a Val allele.

The effects of genotype of GSTP1 on the pharmacokinetics of anticancer drugs have not been studied extensively. A study of 240 women using tissue DNA demonstrated a better survival in those with GSTP1**B* genotype in those treated with chemotherapy [513]. The authors hypothesise that as GSTP1**B* is less active, the elimination rate of chemotherapeutic agents is decreased and therefore prolonged exposure of the drug to the tumour is achieved and a better survival rate could be expected. GSTP1 genotype was associated with OS in a study of 240 women with breast cancer, 189 of who received chemotherapy [513].

1.7.7.4 ABCB1 (Rs 1045642)

ABCB1 (previously known as Multi-drug resistance 1, MDR1) is a member of the ATP-binding cassette transporters that code for the membrane associated protein P-glycoprotein (P-gp). P-gp is a 170-kDa phosphorylated and glycosylated transmembrane protein of 1280 amino acids [525]. It is expressed in intestinal epithelium as the first line of defence preventing xenobiotic absorption and also in liver, renal tubules and breast tissue

[526-528]. It may play a role in the WNT signalling pathway [529] but its main function is as an ATP dependant drug efflux pump for xenobiotic compounds with a broad substrate specificity including anthracyclines, taxanes and tamoxifen[530-532]. Over-expression of ABCB1 conveys resistance to a number of cytotoxic agents [533].

A synonymous SNP in exon 26, 3435C>T has been shown to lead to large inter-individual variation of expression level of P-gp [534]. The T allele has been reported to affect RNA stability and possibly translation leading to decreased protein expression [535-537].

Consistent results are not reported between different studies of the consequences of ABCB1 polymorphisms on pharmacokinetics and pharmacodynamics, suggesting that this functional SNP may behave differently in different tissues [538, 539].

The T allele of 3435 SNP is associated with decreased ABCB1 expression in breast cancer [540]. Individuals homozygous for TT have been shown to be at an increased risk of breast cancer in numerous studies [541-543], perhaps unsurprisingly as TT offers the least protection against accumulation of carcinogens. An Iranian study failed to find any association between genotype and breast cancer [544].

3 small studies assessing anthracycline based neo-adjuvant chemotherapy in breast cancer have associated the TT genotype as having an increased response to treatment, although only one reaches statistical significance [542, 545, 546]. Conversely, one study found an independent association between having at least one C allele and an increased response to treatment[541]. Early relapse is significantly associated with the CC genotype for patients receiving adjuvant anthracycline based chemotherapy [541, 547]. There is a 2.6 fold increase in the risk of relapse after receiving anthracycline-based chemotherapy for patients who have both ABCB1 3435 CC and MTHFR 67CC (discussed later) genotype [547].

Over-expression of P-gp has also been associated with resistance to paclitaxel [548]. There is one study in the literature assessing metastatic breast cancer patients receiving paclitaxel for an association with ABCB1 polymorphisms[549]. Here there is a significant improvement in disease control rate in the CC genotype as compared to the CT. Heterozygosity was an independent predictor of OS with a significant hazard ratio of 3.5.

Although there are conflicting results, it is clear that ABCB1 polymorphism is associated with chemoresistance and possibly OS in breast cancer patients. Further work is needed to assess the role of this genotype in predicting chemotherapeutic resistance.

1.7.7.5 MTHFR 677C>T (Rs 1801133) and 1298 A>C (Rs 1801131)

5,10 methylenetetrahydrofolate reductase (MTHFR) is a pivotal enzyme in folate metabolism that catalyses the irreversible conversion of 5,10 MTHF to 5 MTHF which provides the methyl group for *de novo* synthesis of methioninesynthase and DNA methylation [550]. It regulates the folate pool for synthesis and methylation of DNA. 5-fluorocil (5-fU) inhibits the folate pathway by inhibiting the target enzyme thymidylate synthase (TS). TS catalyses the conversion of deoxyuridylate (dUMP) to deoxythymidylate (dTMP) for which 5, 10 MTH functions as a methyl donor. 5-FdUMP, an active metabolite of 5-fU in combination with MTHFR inhibits TS which leads to greatly reduced levels of dTMP causing reduced DNA synthesis, dUMP misincorporation into DNA and DNA strand breaks; ultimately leading to apoptosis [551, 552].

2 common SNPs that reduce MTHFR enzyme activity in homozygotes have been reported; at position 677 a C to T change causes a Ala to Val substitution in a catalytic domain and leads to a 70% decrease in activity and an A to C change at position 1298 causes a Glu to Ala substitution in a regulatory domain resulting in a 30-40% decrease in function [553-555].

The allele frequency for 677T is between 0.24 and 0.44 in Europeans and Caucasians [556]

and the frequency of the 1298 C allele is 0.18- 0.36 in studies from Europe and North America [557].

Decreased MTHFR activity is associated with genomic DNA hypermethylation and is suspected to partake in carcinogenesis via its effects on DNA methylation and nucleotide synthesis [558]. Epidemiologic evidence shows that folate deficiency is a risk factor for breast cancer development [559, 560] and therefore many studies have studied the effects of MTHFR variation in breast cancer risk. The results vary between different cohorts studied [248, 561-564], A meta-analysis of 18 studies reported that the association between the 677 T allele and the risk of breast cancer showed significant heterogeneity and non-significant associations[565]. However, in pre-menopausal women, the TT genotype was a significant risk factor for breast cancer when compared to CC. There was a lack of association overall for 1298 genotypes.

Both 5-fU and methotrexate (a folate analogue) have been used as part of breast cancer chemotherapy regimens and exert their actions via inhibition of the folate pathway. It has been demonstrated that MTHFR 677 and 1298 polymorphisms regulate the chemosensitivity of breast and colorectal cancer cells to 5-fU and methotrexate *in vitro* [566, 567]. There is a hypothesis that decreased activity of MTHFR will lead to increased levels of intracellular concentration of 5, 10 MTHF and improve the cytotoxicity of 5-fU and therefore that the T allele of 677 and the C allele of 1298 will lead to greater efficiency of 5-fU.

It has been shown that the genotype 677 CC is significantly correlated with early relapse in breast cancer patients treated with chemotherapy (FEC) [547]. The same study demonstrated that the 677 CC genotype in combination with the ABCB1 3435 CC genotype (discussed above) leads to a 2.6 fold increase in early breast cancer. A further study demonstrated the 677 CC genotype had the same effect but only in ER negative tumours

[568]. It is important to note here that only a small proportion of the patients had received a chemotherapy regime containing 5-fU or methotrexate. A large study of Chinese patients the opposite result was found; a non-significant association between the 677 TT genotype and increased risk of death[569]. However, when the data was analysed only for those who had survived at least 2 years, the TT polymorphism was significantly associated with a doubling of the risk of death among those with late stage disease. There was no information by the authors about the types of chemotherapy used.

MTHFR 1298 AC or CC has been associated with an increased risk of poor outcome in breast cancer [568]. When this data has been stratified the C allele frequency is significantly associated with increased risk of dying in ER negative tumours, Caucasians and those receiving no chemotherapy. No association with MTHFR 1298 polymorphism and outcome was reported by some studies [569, 570]. Largiller *et al* [571] found no association with either MTHFR polymorphism and capecitabine therapy response in patients with metastatic breast cancer.

Chemotherapy response in other cancers

The MTHFR 677 TT genotype is associated with greater response to 5-fU in patients with metastatic colorectal cancer [94, 572]. However no association has been found in patients receiving adjuvant 5-fU following colorectal cancer resection[573]. One study has reported that the 1298 AA polymorphism is associated with OS in female metastatic colorectal cancer patients receiving 5-fU, but not male[574], while others have reported no association between this SNP and outcome in colorectal cancer [572, 573].

In 201 French-Canadian children with ALL the 677 T allele was associated with an increased risk of relapse or death [575] but this has not been confirmed in other studies[576]

There has been no association found between the 677 polymorphism and advanced gastric cancer treated with palliative 5-fU chemotherapy [577].

1.7.7.6 Importance of *HER2/neu* Proto-oncogene

Her2/neu, also known as c-erbB2, is a proto-oncogene member of the ErbB family of transmembrane tyrosine kinases. It encodes *HER2/neu* protein which is an 185kDA transmembrane cell surface receptor with intrinsic tyrosine kinase activity [578, 579]. *HER2* serves as a prognostic biomarker as discussed above, and also as a predictive biomarker of response to specific monoclonal antibodies e.g. trastuzumab, targeted against the over activated protein. The predictive use of *HER2/neu* overexpression in relation to trastuzumab, a humanized monoclonal antibody that acts on the *HER2/neu* receptor, has been established [580]. Positive evidence for the use of *HER2/neu* as a predictive marker for a variety of chemotherapy regimens has been obtained through extensive studies but the evidence is controversial [581-583].

Table 1. 4 summarises the clinical trials looking at *HER2* as a predictive factor in either CMF (cyclophosphamide, methotrexate and 5-fU) regimens or anthracycline based chemotherapy.

	HER2/ <i>neu</i> predictive of response	HER2/ <i>neu</i> not predictive of response
CMF Regimens	<p>Stal et al 1995[584] Premenopausal women randomised to adjuvant CMF or radiotherapy. 152 frozen tumour samples assessed. HER2/<i>neu</i> negative benefited from CMF vs. radiotherapy HER2/<i>neu</i> positive highly significant relapse rate after chemotherapy. (RR of distant recurrence for HER2/<i>neu</i> +ve pts for those receiving chemo vs. radiotherapy 3 95% CI (1.1-7.8))</p> <p>Gusterson et al 1992[585] Randomised trial comparing short duration and prolonged CMF Decreased DFS² in HER2/<i>neu</i> +ve group (HR 0.57 95% CI (0.46 -0.72). No effect seen in HER2/<i>neu</i> negative group.</p> <p>Giai et al 1994[586] Retrospective review of 159 pts receiving adjuvant CMF HER2/<i>neu</i> was an independently predictive of a poor response to CMF regimes (p=0.04)</p>	<p>Paik et al 2000[587] 2295 node positive pts randomised to adjuvant AC¹ or CMF. Trial findings that the 2 treatments were not different in terms of overall efficacy HER2/<i>neu</i> negative favoured CMF but not statistically significant HER2/<i>neu</i> positive favoured AC but not statistically significant</p>
Anthracycline based regimens	<p>Muss et al 1994[588] Randomised trial of 2 doses of CAF³. Retrospective IHC review of 397 tumour blocks. In the high dose group HER2/<i>neu</i> +ve had a longer OS⁴ and DFS whilst no dose response seen in HER2/<i>neu</i> -ve.</p> <p>Thor et al, 1998[165]</p>	<p>Paik et al 2000[587] See above Trend towards HER2/<i>neu</i> +ve in AC arm but not statistically significant HR for relapse 0.88 (95% CI 0.65-1.07) HR for survival 0.82 (95% CI 0.63-1.06) Trend not seen in HER2/<i>neu</i> -ve tumours.</p>

<p>Additional 595 blocks from trial reported by Muss et al [588]. Initially same trend observed but not significant, (different IHC antibody used). When cohorts combined (995pts) HER2/<i>neu</i> +ve and higher CAF dose predictive for longer DFS and OS (p=0.001)</p> <p>Paik et al 1998[589] Randomised trial comparing PAF⁵ to PF⁶. Overall trial result PAF superior to PF. 638 tumour blocks examined. Median follow-up 13.5 years. In HER2/<i>neu</i> +ve group PAF superior in DFS (RR 0.60 CI (0.44-0.83) p=0.001) and OS (RR 0.61 CI (0.44 – 0.85) p=0.003). No difference in survivals in HER2/<i>neu</i> - ve group</p> <p>Pritchard et al 2006[590] Randomised trial comparing adjuvant CMF with CEF⁷ Premenopausal node +ve women CEF superior to CMF in HER2/<i>neu</i> +ve women, adjusted HR for OS 2.04 (95% CI 1.14-3.65) p=0.02. No difference for HER2/<i>neu</i> –ve women.</p> <p>Moliterni et al, 2003[591] Randomised trial comparing adjuvant CMF with CMF plus ADM⁸. 507 women 1-3nodes +ve Benefit of ADM in HER2/<i>neu</i> +ve group. OS HR 0.64 (95% CI -0.78- -0.49)</p>	<p>Petit et al, 2001[594] Neo-adjuvant comparison of 2-dose FEC regime, 79 pts. HER2/<i>neu</i> +ve and high dose FEC trend towards better response but not significant.</p> <p>Colleoni et al, 2003[595] Retrospective analysis of 399 pts receiving various neo-adjuvant regimes HER2/<i>neu</i> status not predictive of pathological response.</p> <p>Zhang et al, 2003[596] 97 pts undergoing neo-adjuvant FAC⁹. Weak trend towards a better pathological and clinical response in pts with HER2/<i>neu</i> over expression but not statistically significant.</p> <p>Cardoso et al. 2004[597] 59 pts with LABC or metastatic disease. Anthracycline based chemotherapy vs. taxane based. HER2/<i>neu</i> was not associated with response.</p> <p>Martin-Richard et al, 2004[178] 41 pts receiving neo-adjuvant FAC or FEC. HER2/<i>neu</i> status stable throughout treatment. HER2/<i>neu</i> status not predictive of response.</p> <p>Bonnefoiet al, 2003[598] 187 pts with LABC, 2 neo-adjuvant anthracycline based regimes. HER2/<i>neu</i> not predictive of progression free survival, OS or clinical response.</p>
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	<p>Penault-Llorca et al, 2003[177] Retrospective review of 115 pts receiving neo—adjuvant anthracycline based chemotherapy. Her2/<i>neu</i> +ve independently predicted response to treatment. 4.54 times more likely to have a complete pathological response. HER2/<i>neu</i> status stable throughout treatment.</p> <p>Colomer et al, 2000[592] First line paclitaxel and doxorubicin in 58 metastatic patients. Circulating HER2/<i>neu</i> levels corresponded to levels in primary tumour and predicted for worse response on multivariate analysis.</p> <p>Vargas-Roig et al, 1999[593] Neo-adjuvant, 60 pts with LABC, various chemotherapy regimes. HER2/<i>neu</i> status stable throughout treatment. HER2/<i>neu</i> +ve associated with worse DFS (p=0.0006).</p>	<p>Niskanen et al, 1997[184] 127 pts with metastatic disease and FEC based chemotherapy. HER2/<i>neu</i> status not predictive of response.</p> <p>Rozan et al, 1998,[599] 329 pts randomised to primary FAC chemotherapy or primary radiotherapy. HER2/<i>neu</i> status not predictive of response.</p> <p>Clahsen et al, 1998[175] 441 premenopausal, node-ve pts Randomised to perioperative FAC or nothing. HER2/<i>neu</i> status not predictive of response.</p> <p>Sjostrom et al, 1998[600] 103 pts randomised to different dosing of adjuvant EC. HER2/<i>neu</i> status not predictive of response.</p> <p>Vincent-Saloman et al, 2000[601] 54 pts with inflammatory or poor prognostic breast cancers treated with neo-adjuvant anthracycline based therapy. HER2/<i>neu</i> status not predictive of response.</p> <p>Schneider et al, 2000[602] 48 pts with LABC treated with CAF induction chemotherapy. HER2/<i>neu</i> status not predictive of response.</p>
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Table 1.4 HER2/*neu* as a predictive biomarker

1. Doxorubicin and cyclophosphamide
2. Disease Free Survival
3. Cyclophosphamide, doxorubicin, 5-fU
4. Overall Survival
5. Doxorubicin and L-phenylalanine and 5fU
6. L-phenylalanine and 5-fU
7. Cyclophosphamide, eipirubicin, 5-fU
8. Doxorubicin (adriamycin)
9. 5-fU, doxorubicin, cyclophosphamide

It seems that HER2/*neu* over expression can predict a cohort of patients who will be less responsive to CMF adjuvant chemotherapy compared to HER2/*neu* negative tumours. However, as tumours over-expressing HER2/*neu* are likely to derive some benefit from CMF over no adjuvant therapy, CMF should not be withheld if other treatment regimens are contraindicated [603].

With HER2/*neu* anthracycline based regimes there are numerous studies with less than 150 patients in the neo-adjuvant, adjuvant and metastatic setting that give conflicting results (listed with minimal detail in table 4). It must be noted that these are mostly retrospective studies with small numbers of tumours and are grossly underpowered to detect any meaningful difference in HER2/*neu* expression. The larger studies that have assessed HER2/*neu* status amongst patients partaking in a clinical trial may have more significance. There is no variation in chemotherapy regimes as they are given in a trial protocol and HER2/*neu* testing is more standardised. The Ludwig groups of trials [165, 588] could be considered for hypothesis generation. The first cohort of 397 cases demonstrated that the higher dose of anthracycline was of particular value in HER2/*neu* +ve patients and although this was not confirmed for a second cohort, combined data demonstrated a statistically significant benefit for HER2/*neu* +ve patients. The study of HER2/*neu* within the national surgical adjuvant breast and bowel project (NSAPB) trials initially showed a non-significant trend amongst 1355 patients and then showed a benefit in survival over 13.5 years of follow-up[587, 589]. Although encouraging it must be remembered that these trials were designed with different primary end points and HER2/*neu* assessment has been a retrospective analysis on data collected and hence must be regarded with caution.

It is difficult to compare all results directly as there are multiple methods of measuring HER2/*neu* and multiple definitions for end points of response. It is also possible that metastatic cells behave differently from cells in the primary tumour mass[583] making

advanced breast cancer trials difficult to compare with adjuvant or neo-adjuvant trials. The lack of a clear mechanism by which HER2/*neu* over expression should lead to anthracycline sensitivity also makes for cautious interpretation of results although this may be explained by co-expression of Topo II (see below).

Currently the advice from ASCO (American Society of Clinical Oncology) states that HER2/*neu* may identify patients who particularly benefit from anthracycline based adjuvant therapy but that levels of HER2/*neu* should not be used to exclude patients from this type of treatment [604].

1.3.1.1 HER2/*neu* and predictive value with taxanes

The clinical significance of HER2/*neu* as a predictor of response to taxane chemotherapy remains unclear. Clinical trials have shown that docetaxel and trastuzumab are more efficacious than docetaxel alone in the metastatic setting[605]. It is thought that the over expression of HER2/*neu* induces resistance against docetaxel in vitro as concomitant treatment with trastuzumab results in the sensitization of breast cancer cells to docetaxel[606]. The mechanism is unknown but it has been suggested that that HER2/*neu* overexpression induces docetaxel resistance by inducing CDK1-inhibiting p21, which may result in delay of docetaxel, mediated entry into mitosis and apoptosis [607].

Although the majority of pathological complete responses are observed in patients with HER2/*neu* negative tumours treated with taxanes in the neo-adjuvant setting [608], many studies have failed to find an association between HER2/*neu* over expression and resistance to docetaxel in the neoadjuvant setting [206, 609]. Conversely Durbeq et al [610] did demonstrate in a small study that HER2/*neu* positive tumours had a better response to docetaxel than HER2/*neu* negative tumours in a metastatic setting

With regards to paclitaxel, cell studies have shown that HER2/*neu* overexpression or forced expression does not affect sensitivity [611]. However a moderate sized randomized trial of

adjuvant anthracycline based chemotherapy followed by paclitaxel verses anthracycline based alone has shown that whilst HER2/*neu* negative patients gained no significant benefit from paclitaxel, HER2/*neu* positive patients had significant improvements in 5 year DFS[48].

1.7.7.6.1 HER2/*neu* Codon 655 (Rs 1136201) and -1170 G>C (Rs 1058808)

Structural and functional alterations of HER2/*neu* have been reported in different steps of carcinogenesis including initiation, promotion and progression[612]. The presence of a SNP in the transmembrane coding region at codon 655 encoding either isoleucine (Ile:ATC) or Valine (Val:GTC) was first reported in 1991[613]. It has been demonstrated that the presence of Val stabilises the formation of an active dimer of the protein thus predisposing to an auto-activity of the receptor [614].

The presence of the Ile/Val SNP may be associated with an increased risk of breast cancer development [615] although this is controversial [616-622]. The controversy may be due to the substantial differences in the SNP between ethnic groups [623]. A meta-analysis published in 2009 looking at association with breast cancer risk identified 20 studies with 10,642 case and 11,259 controls[624]. In the overall analysis the Val allele frequency was significantly higher in cases than controls. However, in the subgroup analysis, whilst Val significantly correlated in an Asian population no such association was seen in European or White populations.

HER2/*neu* codon 655 may be considered a susceptibility biomarker for early onset breast cancer risk, especially under the age of 45 [624, 625]and over the age of 60[616]. Naidu *et al* could not demonstrate an association with breast cancer risk but did illustrate that the presence of a Val allele was significantly associated with nodal metastasis and therefore may be a useful marker for tumour prognosis [622]

In a cell-line study, Val isoforms have been shown to express the highest growth capacity and, when injected in to nude mice, developed aggressive tumours that are sensitive to trastuzumab [626]. However, the authors also describe that treatment related cardiotoxicity is associated with the Ile/Val genotype.

Data from dbSNP suggest that the minor Val allele is present in 32.5% of a European population and 24% of a Caucasian population.

A SNP at position 1170 encoding either Alanine (Ala: GCC) or Proline (Pro CCC) has been studied in relation to AML. Specific cytotoxic activity against the Pro peptide and the absence of recognition of the Ala peptide has been demonstrated[627]. dbSNP reports the presence of the minor allele Pro to be 29.2% in a European population.

There are no reports in the literature of SNPs in *HER2/neu* in relation to chemotherapy response of breast cancer or any other solid tumours.

1.8 Aims and Hypothesis

There is a wide diversity of natural genetic variants that could in principle influence the outcome of chemotherapy. We therefore hypothesised that specific variants would be enriched in chemoresponsive groups of breast cancers and aimed to test such association with a longer term view to creating panels of SNP based chemotherapy response biomarkers. The SNPS assessed cover a range of subcellular processes and are summarised in **Table 1.5**.

Pathway	Gene	SNP
Apoptosis	TP53	RS 1042522
	MDM2	RS 2279744
	CDKN1A	RS 1801270, RS1059234
	BCL-2	RS 2279115
	BAX	RS 4645878
Cell Cycle	CCND1	RS 9344
Metastor Genes	XBP-1	RS 2269577
	SPP1	RS 11730582, RS 28357094
Drug Metabolism	GSTP1	RS 1695, RS 1138272
	ABCB1	RS 1045642
	MTHFR	RS 1801133, RS1801131
	CYP2B6	RS 4802101, RS4803419, RS2279345
Predictive SNPs	HER2/ <i>neu</i>	RS 1136201, RS 1058808

Table 1.5 SNPs of interest

2. Chapter 2 - Materials and Methods

2.1 General Methods

2.1.1 Ethical Approval

Ethical approval was obtained from the Cheshire Local Research Ethics Committee (LREC) using National Research Ethics Service NRES forms. Ethical issues concerned the use of case notes from Clatterbridge centre for Oncology (CCO). Available tissue bank material from the Candis Cancer Research Tissue Bank (CCRTB) was not covered by generic consent and although patients who kindly donate tissue to the CCRTB complete a consent form this only allowed future researchers to access their notes held at the Royal Liverpool and Broadgreen University Hospital, which did not include information regarding adjuvant therapy. The LREC considered this and gave permission for the CCO notes to be reviewed by pseudo-anonymisation, achieved through a CCO intermediary (REC ref no.: 09/H1017/96).

2.1.2 NHS Research and development Approval

The study was undertaken in collaboration with CCO NHS Trust. NHS Research and Development (R&D) Committee approval was therefore sought prior to review of case notes at CCO (reference number at CCO - [R & DD343](#)). This was achieved through the integrated research application system (IRAS) application form (IRAS number 24674).

2.1.3 Patient Selection

Patients who had undergone primary breast cancer surgery and consented for tissue to be stored within the CCRTB between 1993 (year of commencement of tissue collection) and 2005 were identified via the CANDIS tissue bank databases. This allowed a minimum of a 5

year follow-up period for those identified up to 2003 and also 2 year follow up for those identified between 2003-2005.

A comprehensive case note review was performed for each patient including patient demographics, surgical details, histology reports and details of adjuvant therapy including hormonal manipulation, radiotherapy and systemic cytotoxic therapy.

2.1.4 Candis Cancer Research Tissue Bank Application

Study approval was sought from the CCRTB once ethical and R&D approval had been obtained.

2.2 Chemotherapy cohort

From 1400 tissue samples held in the cancer tissue bank, 326 were identified as being from patients who had received adjuvant or neo-adjuvant chemotherapy as part of their primary breast cancer treatment. 303 of these had some DNA available for analysis.

For DFS the time to first disease free event was used, a disease free event being classified as local recurrence, distant recurrence or death. In this analysis all cause death was used as the information of cause of death and was not reliable enough to distinguish between breast cancer related deaths and non-breast cancer related deaths.

2.3 ECACC Control Panel DNA

ECACC HRC DNA Panels (supplied by Sigma Aldrich) are DNA samples from anonymous apparently normal, randomly selected subjects. HRC DNA was prepared by the ECACC from lymphoblastoid cell lines derived by Epstein Barr Virus (EBV) transformation of peripheral blood lymphocytes from fresh, single donor blood samples. The HRC1 DNA Panel was supplied in a 96 well plate format. Each well had a 20µl of DNA sample at 100ng/µl concentration.

To prepare control samples for SNP assay the HRC1 96-well plate was removed from -80°C storage to a 4°C fridge, allowing the plate to thaw at 4°C. It was then centrifuged (700g for 30seconds) to ensure all of the liquid from the sample is at the bottom of the wells. Dilution plates were created by adding 9µl of Millipore 0.2µM filtered H₂O to each well of a 96 well PCR and 1µl of the HRC1 DNA samples, maintaining the 96 well placement format. This creates plates of 10ng/µl with 10µl in each well. Plates are sealed with an adhesive lid and centrifuged. These plates were Stored at -80°C until needed.

All work was carried out in the DNA Addition Hood in the Pre-PCR area of the lab to ensure cross contamination did not occur.

2.4 Solutions used

2.4.1 TAE buffer

48.8g of Tris base (tris(hydroxymethyl)aminomethane), 11.4 mL of glacial acetic acid (17.4M) and 3.7 g of EDTA, disodium salt was dissolved in 800ml of deionized water. This was then diluted to 1000ml.

2.4.2 TE Buffer

For 100ml of 1x TE buffer, 1 ml of 1 M Tris base was combined with 0.2ml 0.5M EDTA and pH brought to 8.0 with hydrochloric acid (HCl) and volume made up with double distilled water.

2.4.3 SYBR safe (Life Technologies)

A nucleic acid stain for visualisation in agarose gels that can be used with either blue-light or UV excitation. It is a cyanine dye.

2.4.4 Salt Solution for TA topo cloning

A salt solution is provided by Life technologies – 1.2M NaCl. 0.06M MgCl₂

2.4.5 Super Optimal Broth with catabolite repression (S.O.C.) Medium

To create 1000 ml of S.O.C. medium the following were mixed and then topped up to 1000ml by added de-ionised water.

20 g of 2% Tryptone

5g of 0.5% yeast extract

584mg 10mM NaCl

186mg 2.5mM KCl

952mg 10mM MgCl₂,

3.603g of 20 mM dextrose

The pH was adjusted to 7.0 by the addition of sodium hydroxide. The solution was then filter sterilized through a 0.22 µm filter.

2.4.6 LB Broth

10g Bacto-tryptone, 5g yeast extract and 10g NaCl was added to 800ml of water. NaOH was used to adjust the pH to 7.5. 15g of agar was added and melted into the solution in the microwave. The volume was increased to 1 litre by the addition of distilled water and the autoclave used for sterilization.

2.4.7 Kanomycin

An aminoglycoside bactericidal antibiotic used in LB broth plates to isolate E.coli containing plasmids resistant to kanomycin.

2.4.8 Isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal

In TA cloning (see chp 2.5.5) with a blue-white screen IPTG is used with X-galto identify colonies that have been transformed with the recombinant plasmid

2.4.9 Qiagen Kit solutions

The exact composition of buffers provided in kits by Qiagen is confidential.

2.4.9.1 Buffer RW1

A wash buffer containing guanidine hydrochloride. It removes biomolecules such as carbohydrates, proteins and fatty acids that are non-specifically bound to the silica membrane.

2.4.9.2 Buffer AW2

A Tris-based wash buffer to remove salts; to prepare, 30ml ethanol (96-100%) was added to 13ml Buffer AW2 and stored at room temperature

2.4.9.3 Buffer EB

An elution buffer for DNA preparation containing 10mM Tris-Cl at pH 8.5.

2.4.9.4 Xylene

An aromatic hydrocarbon used as to remove paraffin

2.4.9.5 Ethanol

96-100% used

2.4.9.6 Buffer ATL

An animal tissue lysis buffer for use in purification of nucleic acids. It contains EDTA and dodecyl sulfate. Any precipitate that has formed is dissolved by heating to 70° C with gentle agitation

2.4.9.7 Proteinase k

An enzyme able to digest native keratin. It is used to digest protein and remove contamination from preparations of nucleic acid.

2.4.9.8 Buffer AL

A lysis buffer containing guanidine hydrochloride. Any precipitate that has formed is dissolved by heating to 70° C with gentle agitation

2.4.9.9 Buffer RLT

Is a lysis buffer for lysing cells and tissues prior to RNA/DNA/protein isolation. It contains a high concentration of guanidine isothiocyanate, which supports binding of RNA to the silica membrane. When Buffer RLT was used 10 µl of β-mercaptoethanol was added separately per 1ml of Buffer RLT

2.4.9.10 Buffer RPE

A mild buffer for washing membrane bound RNA whose main function is to remove traces of salts still on the column due to buffers used earlier in the protocol. It requires the addition of ethanol

2.4.9.11 Buffer APP

A novel aqueous protein precipitation solution containing zinc chloride

2.4.9.12 Buffer AW1

A wash buffer with a low concentration of quinidine that requires the addition of 25ml of ethanol (96-100%) to 19ml of Buffer AW1 and stored at room temperature.

2.5 TaqMan PCR Probes

Polymorphisms were genotyped using commercially available TaqMan real-time PCR SNP genotyping assays (Applied Biosystems, Carlsbad, CA). Thermo-cycling and subsequent genotype calling was performed using an ABI 7900HT real-time PCR system (Applied Biosystems).

All probes are stored at -20°C in aliquots in a darkened box to avoid direct exposure and pre aliquoted to avoid multiple freeze thaw cycles.

2.5.1 Annealing Temperature optimisation.

Human Genomic DNA (life technologies) was used to perform a temperature gradient PCR on all SNP probes to identify optimal annealing temperature. PCR was performed under the following conditions, 3 minute at 95 °C, then 15 seconds at 92°C followed by a temperature gradient between 55 and 65 °C repeated 44 times followed by a cool to 4 °C. Rows A-H on a standard 96 well plate corresponded with temperatures 65.0, 64.5, 63.3, 61.4, 59.0, 57.0, 55.7 and 55 °C respectively products ran on a 2% agarose gel (see section 2.5.4).

2.5.2 TaqMan Quantitative PCR reactions

PCR reactions were set up as follows with each SNP probe mix in all used wells of the 96 well plate (MASTERMIX): 4ul RT-PCR Mix (no Sybr,) 0.25ul Probe mix and 3.75ul of water with 20 ng of DNA. The mastermix is described in **table 2.1**

Reagent	Volume
RT-PCR Mix	400µl
Probe Mix	50µl
H ₂ O	350µl

Table 2.1 Mastermix for quantitative PCR reactions (per 96 well plate)

The PCR plate was then sealed and centrifuged briefly.

PCR plate performed under the following conditions, 95°C for 3 minutes, 90°C for 20 seconds and assay specific annealing temperature 55°C – 65°C for 90 seconds.

2.5.3 PCR Plate Set Up

For optimisation experiments on HRC panels, 2 standard, negative, no template controls (NTC) were used. For genotyping of clinical breast samples additional positive controls derived from mixing experiments (see section 4.2.4.1) were added to the plate layout,

figure 2.1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	NTC	NTC
B	S 11	S 12	S 13	S 14	S 15	S 16	S 17	S 18	S 19	S 20	A1 100:A2 0	A1 100:A2 0
C	S 21	S 22	S 23	S 24	S 25	S 26	S 27	S 28	S 29	S 30	A1 90:A2 10	A1 90:A2 10
D	S 31	S 32	S 33	S 34	S 35	S 36	S 37	S 38	S 39	S 40	A1 50:A2 50	A1 50:A2 50
E	S 41	S 42	S 43	S 44	S 45	S 46	S 47	S 48	S 49	S 50	A1 10:A2 90	A1 10:A2 90
F	S 51	S 52	S 53	S 54	S 55	S 56	S 57	S 58	S 59	S 60	A1 0:A2 100	A1 0:A2 100
G	S 61	S 62	S 63	S 64	S 65	S 66	S 67	S 68	S 69	S 70		
H	S 71	S 72	S 73	S 74	S 75	S 76	S 77	S 78	S 79	S 80		

Figure 2.1 Plate layout for diluted clinical samples for storage prior to assay application

S= clinical sample; A1 = Allele 1 ratio %; A2 = Allele 2 ratio %; NTC = negative control

All work was carried out in the PCR Hood (template tamer) to ensure contamination did not occur.

2.5.4 Agarose Gel electrophoresis

Horizontal gel trays were sealed appropriately with autoclave tape and 48 plus control lane well, spaced according to standard 96 well plate dimensions inserted (4-6 per tray). 4 - 12g of SeaKem LE agarose (Cambrex) for gel strengths between 1 and 3 % was weighed into a clean glass bottle and 400ml of TAE electrophoresis buffer added. The lid was placed

loosely on and microwaved until the agarose dissolved. Once cooled to $\sim 50^{\circ}\text{C}$, $4\mu\text{l}$ of 10,000X SYBR safe was added and the resultant mixture poured into the tray to a depth of 3mm. Once set, $10\mu\text{l}$ of a 100bp ladder (molecular weight marker, Invitrogen $1\mu\text{l}/\text{ml}$) was added to the first well. $5\mu\text{l}$ of PCR products were mixed with $5\mu\text{l}$ of loading dye. The gel was immersed in electrophoresis buffer and electrophoresis ran at 125 volts/hour. The gel was then scanned on a flat bed, ultraviolet gel scanner using a blue filter.

2.5.5 TA Topo Cloning

Pre-prepared pCR2.1 vector linearised and covalently bound to Topoisomerase I in (Life Technologies) at $25\text{ ng}/\mu\text{l}$, was used to clone our PCR products of interest. The protruding 3' deoxyadenosine (A) added to the ends of Taq derived PCR products were joined to the single 3' deoxythymidine (T) residues of the linearized vector. For the joining reaction $1\mu\text{l}$ of vector was used to incorporate $4\mu\text{l}$ of PCR product in the presence of $1\mu\text{l}$ of salt solution at room temperature for 15 minutes.

2.5.5.1 Bacterial transformation

To transform the construct into competent E. coli, $2\mu\text{l}$ of the TA TopoCloning ligation reaction (2.4.1) was mixed with One Shot[®] INVF' Chemically Competent E.Coli (Life Technologies) on ice, and incubated for 30 minutes. Cells were heat shocked at 42°C for 30 seconds and returned to ice. $250\mu\text{l}$ of S.O.C. medium was added to the cells which were then incubated at 37°C with shaking at 220 rpm for one hour.

LB broth was then poured into petri dishes and allowed to set. $10\mu\text{l}$ and $50\mu\text{l}$ of bacterial cell suspension were then spread onto separate pre-warmed LB plates containing $50\mu\text{g}/\text{ml}$ of kanomycin, $80\mu\text{l}/\text{ml}$ of IPTG and $50\mu\text{l}/\text{ml}$ of X-gal. Plates were incubated overnight at 37°C to allow colony formation. White colonies (compared to blue) were assumed to have incorporated the PCR product, thus disrupting the LacZ gene and were picked for further analysis.

2.5.5.2 Colony PCR

In order to sequence our SNP region inserts, M13 forward and reverse primers were used for PCR and as sequencing primers. Transformed E.Coli colonies were picked into PCR reactions containing 2µl 10x Buffer, 2 µl dNTP 2mM, 0.8 µl MgCl₂, 0.2 µl Taq polymerase (1unit/µl), 1 µl, M13 primer Forward 10mM, 1 µl M13 primer Reverse 10mM, 12 µl H₂O and PCR performed under the following conditions; 94.5°C for 5 minutes, 32 cycles of 94.5°C and 65°C for 30 seconds each, 72°C for 1 minute followed by a further incubation at 72°C for 10 minutes.

2.6 Sequencing (Sanger Method) of PCR Products

2.6.1 Preparation of PCR products for Sequencing

Primers and pyrophosphate were removed from colony PCR products by Exonuclease I and shrimp alkaline phosphatase in an ExoSAP master mix (GE Healthcare). The ExoSAP reaction contained 5µl of PCR product and 2.5 µl ExoSAP, with the addition of 2.5 ul of water. After mixing, the reaction was incubated at 37°C for 15 minutes followed by 15 minutes at 80°C in the thermal cycler.

2.6.2 Sequencing reaction

Fluorescent DNA sequencing was performed with DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Biosciences) and analysed on a MegaBACE 1000 (Amersham Biosciences). A reaction containing 4 µl sequencing reaction mix (DYEnamic ET reagent premix), 0.5 µl primer (forward or reverse), 1.5 µl water and 4 µl of the ExoSAP sample was ran in a thermal cycler for 25 cycles of 95°C for 20 seconds, 50°C for 15 seconds and 60°C for 1 minute.

2.6.2.1 Removal of Dye Terminators from sequencing reactions

Fluorescent terminators and buffer were removed from the sequencing reactions using a 96 well Genclean filtering plate removed from 4°C storage and allowed to rest at room temperature for 30 minutes. 100µl of filtered water was added to each well and centrifuged at 910g for 5 minutes at 20°C. This step was repeated twice. 15 µl of the sequencing reaction was then added to the centre of the resin well, an opaque Robbins plate added to the collection plate and centrifuge repeated under the same conditions.

2.6.2.2 Sequence Reaction Analysis

The Robbins plate containing labelled DNA was then ran on the MegaBACE, which uses linear polyacrylamide (LPA) separation matrix in capillary electrophoresis, to enable read lengths in excess of 800 bases in approximately 2 hours in an automated fashion. Narrow bore capillaries are filled with LPA and an electric field causes the DNA fragments migrate into and through the capillaries. Laser excitation and an optical detection system identifies the dye-labelled DNA fragments as they migrate past the detection window. Base calling was performed using the UTAH base-caller component from Cimarron Software

2.7 Tumour DNA from Candis Cancer Research Tissue Bank

2.7.1 Use of previously extracted DNA

A proportion of the samples requested from the CCRTB had extracted macromolecules available. Samples were provided with concentrations, which were used to dilute samples to 100ng/µl for routine stocks.

2.7.1.1 DNA concentration and purity.

DNA concentrations and purity were estimated in 1.5 ul samples, diluted if necessary using the NanoDrop ND-1000 spectrophotometer.

To measure A_{260} , A_{280} and A_{320} values. TE buffer was used as the reference.

Each DNA sample was measured in duplicate for confirmation. The A280/A260 ratio and A280/A230 ratios were used as a measure of DNA purity with values >1.8 the aim where possible. Samples derived from paraffin in particular were less likely to have higher purity and lower values were accepted.

2.7.2 Extraction of Macromolecules from Frozen Tissue Blocks

Frozen tissue blocks for samples where DNA was not already available were used to extract DNA, RNA and protein using ion exchange chromatography (the Qiagen Allprep DNA/RNA/Protein Mini Kit). The maximum binding capacity was 100µg RNA for the RNeasy spin column and 100µg DNA for the Allprep DNA spin column. 16-30mg of breast tissue was processed per column.

2.7.2.1 Tissue Lysis and homogenisation

A Tissue Lyser (Qiagen) was used for tissue disruption and homogenisation. 5mm stainless steel beads were cleaned and sterilised by flaming in 70% ethanol. For each sample, a bead was then placed in a 2ml Eppendorf Tube. Each tube plus bead was weighed separately and cooled to -80°C for at least 2 hours. A sample of the tissue to be processed was sliced into one of the tubes on ice and the tube re-weighed immediately, to determine the weight of tissue. Buffer RLT containing β-mercaptoethanol (β-ME) was added to each tube. For 16 – 30 mg tissue 600µl was added and for 31-60 mg 1200µl was added. Tubes were immediately placed in the rack for the tissue lyser ensuring equal distribution between the 2 holders and the tissue lyser operated for 2 minutes at 20Hz. The tubes were then rearranged swapping the outermost for the innermost to average disruption and the tissue lyser operated for a further 2 minutes at 20 Hz. These steps were repeated until no further tissue could be solubilised. Higher frequencies were used if necessary.

After homogenisation of the sample in Buffer RLT (+ β-ME) the lysate was centrifuged for 3 minutes at full speed (>12K x g). The supernatant was transferred to the Allprep DNA spin

column placed in a 2ml collection tube and centrifuged (8000 x g) for 30 seconds. The DNA spin column was then placed in a new 2ml collection tube and stored in the fridge at 4°C for DNA purification within a few hours.

2.7.2.2 Total RNA purification

430µl of 100% ethanol was added to the flow through and mixed by pipetting. 700µl of this sample was transferred to an RNeasy spin column and placed in a 2ml collection tube and centrifuge (8000 x g) for 30 seconds. The flow through was removed to a new 2ml collection tube for later protein purification. This was repeated for the remaining flow through and the eluate combined with the previous eluate for protein purification.

The optional column DNase digestion was then performed. 400µl of Buffer RW1 was added to the RNeasy spin column, left at room temperature for 1 minute and centrifuged for 30seconds. The flow through was discarded. 10µl of DNase I stock solution was added to 70µl of Buffer RDD and mixed gently by inverting the tube. The mix was then added directly to the RNeasy spin column membrane and the column incubated at 30°C for 15 minutes. 350µl of Buffer RW1 was then added to the column and centrifuged for 15 seconds at >8000 x g. The flow through was discarded. This step was repeated using 400µl of Buffer RW1.

500µl Buffer RPE was added to the RNeasy spin column to wash the membrane, which was then centrifuged (>8000 x g) for 30 seconds and the flow through discarded. A further 500µl of Buffer RPE was added and the column centrifuged (>8000 x g) for 2 minutes before transfer of the spin column to a new 2ml collection tube to ensure no carry-over of ethanol. The spin column was then centrifuged (>8000 x g) for a further 1 minute. Using a 10µl pipette tip any residual liquid was removed from the rim of the membrane and the spin column re-spun at >8000 x g for 1 minute.

In order to elute the RNA the RNeasy spin column was transferred to a new 1.5ml collection tube and 50µl of RNase free water added directly to the spin column membrane and left at room temperature for 1 minute. The column was then centrifuged (>8000 x g) for 1 minute. This step was repeated. The elute was immediately put on ice and the quantity and quality of RNA assessed using the Nanodrop 1000 Spectrophotometer and the Agilent Bioanalyser respectively. Depending on the results of quantitation, eluates from samples that have been processed using more than one column were recombined prior to storage at -80°C.

2.7.2.3 Total Protein Precipitation

Buffer APP (1000µl) was added to the flow through collected for protein precipitation during RNA purification, mixed vigorously and incubated at room temperature for 10 minutes to precipitate protein. This was then centrifuged at full speed for 10 minutes and the supernatant carefully decanted. 500µl of 70% ethanol was then added to the protein pellet and centrifuged at full speed for 1 minute. Decanting or pipetting then removed the supernatant and the ethanol wash repeated. After removing the bulk of the supernatant the tube was re-centrifuged and residual liquid removed by pipetting. The pellet was placed at 37°C until dry and then stored at -80°C for future use.

2.7.2.4 Genomic DNA purification

Buffer AW1 (500µl) was added to the Allprep DNA spin column stored at the start of RNA purification, centrifuged (8000 x g) for 30 seconds and the flow through discarded. Buffer AW2 (500µl) was then added to the DNA spin column and centrifuged at full speed for 2 minutes to wash and dry the column membrane. A 10µl pipette tip was then used to remove any residual liquid from the rim of the membrane and the sample re-spun at >8000 x g for 2 minutes to remove residual drops of liquid. The DNA spin column was then

transferred to a new 1.5ml collection tube. Buffer EB (100µl), preheated to 70°C was then added directly to the spin column and incubated at room temperature for 2 minutes. The column was then centrifuged (8000 x g) for 1 minute to elute the DNA. Further Buffer EB (100µl) was added and these steps repeated to elute further DNA.

If the A260/A230 ratio was <1.8 then ultra filtration using a microcon filtration device (Rose Microcon® Centrifugal Filter Unit) was used order to further purify and optionally concentrate the DNA. The microcon was washed by centrifuging through 400µl of purified water at 12,500 x g for 5 minutes. This step was repeated twice. The DNA sample was then added and the volume made up to approximately 400µl and centrifugation performed at 12,500 x g for 5 minutes. Two further washes with 0.2 x EB took place. The liquid was recovered by inverting the filter to a fresh tube and centrifuging at 300 x g for 1 minute. If the sample was still considered to be too dilute then a Genevac (GE Healthcare) at 37°C was used to decrease the volume further. DNA was stored at -80°C until required.

2.7.3 Extraction of DNA from Paraffin Embedded Tissue Blocks

DNA was extracted from paraffin blocks provided in shavings from the tissue bank using specially optimized lysis conditions (QIAamp DNA FFPE Tissue kit). The tissue bank provided shavings from samples in paraffin blocks.

Sections of paraffin block 10 µm thick were dissolved by vortexing in 1ml of xylene in 2ml eppendorf tubes for 10 seconds. The supernatant was removed and 1ml of 100% ethanol added to the pellet and mixed to extract residual xylene from the sample. The supernatant was removed and excess ethanol removed using a pipet tip. The tubes were then incubated at 37°C to evaporate residual ethanol. The pellet was then suspended in 180 µl of Buffer ATL and 20 µl of proteinase K added and mixed to digest proteins.

An incubation period of 1 hour at 90°C to partially reverse formaldehyde modification of nucleic acids was then undertaken.

A mixture of 200 µl Buffer AL and 200 µl of ethanol was added and mixed. The entire lysate was then added to the QIAamp MinElute column in a 2ml collection tube and spun at 6000 x g for 1 minute. The flow through was discarded and 500 µl of Buffer AW1 added to the column and spun at 6000 x g for 1 minute. Once again the flow through was discarded and then 500 µl of Buffer AW2 added and spun for 1 minute at 6000 x g. The column was then placed in another clean 2ml collecting tube and centrifuged at 20,000 x g for 3 minutes to dry membrane completely. This prevents any ethanol carry-over, which may interfere with downstream applications.

A clean 1.5 ml eppendorf tube was then used for the column and 100 µl of Buffer ATE was applied to the centre of the membrane. After incubating for 1 minute the columns were spun at 20,000 x g to collect the DNA solution.

2.8 Statistical Methods

Data was collected using Microsoft Excel (2009-2016) based databases, always using the most up to date version available. Statistical analysis was originally performed using NCSS (NCSS 9 Statistical software (2013). NCSS, LLC. Kaysville, Utah USA, ncss.com/software/ncss.) and subsequently using SPSS (IBM SPSS for Macintosh).

Associations with tested using Chi square. Survival curves were produced using censored Kaplan Meier's with Mantel Cox log ranks. Bonferroni correction was considered but given the large number of potential variables (3 genotypes per SNP and chemotherapy type) it was thought to be too stringent and not used (see discussion). A significance value of $p < 0.05$ was therefore accepted.

The χ^2 test for deviation was used for Hardy-Weinberg proportions with one degree of freedom. The 5% significance level for 1 degree of freedom is 3.84.

3. Chapter 3 - Clinical Cohort

3.1 Case note analysis

Samples were analysed from 303 patients. The mean and median age of the entire cohort was 50.6 and 49.5 respectively with a range from 27.3 to 75.8 (standard deviation 10.4). The median follow up was 83.9 months ranging from 2.4 to 208.5. The study period of 1993 to 2005 included the transition to taxane containing regimes and so multiple chemotherapy regimes were used within this cohort changing according to clinical guidelines at a given time. Many patients were also involved in the clinical trials previously discussed. The type of chemotherapy given changed over the study period with the addition of anthracyclines to CMF alone regimes as well as the appearance of taxanes in the latter years, **figures 3.1** and **3.2**. In the first 6 years of the study period 65% of patients received CMF chemotherapy compared to the later 6 years where 59% of patients received anthracycline plus CMF chemotherapy.

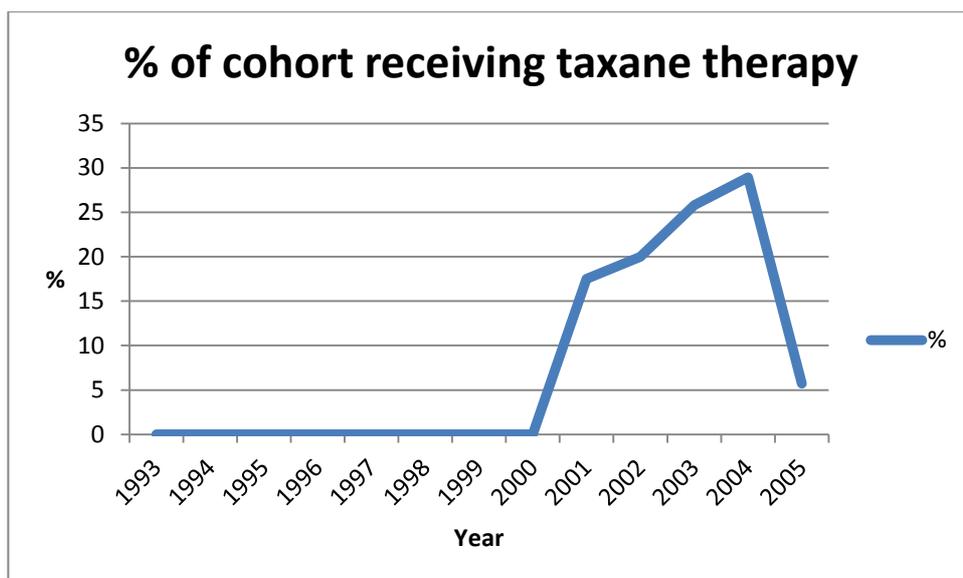


Figure 3.1 % of cohort, grouped by year, receiving taxane therapy.

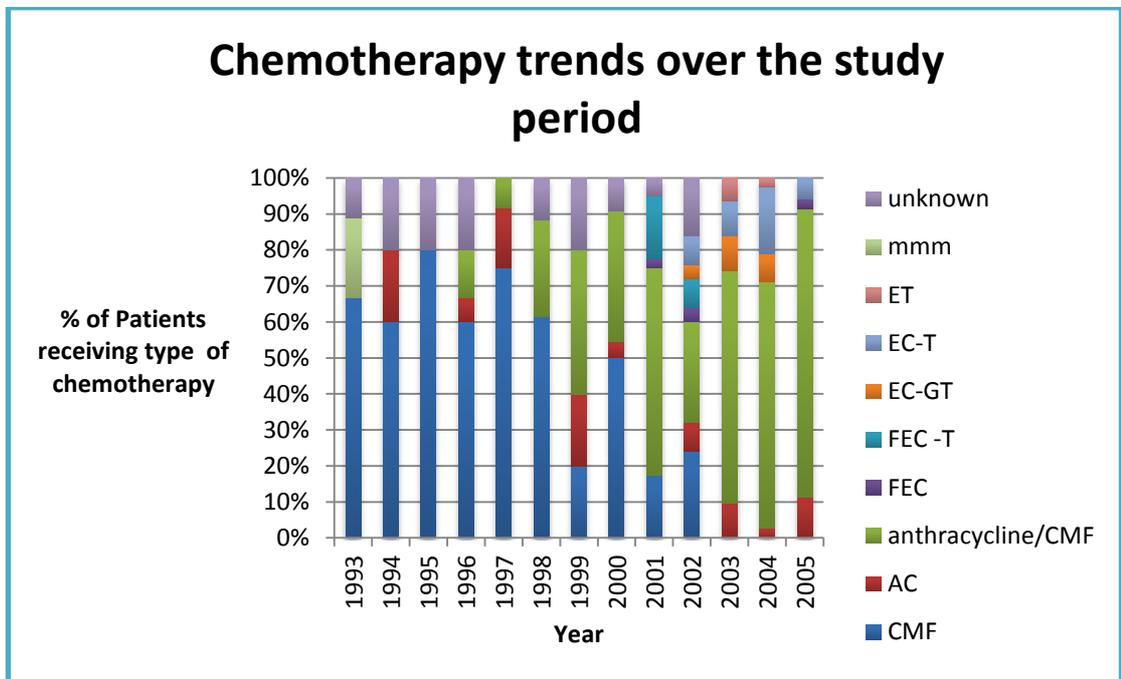


Figure 3.2 Trends in chemotherapy regimes used over the study period

MMM – Mitomycin, methotrexate, mitozantrone

ET – anthracycline* and taxane

EC-T –anthracycline, cyclophosphamide and taxane

EC-GT –anthracycline, cyclophosphamide, gemcitabine and taxane

FEC- T – 5-fU, Epirubicin, cyclophosphamide and taxane

FEC - 5-fU, Epirubicin, cyclophosphamide

Anthracycline/CMF – Anthracycline, cyclophosphamide, methotrexate, 5-fU

CMF - cyclophosphamide, methotrexate, 5-fU

*Anthracycline used usually epirubicin

Further analysis therefore focused on a comparison of anthracycline verses non-anthracycline containing regimes and taxane verses non-taxane containing regimes.

3.1.1 Overall Survival and Disease Free Survival

We have demonstrated that within our cohort of breast cancer cases undergoing chemotherapy the known prognostic factors remain important (**tables 3.1 and 3.2**). As expected the presence of lymphovascular invasion, lymph node metastases, and ER negativity along with tumour size, grade, stage and prognostic grouping were all associated with poorer outcome. This shows that even with chemotherapy, bad cancers do worse.

Table 3.1 summarises the associations between various clinicopathological features and overall survival.

Variable	Category	Count	5 year survival (%)	10 year survival (%)	Log rank p value	Median survival (Months)
Method of detection	Screening	18	81	-	0.88	Not reached
	Symptomatic	174	79	67		174.1
	Missing	111				
Breast Surgery	Mastectomy	182	79	66	0.31	174.1
	WLE	107	83	73		Not reached
	Missing	14				
Lymphovascular Invasion	Absent	130	91	84	<0.0001	Not reached
	Present	152	71	60		166.4
	Missing	21				
ER status	Negative	108	69	65	0.005	Not reached

	Positive	167	90	74		174.1
	Missing	28				
HER 2 status	Negative	93	82	51	0.49	131.4
	Positive	27	88	-		Not reached
	Missing	183				
LN Status	Negative	96	88	87	<0.0001	Not reached
	Positive	196	76	60		146.5
	Missing	11				
Anthracycline treated	No	111	76	68	0.3	166.4
	Yes	181	82	-		Not reached
	Missing	11				
Taxane treated	No	260	80	81	0.83	174.1
	Yes	32	81	-		Not reached
	Missing	11				
Menstrual status	Peri-menopausal	8	-	-	0.53	Not reached
	Post-menopausal	145	82	73		174.1
	Pre-menopausal	111	75	63		166.4
	Missing	39				

Grade	1	7	100	100	0.04	Not reached
	2	113	87	73		Not reached
	3	170	70	65		174.1
	Missing	13				
T-Stage	1	53	81	74	0.03	177.3
	2	187	83	71		Not reached
	3	48	70	52		146.2
	Missing	15				
Stage	1	21	-	-	<0.0001	Not reached
	2a	90	83	81		Not reached
	2b	136	77	65		146.5
	3a	37	61	41		96.7
	Missing	19				
NPI prognostic group	Excellent	1	-	-	<0.0001	Not reached
	Good	7	-	-		Not reached
	Moderate	148	90	83		Not reached
	Poor	129	68	52		135.4

	Missing	18				
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Table 3.1 Overall survival associated with clinicopathological features.

.An example of Kaplan Meier Curves for lymph node status is shown (Figure 3.3).

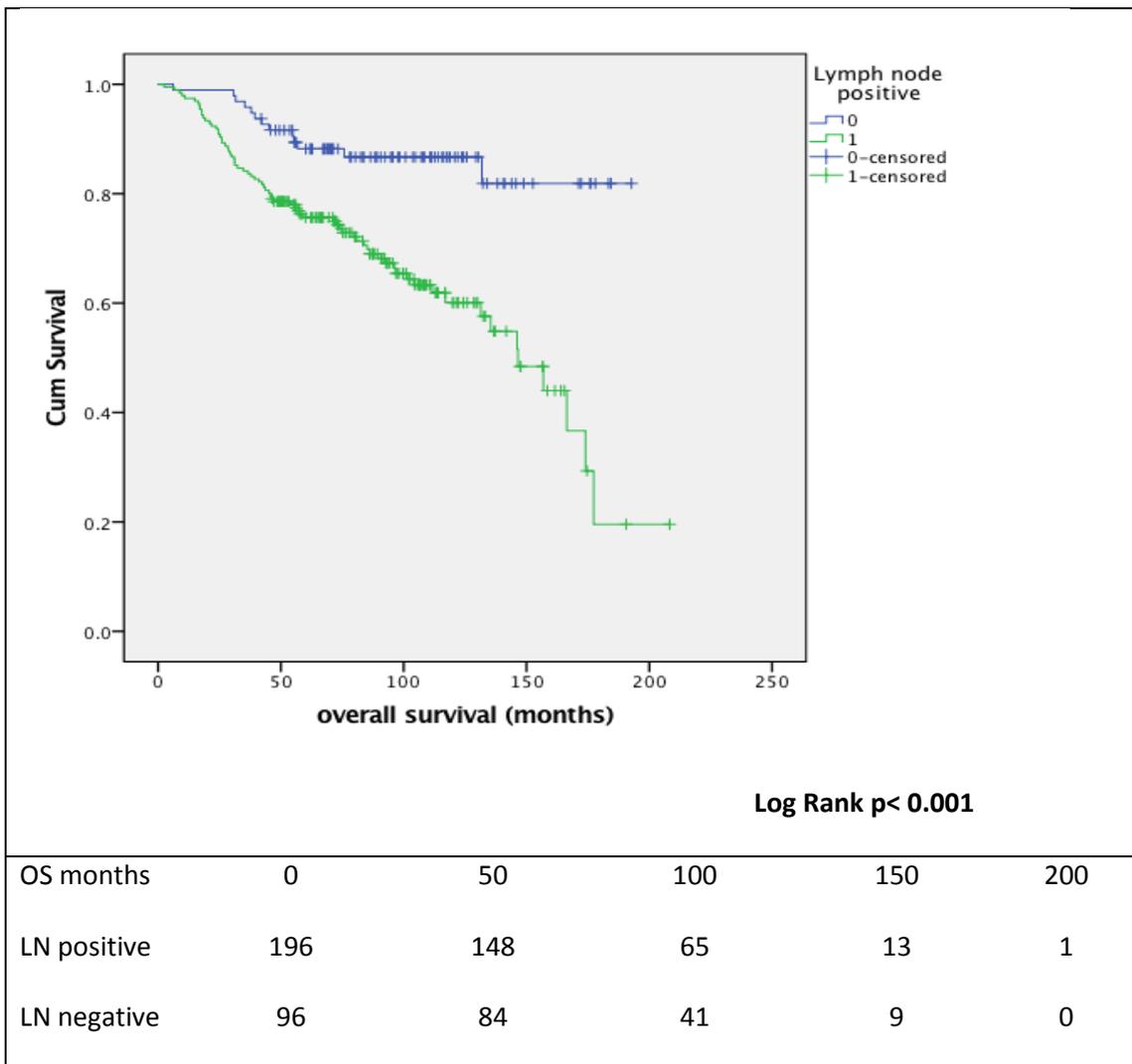


Figure 3.3 OS of cohort grouped by lymph node positivity with numbers at risk tabulated below

Variable	Category	Count	5 year	10	Log	Median
----------	----------	-------	--------	----	-----	--------

			DFS (%)	year DFS (%)	rank	DFS
Method of detection	Screening	17	82	-	0.4	n/a
	Symptomatic	167	73	61		156.9
	Missing	119				
Breast Surgery	Mastectomy	184	72	60	0.48	150.2
	WLE	110	76	68		156.9
	Missing	9				
Lymphovascular Invasion	Absent	131	88	81	<0.0001	156.9
	Present	156	61	47		90.3
	Missing	16				
ER status	Negative	109	67	64	0.25	146.5
	Positive	171	80	64		150.2
	Missing	23				
HER 2 status	Negative	93	70	41	0.5	90.4
	Positive	28	74	-		n/a
	Missing	182				
LN Status	Negative	98	82	81	0.0005	183.4
	Positive	199	69	54		150.2
	Missing	6				
Anthracycline treated	No	115	69	60	0.26	150.2
	Yes	181	76	64		148.1
	Missing	7				
Taxane treated	No	260	75	65	0.05	156.9

	Yes	32	65	-		102.1
	Missing	11				
Menstrual status	Peri-menopausal	8	-	-	0.5	n/a
	Post-menopausal	145	76	69		148.1
	Pre-menopausal	115	68	55		156.9
	Missing	35				
Grade	1	7	100	-	0.04	n/a
	2	115	82	67		n/a
	3	173	67	59		150.2
	Missing	8				
T-Stage	1	53	73	69	0.008	n/a
	2	190	77	66		156.9
	3	50	64	44		90.3
	Missing	10				
Stage	1	21	-	-	<0.001	n/a
	2a	91	78	75		183.4
	2b	139	75	61		156.9
	3a	38	55	32		83.1
	Missing	14				
NPI prognostic group	Excellent	1	-	-	<0.0001	n/a
	Good	7	-	-		n/a
	Moderate	151	83	75		183.4

	Poor	131	62	47		135.4
	Missing	20				

Table 3.2 DFS associated with clinicopathological features

3.1.3 Comparisons of Chemotherapy Regimes Given

3.1.3.1 Anthracyclines

Table 3.3 summarises the comparison of clinicopathological features between those receiving or not receiving anthracycline-based chemotherapy. Although OS and DFS were no different in those receiving and not receiving anthracyclines (**tables 3.1 and 3.2**), there were statistically significant differences in T stage and overall breast cancer stage between the two groups. Those receiving anthracycline were more likely to have a larger T stage ($p = 0.0000$) and a higher breast cancer stage ($p=0.0021$), **table 3.3**.

Variable	Category	Total	No Anthracycline count	%	Anthracycline containing count	%	Chi square p value
Method of Detection	Screening	17	4	24	13	76	0.24
	Symptomatic	176	67	38	109	62	
	Missing	90					
Menstrual Status	Peri-Menopausal	9	5	56	4	44	0.19
	Post-Menopausal	142	46	32	96	68	
	Pre-menopausal	123	50	41	73	59	

	Missing	29						
Grade	1	7	3	43	4	57	0.24	
	2	115	32	28	83	72		
	3	172	67	37	115	63		
	Missing	9						
T Stage	1	52	32	62	20	38	0.00	
	2	189	60	32	129	68		
	3	52	10	19	42	81		
	Missing	10						
Lymphovascular invasion	Absent	135	43	32	92	68	0.59	
	Present	161	56	35	105	65		
	Missing	7						
ER Status	Negative	117	44	38	73	62	0.11	
	Positive	178	51	29	127	71		
	Missing	8						
Lymph Node Status	Negative	94	33	35	61	65	0.68	
	Positive	208	68	33	140	67		
	Missing	1						
Stage	1	20	13	65	7	35	0.00	
	2a	96	39	41	57	59		
	2b	145	40	28	105	72		
	3a	40	9	23	31	78		
	Missing	2						
NPI prognostic	Excellent	1	0	0	1	100	0.14	
	Good	5	4	80	1	20		

grouping	Moderate	157	50	32	107	68	
	Poor	139	47	34	92	66	
	Missing	1					

Table 3.3 Comparison of clinicopathological features between anthracycline and no anthracycline containing regimes.

3.1.3.2 Taxanes

Unlike anthracyclines and perhaps because of the smaller numbers involved (32), there were no significant differences in between those receiving taxanes and not receiving taxane chemotherapy.

3.2 Discussion of chemotherapy cohort

We have demonstrated a cohort that is comparable to other clinical groups and whose outcome is not unexpected. Anthracycline based chemotherapy was received by a large proportion of our group in keeping with current practice. If a patient is considered fit enough then an anthracycline is considered standard therapy for anyone with node positive disease. Reflecting their more toxic nature, anthracyclines were more likely to be given in our cohort if advanced stage was present.

It is today's practice to give taxanes to patients with poor prognostic features. When the majority of patients in our cohort were undergoing their treatment taxanes were not routinely given leaving us with only 10% of samples from patients who received a taxane. There were no prognostic factors associated with the receipt of a taxane. The type of regime given did not affect outcome, suggesting room for improvement in choice of drug used for individual patients.

4. Chapter 4 – SNP assay development

4.1 Methods of SNP genotyping

Given that there are over 1.5million SNPs reported, reliable methods of detection are required. SNP detection can be genome wide or, more suitable for our hypothesis driven needs, candidate gene based. **Table 4.1** summarizes some of the current methods of genotyping available. There are 3 basic types: allele specific hybridisation whereby a nucleic acid probe is designed to distinguish between alleles, enzyme based whereby the properties of selected enzymes are used to produce different reaction products that distinguish between alleles and physical methods that distinguish between the physical properties of alleles, for example their melting temperature.

Genotype detection mechanism	Method
Allele-specific hybridization	<i>Taqman</i> real time PCR
	Dynamic allele specific
	Molecular beacons
Enzyme based	Restriction fragment length polymorphism
	Flap endonuclease
	Primer extension
	Oligonucleotide ligase
Physical methods	Single Strand conformation polymorphism
	Temperature gradient gel electrophoresis
	Denaturing high performance liquid chromatography
	High resolution melting of the entire amplicon

Table 4.1 Summary of representative methods for SNP genotyping

For allele specific hybridization approach, one allele specific probe per allele is hybridised to its target sequence during real time polymerase chain reaction (qPCR). Typically, each probe is double dye labelled, consisting of, a reporter at the 5' end and a quencher at the 3' [628]. The quencher uses fluorescence resonance energy transfer (FRET) to prevent the emission of detectable energy from the reporter when excited by a light source until the accumulation of complementary target sequences in the PCR separates the quencher and the reporter on hybridisation to the probe. Each allelic specific probe is labelled with a uniquely coloured reporter dye to enable differentiation of SNPs within alleles. Variants of the method include: Molecular Beacons, single stranded, hairpin oligonucleotides that

become fluorescent when they bind to perfectly complementary nucleic acids and TaqMan, where the 5' quencher is removed by Taq Polymerase during PCR if hybridised to the target [629-632].

As no enzymes are involved in any of the hybridization methods they are the simplest methods for genotyping. The difficulty and challenge is designing a probe that produces robust allelic discrimination.

Enzyme based methods include: Restriction fragment length polymorphisms which exploit differences in the presence of restriction sites determined by the presence of specific SNPs within the recognition sequence for the enzyme, detected by size separation of the possible products[633]; flap endonucleases, which allow allele specific invader assays with a fluorescence readout according to correct probe alignment in a 3 probe model. Each allele specific probe is complementary to the 5' region of the SNP site with an overhang and the invader probe is complementary to the 3' region of the polymorphic region creating a 3 dimensional structure that is recognized by cleavase to release FRET based fluorescence [634]; primer extension reactions where common or specific primers incorporate allele specific nucleotides to allow differentiation by mass or fluorescence [635]. Although enzyme based methods often require no PCR amplification they are slower processes requiring significant post PCR processing and large amounts of target molecule to generate detectable signals.

The physical properties of DNA allow for other post amplification SNP analysis methods. Single strand conformation polymorphism (SSCP) involves denaturing an amplified target DNA region to become single stranded which then folds into a 3 dimensional structure. Given that most single base pair changes will alter the shape of single strand DNA, subsequent non-denaturing gel electrophoresis utilizes the different electrophoretic mobility despite having identical number of nucleotides [636]. Temperature gradient gel

electrophoresis relies on the variations in nucleotide sequence having minor differences in thermal melting profiles [637]. High resolution melting of the entire amplicon uses high resolution software to plot melt curves in precisely warmed intercalating dye PCR reactions as T_m is reliant on sequence. Dynamic allele-specific hybridization (DASH) where one PCR primer is biotylated allows capture of the PCR products by a streptavidin coated solid support for probing with labelled allele specific probes. A single base mismatch between the fluorescence probe and the target results in a decreased melting temperature (T_m) that can be detected for pairs of alleles whose T_m differs between 5 and 9 degrees [638].

DNA microarrays are a multiplex technology with an arrayed series of thousands of spots of DNA oligonucleotides, each containing picomoles of a specific DNA sequence (probes). Using fluorescence, probe/target hybridisation is detected and quantified. Signal generation is by hybridisation and enzymatic cleavage or extension of probes [639]. Surface engineering allows probes to be attached to solid surface usually by covalent bond to a chemical matrix. The solid surface may consist of glass or silicon chips or microscopic beads. This allows simultaneous collection of data and quantification of target material bound to probes on the solid surface.

The basic principles of the convergence of DNA hybridisation, fluorescence microscopy and solid surface DNA capture can also be used to detect SNPS provided that the three mandatory components are met. The array must contain immobilised nucleic acid sequences, one or more labelled allele specific oligonucleotide probes and a detection system that records and interprets the hybridisation signal. Recently a method has been developed using pooled DNA samples that allows for scaling up in both number of SNPs and pooled samples[640].

Given that we had multiple hypothesis driven SNPs to analyse in over 300 samples we needed a reliable, robust method that could be tested in an efficient manner. Our first

concern was the lack of normal genomic DNA available to us and the consequence of loss of heterozygosity (LOH) for scoring using DNA extracted from tumour samples containing varying admixtures of normal cells. We therefore needed a method that would allow for control of this. We believed that using real time PCR with Taqman fluorescent probes would allow optimisation of individual probes and quantification of the impact of differing degrees of tumour / normal DNA admixture in the presence of LOH giving us confidence that any reasonable LOH could be scored.

4.2 Taqman assay

Taqman assays contained sequence specific forward and reverse primers to amplify the polymorphic sequence of interest. HEX and FAM dye labelled probes detect specific SNP targets. In addition they utilise minor groove binder (MGB) technology at the 3' end to bind to the helix minor groove improving hybridisation by stabilising the probe-template complex. There is also a non-fluorescence quencher to eliminate background fluorescence. Pilot studies were performed with the SNP Rs 1801131 (MTHFR 1298)

4.2.1 Rs 1801131 Annealing temperature optimization

Temperature gradient PCR using genomic control DNA, according to the standard conditions described in Sections 2.5.2 was performed to determine the optimum annealing temperature between 55°C and 65°C consistent with unique products per allele. Identical reactions using 20 ng of DNA were set up in each well except for D5, which contained water instead of DNA as a negative control. 3ul each of the PCR products were subject to gel electrophoresis on a 2% agarose gel with a 100bp ladder. 61.4 °C was ideal which was consistent with manufacturer's recommended annealing temperature. 61.4°C was therefore used as the standard annealing temperature in further experiments.

4.2.3 Standard Assay Format

SNP Taqman assays allow detection of homozygote and heterozygote samples for a given SNP using fluorescent probes in a real time PCR reaction. A uniform assay format and use of a close tube system was developed to allow a highly parallelised work flow to be set up, minimise risk of contamination, eliminate post-PCR processing thus reducing sources of error, and maximising use of precious DNA samples. Testing was performed in duplicate with each probe. This was especially important for samples with poorer quality DNA (for example extracted from paraffin blocks). Predesigned assays from Applied Biosystems were used as far as possible and each underwent rigorous validation and optimization before scoring. Separate replica plates for each assay and also the sample DNA were set up and stored before performing the assays. DNA and replica plates were then combined for each assay in turn as a “turn handle” process.

4.2.4 SNP Rs 1801131 genotype analysis for the ECACC HRC DNA Panel

ECACC HRC Panel DNA samples were used to optimise allele calling for the Rs 1801131 SNP Taqman probe.

94 samples from the panel were subject to Taqman PCR according to the standard assay format, plus 2 negative controls and 60°C annealing temperature determined above.

Relative fluorescence (RFU) was plotted versus cycle number (time). Successful amplifications were seen as sigmoid curves as expected, **Figure 4.1**. Baseline thresholds were set separately for the HEX (Vik) probe and the FAM probe. FAM and HEX on the example used according to the lowest values consistent with no amplification having occurred as represented by negative control PCR reactions, which appear as a linear, horizontal line on the same graph.

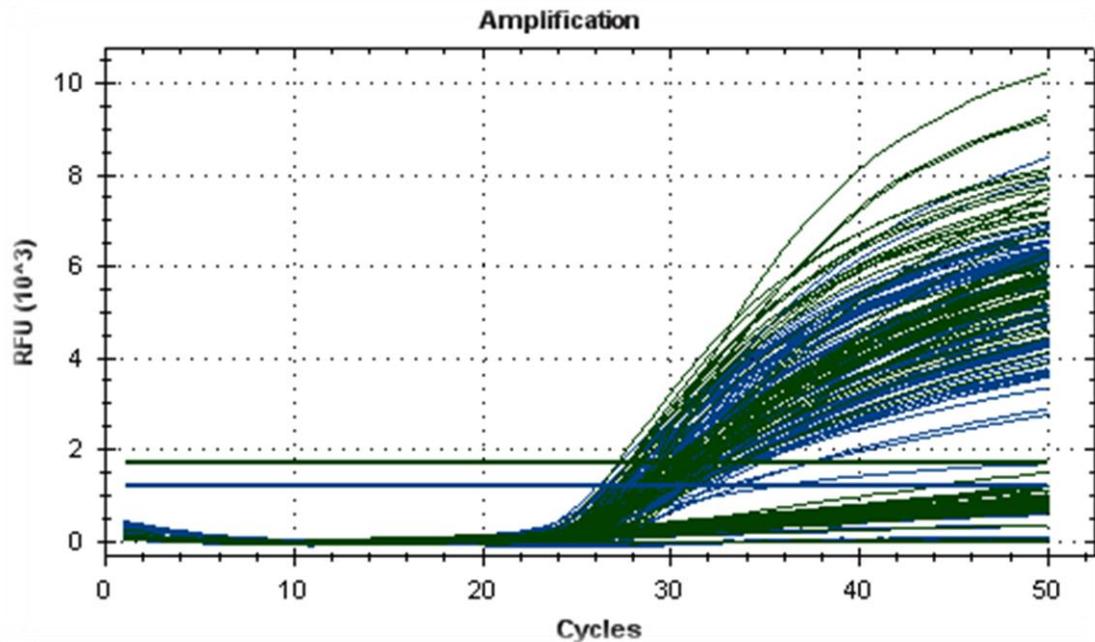


Figure 4.1 Amplification curve for RS 1801131 using 96 well HRC panel

RFU=fluorescence of reporter dye

The base lines have been set, based on negative controls, to eliminate any non-sigmoid curves

A successfully amplified product for both HEX and FAM probes indicates a Heterozygote. A successfully amplified product for HEX but not FAM or vice versa indicates a homozygote for either Allele 1 or 2 respectively.

Threshold cycle (Ct) is the fractional number at which fluorescence passes the threshold; that is the intersection between amplification curve and a threshold line and was used to compare the relative amounts of the 2 alleles in the samples. The value increases with decreasing amount of template used. For each sample we could calculate the Ct for allele 1 (Ct1) minus the Ct for allele 2 (Ct2) which gave us 2 or 3 distinct groupings for each homozygote and heterozygote, **figure 4.2**.

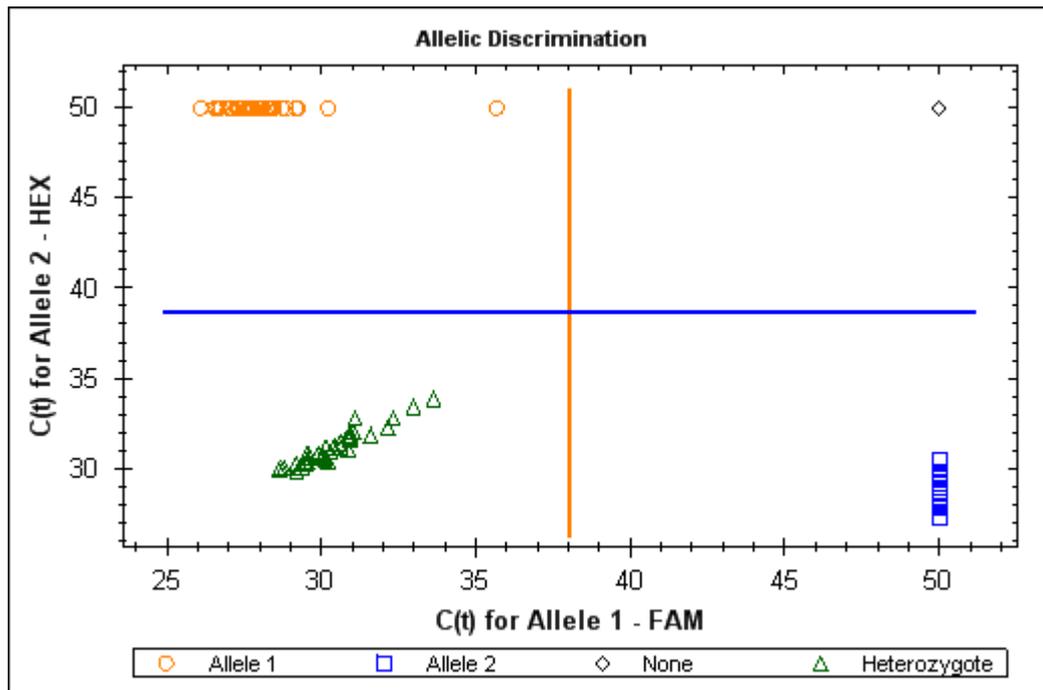


Figure 4.2 Genotype groupings of probe RS1801131 using the 96 well HRC panel

These corresponded to our candidate genotypes, homozygote for one allele, homozygote for the alternative allele or heterozygous. In this case the groupings were discrete with tight error bars, **Figure 4.3** and genotypes could be called automatically, **Table 4.2**.

	<u>Ct1-Ct2 range limits</u>		
	Heterozygote	Allele 1	Allele 2
Rs1801133	(-5.4)-(-12)	(-22.4)-(-16)	(9.5)-(25)

Table 4.2 Numerical representation of genotype calls for RS 1801131 by calculating Ct1-Ct2

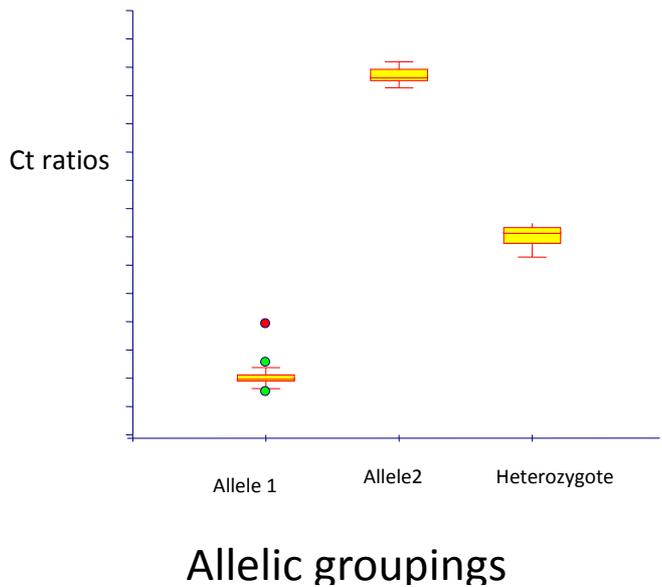


Figure 4.3 Box plot demonstrating discrete groupings of genotypes using ct1-ct2.

Error bars represent 1.5 of the interquartile range.

4.2.4.1 Setting Limits of Detection for SNP RS 1801131 Taqman allele calling.

Tumour DNA was used for the genotype analysis and therefore it was possible that Loss of Heterozygosity may have occurred, giving rise to apparent homozygosity when in fact a heterozygous sample was present. Previous experience in our group with CCRTB breast samples has shown that these samples contained approximately 70% tumour cells. We aimed to be able to detect LOH down to 10% allowing a large overlap and reliability of our results. In practice, normal cells within the tumour sample were expected to give rise to both alleles and only high tumour cellularity (high proportion of tumour cells greater than 90 % would be expected to cause miscalling. Nevertheless, we determined the limits of detection for each allele by mixing DNA from opposite homozygous samples in different ratios.

Using the results from the HRC panel samples, a homozygote for each allele was mixed in the following ratios - 0:1, 0.05 to 0.95, 0.1 to 0.9, 0.5 to 0.5, 0.9 to 0.1, 0.95 to 0.05 and 1:0 to cover loss of either allele. Standard reactions were performed for each mixed sample as

described above section 2.5 and the Ct values for each allele per mixture plotted as above, see **Figure 4.4**.

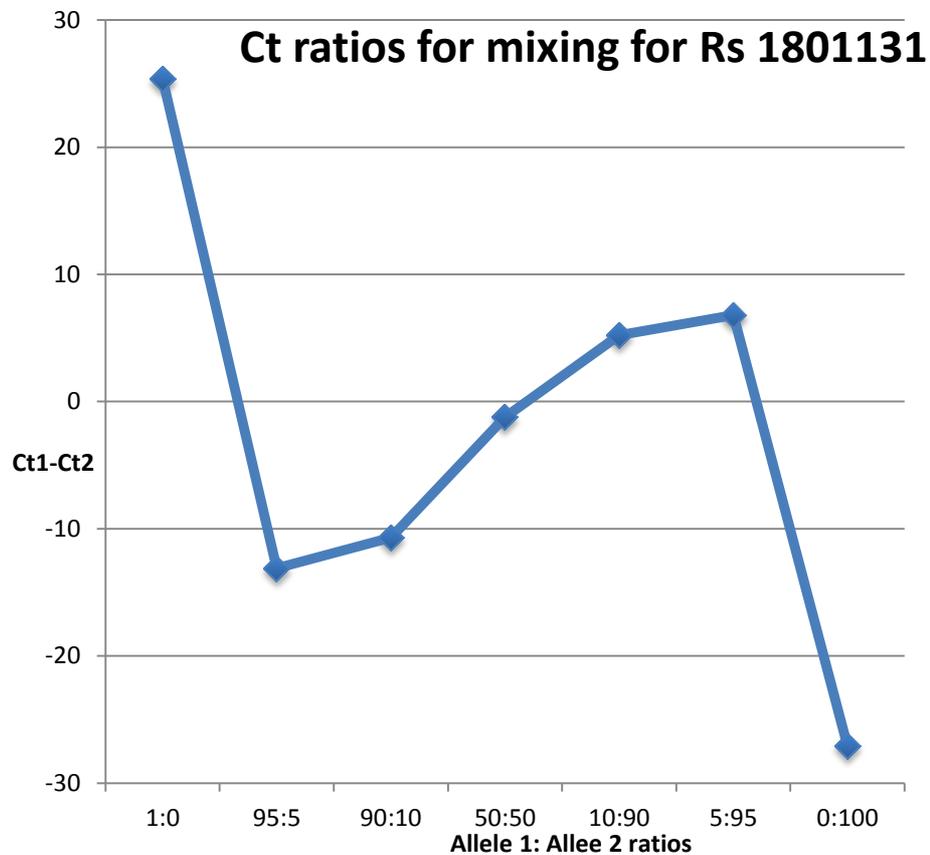


Figure 4.4 Ct1-Ct2 for controlled proportions of allele1:allele2.

These experiments were performed in duplicate. Statistical normality was confirmed and using 2 tailed T-test we were able to identify a heterozygote at the 95:5 mix for allele 1 ($p < 0.00$) and the 90:10 mix for allele 2 ($p < 0.00$). Positive controls were therefore used at the 10% level for both alleles.

To make the controls the volume required of each homozygote was 200 μ l. From the 200 μ l stock of each homozygote the following action is taken:

- 80 μ l of each is stored as aliquots of 10 μ l for the 100% Allele1/2 controls
- 40 μ l of each is combined and stored as aliquots of 10 μ l for the 50% Allele 1/50% Allele 2 controls

- 72µl of Allele 1 is combined with 8µl of Allele 2 and stored as 10µl aliquots for the 90% Allele 1/10% Allele 2 controls and vice versa

These 10µl aliquots were then stored at -80°C at a DNA concentration of 10ng/µl in 0.5ml eppendorf tubes at -80°C for single use in SNP assays. One Real Time-PCR run required 4µl of each control (2x2µl) per plate.

Storage of samples at -80°C in this format allows future easier PCR plate preparation.

In this case it can be seen that although groupings in Figure 4.3 correspond to a narrow ct ratio band for heterozygotes after the mixing experiments values corresponding to ratios of -10.4-5.4 could be clearly distinguished giving is confidence that in the likely event of tumour cellularity not exceeding 90%, alleles could still be called correctly.

4.2.4.2 Allele Call validation

Allele calls were made for SNP RS1801131 on the basis of the Taqman assays. We therefore chose selected cases and sequenced their amplified DNA to confirm the presence of the expected bases at mRNA position 1298. Nine samples (3 heterozygote and 3 of each homozygote) were selected and subject to PCR as section 2.5.2. Amplicons were cloned and sequenced by TA cloning (Chapter 2.5.5). Initial sequence analysis identified the target sequence, which was used to design PCR primers, in this case insert primer sequences for nested PCR, suitable for base calling. Each allele was found to be present as expected,

Figure 4.5

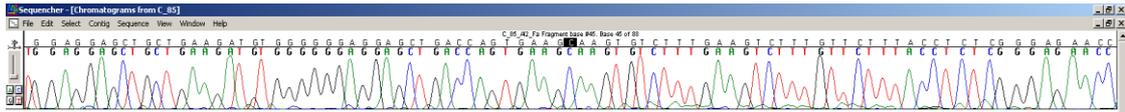


Figure 4.5 Sequencing reaction for MTHFR 1298 A>C RS 1801131

4.2.5 Test of Assay on CLL DNA

A real time PCR reaction was performed on DNA extracted from venous blood from a selection of patients with chronic lymphocytic leukaemia (CLL). This ensured that the assay was reliable on extracted DNA. The assay for SNP Rs 1801131 was successfully run on this DNA as shown in **figure 4.6** demonstrating the results of one SNP.

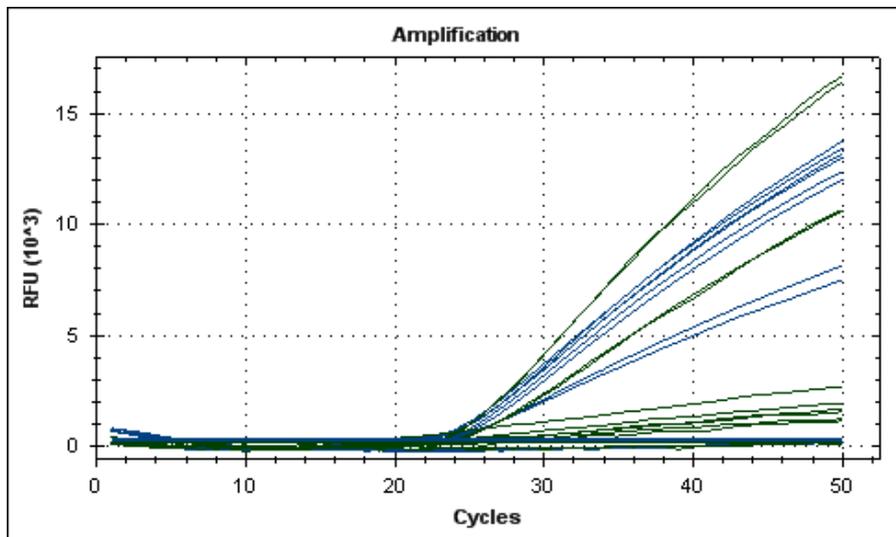


Figure 4.6 Amplification curves of DNA extracted from CLL samples for SNP RS 1801131

SNP panel analysis

The analysis performed above for SNP RS1801131 was repeated for the remainder of our SNP panel. For all remaining SNPs except Rs 1801270 and Rs 4802101, heterozygotes and 2 homozygotes were successfully amplified, cloned, sequenced and tested on CLL DNA. For these exceptions where a homozygote for one of the alleles was not identified from the HRC panel further work was performed in order to set limits for heterozygote limits.

4.2.6 Setting allele call detection limits in the presence of only one variant of homozygote

Three heterozygotes for Rs 1801270 and Rs 4802101 were selected from the HRC panel.

For these samples their PCR product was used in a TA cloning assay as described above (section 2.5.5).

We have previously used 20ng of genomic DNA in all our real time-PCR assays. This cloned PCR product is concentrated compared to the genomic DNA making it incomparable on real time PCR runs. To overcome this, serial dilutions of cloned product were made and run alongside known genomic concentrations to enable optimal real-time PCR curves to be obtained and subsequent optimal concentrations defined to create positive controls.

Probe	Optimal Concentration Allele 1 (ng/ μ l)	Optimal Concentration Allele 2 (ng/ μ l)
GSTP1 Rs1138272	1x10 ⁻⁵	1x10 ⁻⁵
CYP2B6 Rs4802101	1x0 ⁻⁴	1x10 ⁻⁵

Table 4.3 Optimal DNA concentrations for cloned PCR products

The appropriate mixes of homozygotes were made up for each probe as described in section 4.2.4.1 to provide positive controls.

4.5 Summary Results for SNP assay optimisation.

Using the above optimization experiments it was possible to define limits for homozygote and heterozygote calls for each SNP. **Table 4.4** summarises the results for each SNP.

SNP	Ct1-Ct2 range limits				Statistical Analysis (Two tailed t- Test) obtained during assay development			
	Heterozygote**	Allele 1***	Allele 2***	Heterozygote limits from assay development**	Significance of 5% Allele 2/95% Allele 1*	Significance of 5% Allele 1/95% Allele 2	Significance of 10% Allele 2/90% Allele 1	Significance of 10% Allele 1/90% Allele 2
CDKN1A (3'UTR) rs1059234	(-6.1)-(-3.82)	(-24.97)-(-20)	(18.38)-(20.28)	(-11.4)-(4.6)	0.000	0.000	N/A	N/A
BAX rs4645878	(1.27)-(11.17)	(-22.65)-(-19.2)	(16.70)-(-25.6)	(-2.8)-(11.2)	0.000	0.000	N/A	N/A
BCL2 rs2279115	(-12.4)-(-4.5)	(-22)-(-17.2)	(13.4)-(20.1)	(-10.1)-(8.23)	0.045	0.161	N/A	0.020
GSTP1 rs1695	(0.6)-(2.34)	(-22.3)-(-18.9)	(21.4)-(23.7)	(-2.4)-(13.2)	0.000	0.003	N/A	N/A
ABCB1 rs1045642	(-0.9)-(-0.24)	(-23.7)-(-22.5)	(22.4)-(24.8)	(-4.3)-(9.5)	0.000	0.000	N/A	N/A
MTHFR Rs1801133	(-5.4)-(-12)	(-22.4)-(-16)	(9.5)-(25)	(-16.4)-(7.5)	0.051	0.091	0.015	0.006
MTHFR Rs1801131	(-4.8)-(0)	(-23.9)-(-14.3)	(19.1)-(22.8)	(-10.4)-(5.4)	0.072	0.010	0.022	N/A
CYP2B6 rs4803419	(-6.4)-(4.9)	(-24)-(-22.5)	(19.5)-(26.6)	(-15.2)-(6.7)	0.006	0.029	N/A	N/A
P53 rs1042522	(-2.7)-(5.7)	(-26.5)-(-20.8)	(18.9)-(26.6)	(-8.1)-(6.7)	0.017	0.045	N/A	N/A
Her2 rs1058808	(-9.6)-(-6)	(-23.1)-(-20)	(15.1)-(18.4)	(11.9)-(-15)	0.042	0.033	N/A	N/A
CDKN1A rs1801270	(-8)-4.3	(-25.2)-(-8)	10.1-15	(-5.9)-(13.3)	N/A	N/A	0.018	0.004
GSTP1 rs1138272	(-2)-4.8	(-18)-(-10.8)	20.7-26	(-4.5)-4.8	N/A	N/A	0.001 (cloned)	0.003 (cloned)
CYP2B6 rs4802101	(-1.2)-12	(-14)-(-17.6)	24-26	0.65-12.5	N/A	N/A	0.000 (cloned)	0.004 (cloned)
Her2 rs1136201	(-1.6)-3.1	(-18.8)-(-14.5)	15.1-19.2	(-5.7)-8.5	0.001	0.028	N/A	N/A
Topo2a rs6175634 2	(-3.2)-(-2.5)	(-20.7)-(-16.1)	18-18.2	(-7.8)-5.6	N/A	N/A	0.015	0.001
Cyclin D1 rs9344	(-0.3)-1.1	(-16.1)-(-2)	10.7-17.3	(-13.2)-1.1	0.059	0.001	0.009	N/A
SPP1 rs1173058 2	(-4.8)-(-1.5)	(-20.5)-(-17.1)	13.4-19.8	(-11.1)-7.7	0.000	0.001	N/A	N/A

SPP1 rs2835709 4	(-4.8)-0.15	(- 17.9)- (-9)	13.4- 17.4	(-13.6)-3.4	0.019	0.000	N/A	N/A
XBP1 rs2269577	(-5.1)-(-0.5)	(- 18.3)- (-9.4)	8.4- 17.6	(-6.4)-5.9	0.097	0.000	0.000	N/A
Cyclin D1 rs678653	(-0.7)-0.94	(- 14.8)- (-3.3)	9.9-15	(-11.2)-4.7	0.108	0.002	0.013	N/A

Table 4.4 Results from optimisation of assays

*A statistically significant result (i.e. recognition of the presence of a minor allele within a mix at the 5% and/or 10% level) is an indication of the assays sensitivity for the allele. The 10% level was tested if the 5% level was unsuccessful.

** Inclusion of Allele 1 and Allele 2 controls mixed in varying proportions e.g. 95%:5% allows assessment of the sensitivity of individual PCR experiments and therefore assessment of the sensitivity of detection of potential heterozygotes in individual clinical samples. These samples could show LOH and thus have uneven allelic profiles, differing from the 50% Allele 1 and 50% Allele 2 heterozygote that were identified through the HRC1 panel. This analysis enables us to widen the heterozygote Ct range limits to include those showing only a minor second allele presence.

*** Ratios developed using analysis of the HRC1 panel (normal DNA)

5. Chapter 5 – Association of SNP Variants with Clinical Outcome

Our central hypothesis that chemotherapy response would be affected by genetic variants was tested for our breast cancer series (see chapter 2 section 2.2) in a pathway-focused approach using the panel of variants expected to be associated with clinical outcomes (Chapter 1) and the corresponding standard SNP assays developed in Chapter 4 above. We compared the allele frequencies in our selected cohort of chemotherapy treated patients (discussed in chapter 3) for associations with known prognostic factors (as described in chapter 1) as well as patient outcome. The rationale for choosing individual SNPs has been discussed in chapter 1.

5.1 Apoptotic pathway SNPs

5.1.1 Allele Frequency

We assessed SNPs in four genes within the TP53 dependent apoptotic pathway. **Table 5.1** summarises the assay success and allele frequencies for each SNP. Allele calls for 100% of the cases was not achieved for any of the assays. DNA from paraffin blocks in particular was inefficient being unsuccessful for 5% of SNPs from 87 samples. All of the samples from the cohort gave a reliable call for at least one of the SNPs within our panel. Duplicate assays were performed for each sample. Where an allele was not scored, a further assay with double concentration of template DNA was attempted.

SNP	Number genotyped (%)	Allele 1 homozygote (%)	Allele 2 homozygote (%)	Heterozygote (%)	Major allele (%)	Hardy Weinberg X2 value
TP53 codon 72 (RS 1042522)	296 (98)	CC 11 (4)	GG 207 (70)	CG 78 (26)	G (83)	0.917
MDM2, inton-309 (RS 2279744)	280 (92)	GG 29 (11)	TT 138 (49)	GT 113 (40)	TC (69)	0.783
BCL2 - 938 C>A (Rs 2279115)	297 (98)	AA 88 (30)	CC 56 (19)	AC 153 (51)	A (55)	2.287
BAX -248 G>A (Rs 4645878)	290 (96)	AA 6 (2)	GG 222 (77)	AG 62 (21)	G (87)	0.624

Table 5.1 Summary of assay success and allele frequency in apoptotic pathway SNPs using 303 breast cancer cases

Hardy Weinberg- equilibrium was reached for all SNPs. Allele frequencies for the population data from dbSNP and previously published allele frequency studies was compared to the results with our cohort, **table 5.2**. We compared the allele frequency similarities with other cohorts to give us guidance on whether our findings would be applicable to other populations. The p53 SNP studied has a 'G' as the major allele in all groups studied with the exception of a Japanese study[226], in keeping with our finding.

However the proportion of major allele differed significantly from our population; our cohort had a significantly higher percentage major allele than 3 healthy populations and Chinese, Finnish and Brazilian breast cancer populations. However there was no difference when compared with an Italian series of unselected breast cancers. A recently published meta-analysis suggests that this particular SNP is not associated with breast cancer risk[641] and the wide variation in this SNP between ethnicities is well documented [642-645] . It is perhaps not surprising then that, although we can be reassured by having the same major allele, the proportions are so variable amongst various populations. It is interesting to note the population most similar to ours, i.e. European breast cancers have a similar genotypic profile.

The allele distribution for MDM2 -309 is more variable with 4 of the 7 studies analysed having a T major allele, in keeping with our findings, and the remainder a G major allele. For those with the same major allele as our breast cancer cohort there was no significant difference in the proportion in 2 healthy populations and a Scottish series of unselected breast cancers. There was a significantly lower proportion of the T allele than a study of African /American breast cancer patients. Again, this wide variation of allele frequency amongst different ethnicities is well documented[646]. This most recent meta-analysis suggests a link between the GG and TG variants and increased cancer risk, which is more significant in Asian populations than European.

BCL2 -938 allele frequency has less variation with the major allele each time being between 53-56 %. Our population is comparable with the HRC panel chosen (see chapter 2) and both the healthy and breast cancer-affected group of a Caucasian study. We can therefore be somewhat confident that our population represents a typical European breast cancer cohort for this particular gene.

Although BAX -248 G>A has not previously been studied in a breast cancer population we have been able to compare to 6 reportedly healthy populations. There is no variation in the major allele (G), however, the proportions vary somewhat with the HRC panel, UK and American populations differing significantly,

SNP	Source	Population	Cohort (N)	Major Allele (%)	P value*
P53 codon 72		HRC panel	Healthy (94)	G (72)	0.00
	dbSNP	Global	Healthy (176)	G (65)	0.00
	dbSNP	European	Healthy (120)	G (77)	0.05
	Xu[220]	Chinese	Primary Breast Cancer (401)	G (51)	0.00
	Tommiska [221]	Finnish	Unselected breast cancers (858)	G (73)	0.00
	Vannini [222]	Italian	Advanced breast cancer (40)	G (78)	0.23
	Vieira [224]	Brazilian	Unselected breast cancer (113)	G (76)	0.03
	Xu[225]	Chinese	Operable breast cancer receiving neo-adjuvant chemotherapy (110)	G (54)	0.00
	Toyama[226]	Japanese	Primary breast cancer (557)	C (63)	n/a
MDM2, intron -309		HRC panel	Healthy (94)	T (69)	0.95
	dbSNP	Global	Healthy (72)	T (76)	0.11
	dbSNP	Chinese/Japanese	Healthy (88)	G (56)	N/A
	Sun [253]	Taiwanese	Unselected breast cancers (124)	G (53)	N/A
	Paulin [254]	Scottish	Unselected breast cancers (299)	T (64)	0.08
	Toyama [226]	Japanese	Unselected breast cancers (557)	G (57)	N/A
	Boersma [250]	African/American and Caucasian	Unselected breast cancers (290)	T (78)	0.00
BCL2 -938 C>A		HRC panel	Healthy (94)	A (54)	0.85
	dbSNP	Global	Healthy (150)	C (54)	N/A
	Bachmann [276]	Caucasian (German descent)	Healthy (120)	A (56)	0.84
	Bachmann [276]	Caucasian (German descent)	Unilateral invasive breast cancer (274)	A (53)	0.47
BAX -248 G>A		HRC panel	Healthy (94)	G (81)	0.04
	dbSNP	European	Healthy population (226)	G (87)	0.95
	Starczynski [291]	UK	Healthy population (135)	G (93)	0.01
	Nuckel[647]	German	Healthy population (95)	G (91)	0.14
	Skogsberg[648]	Scandinavian	Healthy population (207)	G (88)	0.69
	Chen [280]	Texas	Healthy population (934)	G (80)	0.00

Table 5.2 Apoptotic pathway SNP frequencies in our chemotherapy treated breast cancer cohort compared with published frequencies

*p value represents a t-test comparing with major allele of our chemotherapy cohort.

5.1.2 Association of Apoptotic pathway SNPs and Clinicopathological features

Each apoptotic pathway SNP allele frequency was compared with all of the clinicopathological features previously described. The statistically significant findings are summarized in **table 5.3**. TP53 codon 72 was noted to be highly associated with NPI prognostic grouping, with heterozygosity corresponding to the favourable outcome group. Many other studies have been unable to find associations with clinicopathological features and TP53 codon 72 genotype [220, 221, 224-226], however none of these studies evaluated the NPI grouping. As discussed in chapter 1 the combination of grade, size and nodal status performs better as a prognostic tool than the individual features on their own. In our group we had smaller numbers of patients in good prognostic groups as this is a chemotherapy treated cohort and chemotherapy is reserved for poorer prognostic tumours so this finding needs to be interpreted with caution. It could be hypothesized that the presence of both alleles is protective for risk of cancers predisposed for a poorer outcome.

The BAX genotype was associated with patient age and therefore also to menopausal status as the majority of older ladies will be postmenopausal. Heterozygotes were more likely to be under 50 and pre-menopausal. Although there are no published studies of BAX -348 G>A and breast cancer, given that the allele distribution is so variable amongst other published cohorts and our control population of the HRC DNA panel our results cannot be used to comment on association with breast cancer risk (**table 5.2**). However, it may be that heterozygotes are at risk of developing their cancer at an earlier age. As breast cancer can be detected by screening then further study is warranted. If found to be true, then there may be a role for heterozygotes to commence screening early; particularly with the increasing use of MRI for screening in high risk pre-menopausal women.

There were no associations found between the BCL2 -938 C>A genotype and any clinicopathological features.

SNP	Variable		Genotype count (%)			P
TP53 codon 72			CC	GC	GG	
	NPI prognostic group	Excellent	1 (100)	0 (0)	0 (0)	0.000003
		Good	0 (0)	5 (71)	2 (29)	
		Moderate	8 (5)	39 (26)	102 (68)	
		Poor	2 (2)	32 (24)	98 (74)	
Bax			AA	GA	GG	
	Age	<50	4 (3)	43 (29)	103 (69)	0.005
		>50	2 (1)	19 (14)	119 (85)	
	Menopausal Status	Peri- menopausal	0 (0)	0 (0)	8 (100)	0.006
		Post- menopausal	2 (1)	22 (15)	119 (83)	
		Pre- menopausal	4 (4)	36 (32)	73 (65)	

Table 5.3 Summary of significant clinicopathological and apoptotic SNP associations

5.1.4 Apoptotic pathway SNPs and outcome

5.1.4.1 TP53 codon 72 and outcome

Outcome was associated to TP53 genotype. On initial analysis, cases having the GG variant had on average the worst DFS ($p = 0.03$) but not OS ($p=0.13$). OS is not restricted to cancer specific deaths, reducing the power of the study to test significance. DFS may be more useful for this group. The Kaplan Meier curves for CC and GC variants of the codon 72 genotype were very similar and so they were combined for comparison to GG genotype as shown in **Figure 5.1** for OS and DFS with the data summarised in **table 5.4**

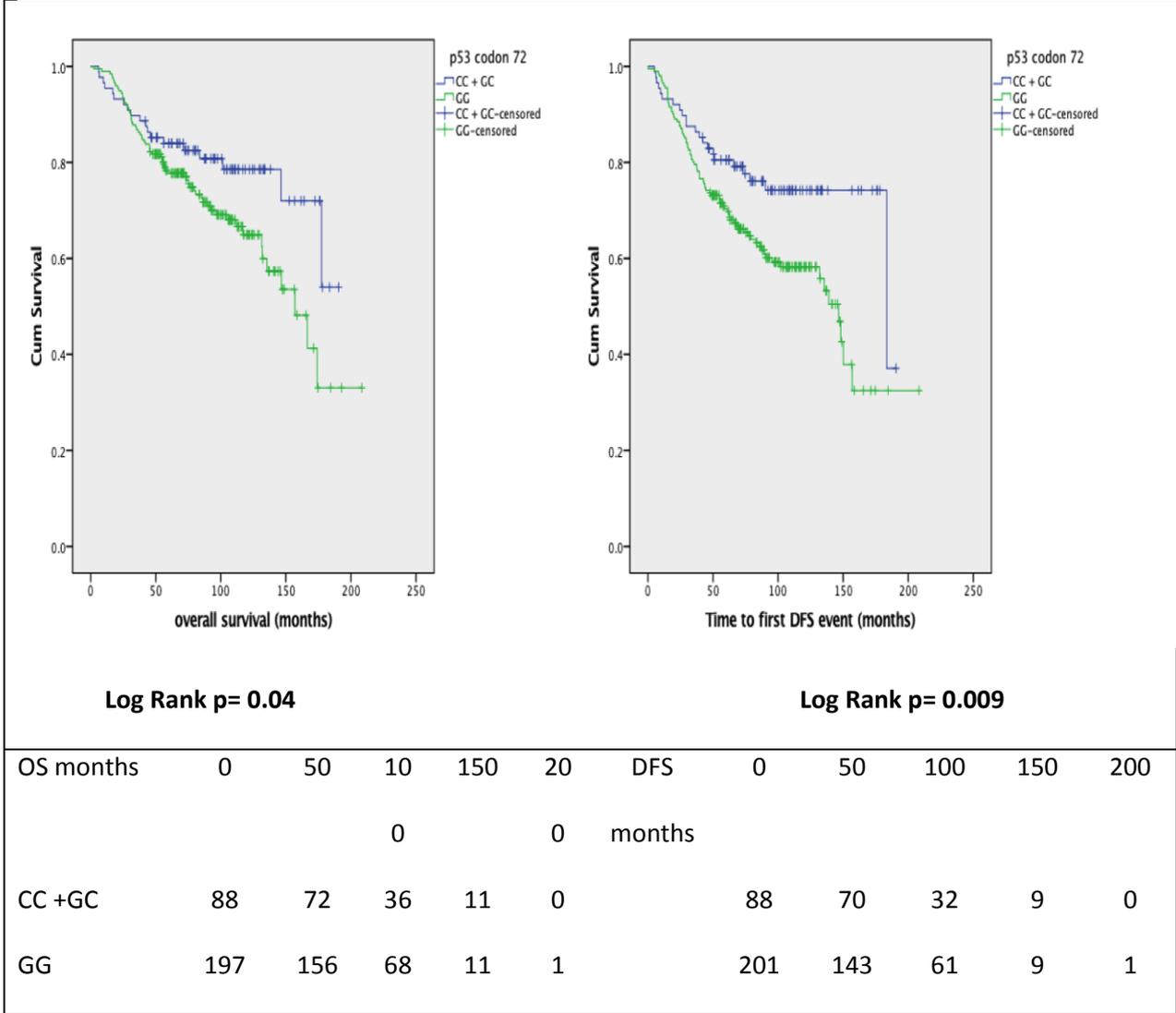


Figure 5.1 Kaplan Meier curves demonstrating the OS and DFS for TP53 codon 72 genotype variations comparing homozygotes for GG to other variants with numbers at risk tabulated below

	CC	GG	GC	Log rank Mantel Cox p value
5 year OS (%)	91	78	83	0.13
10 year OS (%)	-	65	79	
Median OS (months)	n/a	156.9	n/a	
5 year DFS (%)	91	70	79	0.03
10 year DFS (%)	-	58	74	
Median DFS (months)	n/a	146.5	183.4	

Table 5.4 Summary of OS and DFS according to TP53 codon 72 genotype

The GG variant (Arg/Arg) of TP53 codon 72 variant has significantly worse OS and DFS than other variants combined. As previously discussed the GG genotype has a greater apoptotic potential both in the presence and absence of chemotherapies [211-213, 215, 649]. This may be influenced by the presence of the SNP in sequence coding for a proline-rich domain that is important for the apoptotic function of TP53[220]. Alternatively, the significance may be related to enhanced mitochondrial localisation of Arg/Arg variant TP53 compared to the CC (Pro/Pro) variant[649]. Previous studies have reported that the CC variant has a poorer outcome in breast cancer, fitting with the theory that GG is more sensitive to apoptosis. However, we have demonstrated that in this select group of breast cancers, the GG variant has a worse prognosis. It is not immediately obvious why this may be the case particularly as our patients all received chemotherapy and the point is explored further in 5.5 SNPs in Metastor Genes.

We next looked at genetic variation and specific chemotherapy regimes used to see if we could gain extra information on individualised response to treatment. As previously discussed (Chapter 3.1) for analysis we have narrowed our chemotherapy regimes down to those containing anthracycline or taxane. Continuing the comparison of GG variation against other variants, there was no difference in OS between genotype groups when stratified to receiving anthracyclines (log rank p=0.13) or not (log rank p=0.2). There was a non-significant trend for GG variants receiving anthracyclines to have a poorer

outcome, which was not apparent in those who did not receive them and this became slightly more apparent when DFS was analysed, in keeping with results for the complete group (**figure 5.2**).

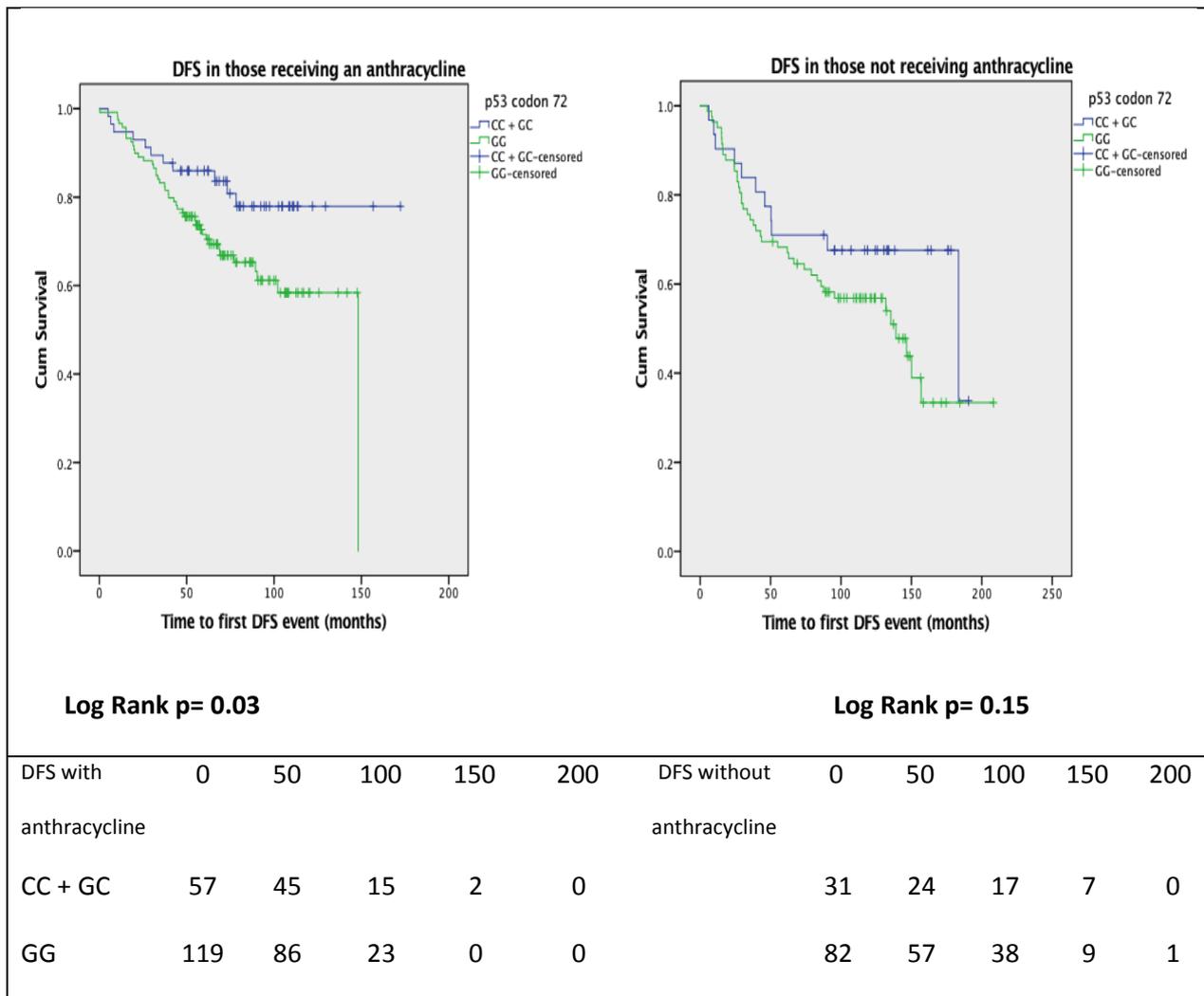


Figure 5.2 DFS for p53 codon 72 variants stratified according to anthracycline receipt with numbers at risk tabulated below

As there was no relationship between chemotherapy received and codon72 variants for the group as a whole and the GG variants is associated with a poorer outcome it would appear that receiving an anthracycline may not improve outcome for these patients. It is difficult to be anything other than speculavie here however, as the statistical difference is small and due to the size of the cohort it is not possible to standarise for other influences such as NPI. Although the number of patients in our cohort

receiving taxane based therapy is small, it was still possible to stratify according to whether a taxane was received; GG variants had the better outcome when a taxane was given (OS no taxane log rank p= 0.02 vs. taxane log rank p=0.27 and DFS no taxane p=0.01 vs. taxane log rank p=0.6) **figure 5.3**. Perhaps cells harbouring the GG variant genotype are particularly sensitive to taxanes.

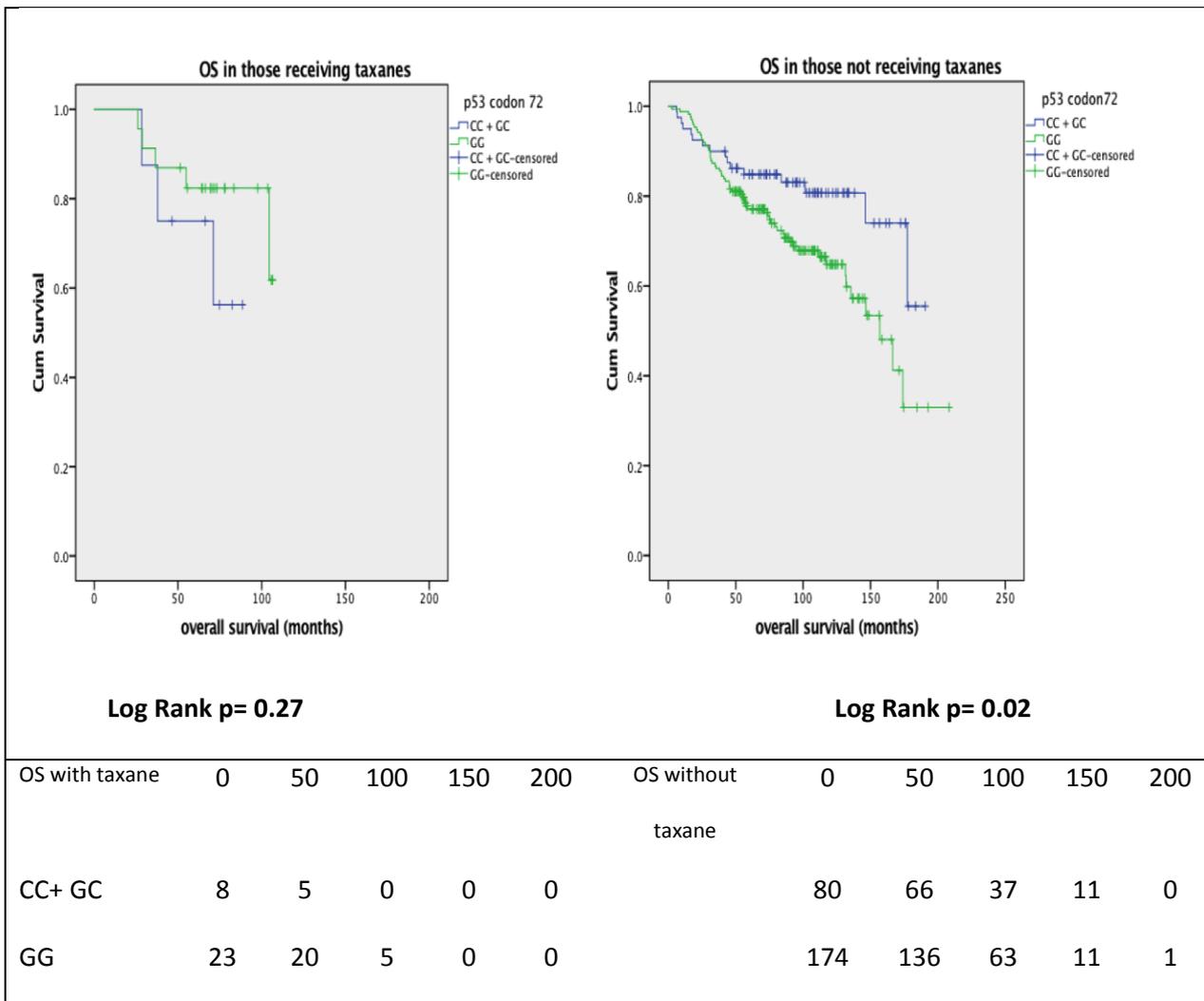


Figure 5.3 OS for p53 codon 72 variants stratified according to receipt of taxanes with numbers at risk tabulated below

There have been two previous studies suggesting that cases homozygous for the C allele may be less sensitive to anthracycline containing chemotherapy regimes [220, 225] yet our data found the opposite. There was no real difference in trends whether anthracycline was received or not.

We have found that if taxane therapy was given then the apparent separations in the groups disappear. Here, having a GG polymorphism does not correlate with worse outcome. Of course the numbers receiving a taxane are small so this should be interpreted with caution. No previous studies report on TP53 codon 72-genotype and adjuvant taxane responsiveness in breast cancer so further work is required to investigate these findings. It is generally believed that taxanes induce apoptosis via a TP53 independent pathway so our association is interesting, although TP53 status may also influence cell- cycle progression.

Given that our findings were opposite to previous studies in a number of cases described above, we did go back to our original assay validation and repeated sequencing on a number of samples, which confirmed that we had made our allele calls correctly.

5.1.4.2 MDM2, intron -309 and outcome

There were no associations between MDM2 intron -309 variants and OS or DFS despite the T allele being correlated with T stage. As previously discussed T stage on its own is prognostic in our cohort but the association with this allele was not strong enough to predict survival. There remained no associations when cases were stratified according to chemotherapy regime received. Although the GG genotype has been associated with poor prognosis in a Scottish breast cancer population, OS and DFS were not analysed in this study [254]; the GG variant was associated with higher-grade tumours, node positivity and higher NPI scores. Although not known to be a risk factor for breast cancer, it would seem that MDM2 intron-309 variants may influence traditional pathological prognostic markers although to date there is no suggestion that the relationship is powerful enough to influence outcome or response to chemotherapy.

5.1.4.3 BCL2 -938 C>A and outcome

When BCL2 -938 variants were analysed for association with survival there was no significant difference (OS log rank $p=0.12$, DFS log rank $p=0.2$). There was a trend for the AA variant to have poorer outcomes compared to AC and CC combined, the latter having shown similar survival curves.

The AA genotype was associated with a worse OS compared to other genotypes (log rank $p=0.04$) and a non-significant poorer DFS (log rank 0.08), **figure 5.4**.

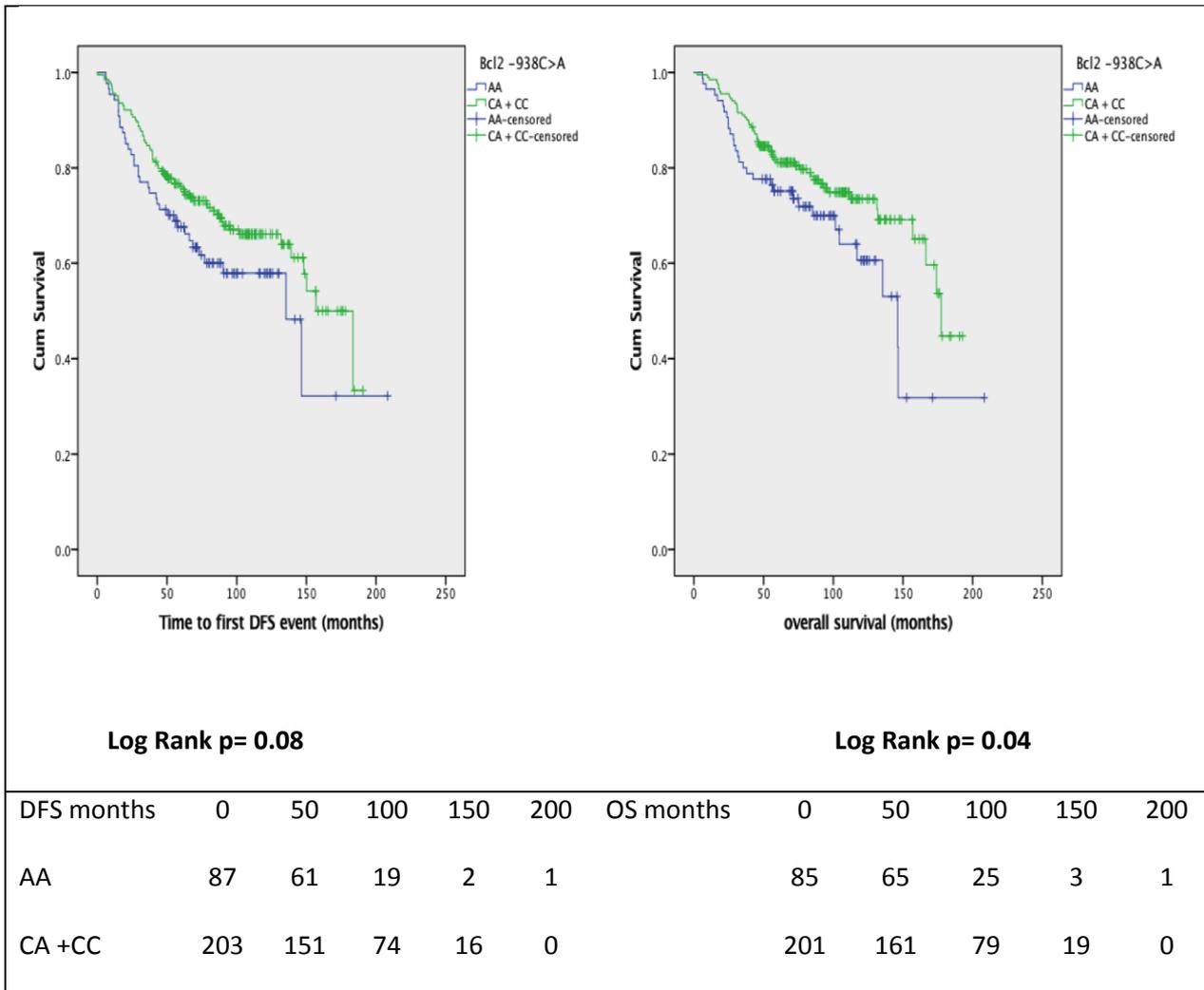


Figure 5.4 DFS and OS of BCL2-938 AA variant compared to CA and CC with numbers at risk tabulated below

Whether anthracycline treatment was given or not did not affect the survival curves for cases with the AA variant continuing to perform worst, although the differences were statistically non significant, perhaps due to smaller numbers making the analysis less powerful (No anthracycline OS log rank $p=0.12$, anthracycline $p= 0.17$). The same trend is seen when stratifying for taxane therapy compared to when no chemotherapy treatment is given. The cases with AA variants have a non-significant poorer

outcome (OS log rank $p = 0.33$, DFS log rank $p = 0.37$). However, when taxane therapy is given those with a C allele have significantly improved outcome, even for breast cancer specific DFS (OS log rank $p = 0.001$, DFS log rank $p = 0.03$), **figure 5.5**.

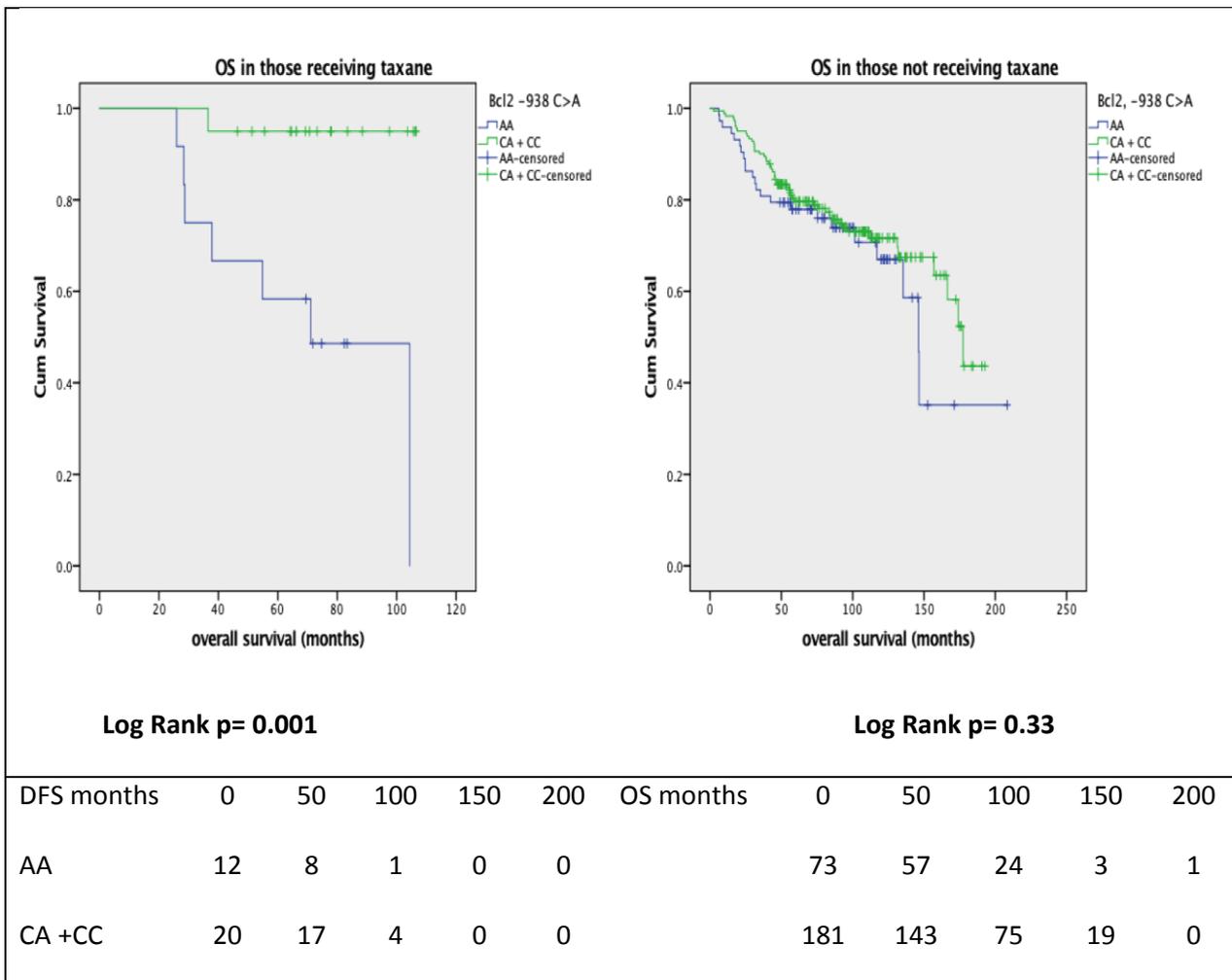


Figure 5.5 OS for BCL2 -938 variants stratified according to taxane therapy with numbers at risk tabulated below

As there were no differences in the taxane versus no taxane groups on analysis of the clinicopathological data, this may suggest that the presence of a C allele improves the responsiveness to taxane therapy.

Bcl-2's role in breast cancer may be tissue specific and in breast cancer an increased expression of the protein has been linked with a favourable outcome [650, 651]. The SNP we have studied is located in the inhibitory P2 promoter and the presence of a C allele increases P2 promoter activity and binding of nuclear protein [273, 274]. As the P2 promoter is suppressive this increased activity decreases the expression of the BCL-2 protein; The C allele therefore was first demonstrated to decrease expression whilst the A allele increased expression. As increased expression has been shown to be favourable in breast cancer we hypothesised that a homozygous A genotype would be associated with improved survival. The opposite was found.

Bachmann et al [276] are the only group to have published on BCL-2 polymorphism and protein expression in breast cancer. They were unable to demonstrate an association between BCL-2 genotype and protein expression levels as determined by IHC in their cohort of 274 cases of unilateral invasive breast cancer. They did not find any association between protein expression and survival in the group as a whole. However, they did show that whilst not significant in node negative breast cancer patients, a high BCL-2 protein expression was independently prognostic in lymph node positive patients.

Confusingly, the genotype in this lymph node positive group did not correlate with survival but it did in the lymph node negative group where those with a CC genotype fared worse.

In further analysis of our cohort there was no correlation with genotype and survival in the lymph node negative group (log rank 0.34) and only a trend towards homozygous A cases having poorer survival in the lymph node positive group (log rank 0.08).

It is therefore difficult to draw conclusions from these two seemingly opposing findings. However it must be remembered that they are potentially different populations as 51% of the Bachmann population was lymph node negative compared to only 31% of ours. It would be interesting to examine the BCL-2 expression levels in our data set and see if there is any correlation between expression and genotype in a chemotherapy treated cohort.

It may be that our population has received chemotherapy, which is influencing our outcomes. BCL-2 has previously been shown to be down regulated in paclitaxel resistant cells. BCL-2 can be phosphorylated at serine residues and this phosphorylation is associated with a loss of function[652]. The treatment of some prostate cancer cells with taxanes has induced BCL-2 phosphorylation and apoptosis whilst BCL-2 negative prostate cancer cells treated with taxane do not undergo apoptosis [105, 653]. That is, in hormone refractory prostate cancer, BCL-2 overexpression in cells appears to be related to increased sensitivity of taxane-induced apoptosis.

BCL-2 provides an additional target to taxane therapy separate to its anti microtubule effects. It is an important component of the mitochondrial targets of taxanes and one hypothesis is that taxanes bind directly to BCL-2 thereby blocking its anti-apoptotic function [654]. Taxane resistant cell lines display consistent down regulation of BCL-2. Although controversial, it has also been suggested that BCL-2 overexpression can prevent chemotherapy induced apoptosis and contribute to drug resistance[654]. This would certainly be in keeping with our findings of the homozygous A genotype, perhaps associated with over expression, being associated with taxane resistance.

BCL-2 overexpression has been associated with chemoresistance [655, 656] and if our findings can be repeated and shown to be related to protein expression then it leaves scope for the use of novel therapies in breast cancer patients. BCL-2 protein inhibitors have been developed. BCL-2 antisense (oblimersen sodium) was the first to clinical trials and results show chemo-sensitising effects when combined with conventional chemotherapy drugs in CLL translating to improved survival[655]. There has also been promise in breast cancer[657].

The literature suggests that Bcl-2 overexpression induces chemoresistance in breast cancer cell lines. However, high levels of Bcl-2 have been associated with favourable prognostic factors, suggesting that Bcl-2 may not be an appropriate target in breast cancer. However, given the now common use of taxanes in breast cancer chemotherapy regimes, there is certainly need for further study into BCL-2 and taxane response and the influence SNPs may play in selection for treatment.

5.1.4.4 BAX -248 G>A and outcome

There was no difference in OS or DFS according to BAX -248 G>A variation. The numbers in the homozygous A group were small and the survival curves for homozygous G and heterozygous GA followed the same path. However, when the data is stratified according to whether or not the patients have received anthracycline-containing regimes the curves start to become divergent. The heterozygotes were more likely to be in the under 50 and pre-menopausal group with a tendency to increased risk of LVI, all poorer prognostic features, but those receiving an anthracycline had the same survival as other variations (**figure 5.6**). However, heterozygotes that did not receive anthracycline therapy had a tendency towards poorer survival. The same was true for DFS (log rank $p=0.09$ and $p=0.02$ respectively).

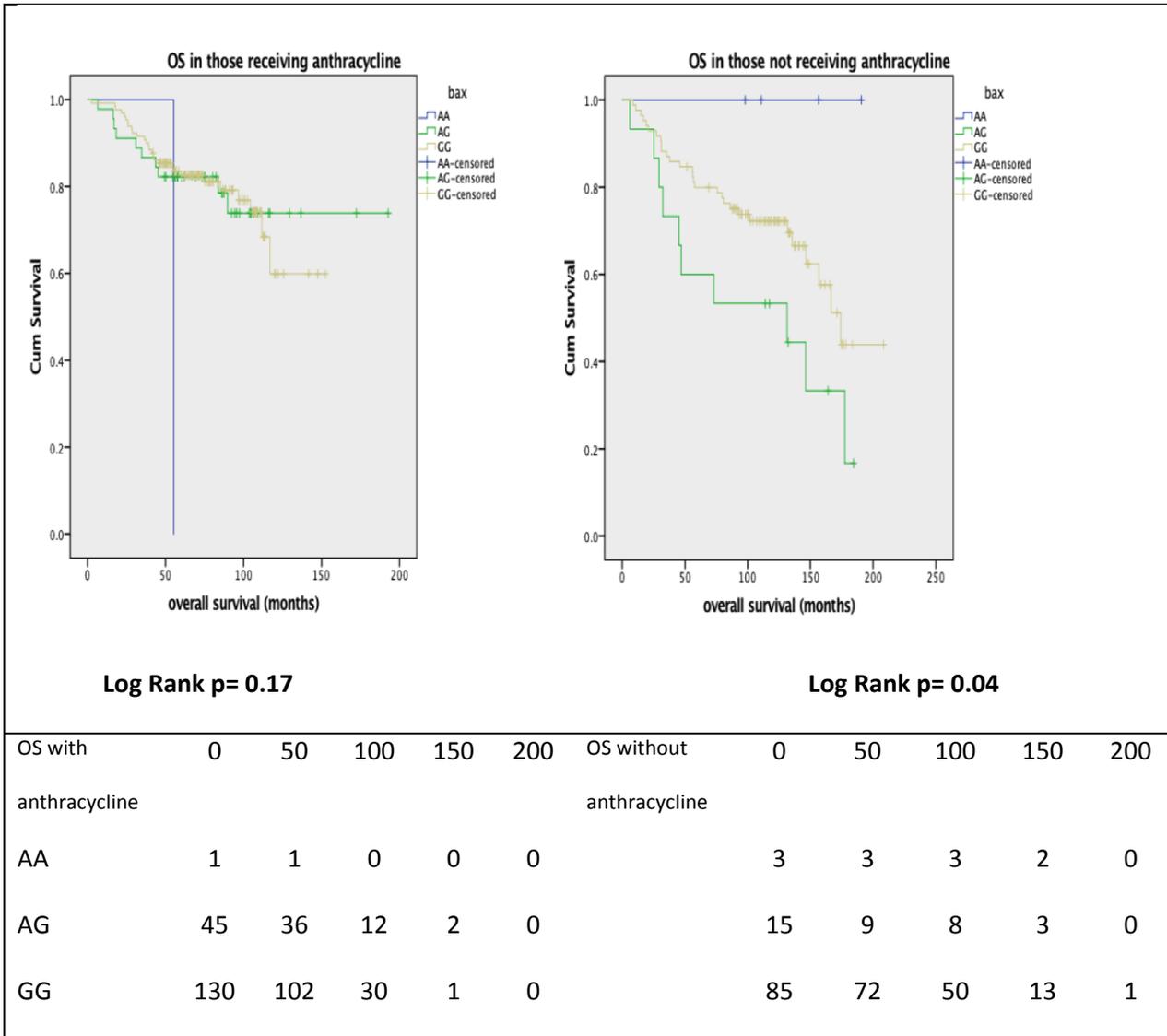


Figure 5.6 OS for BAX -248 variants stratified according to anthracycline therapy with numbers at risk tabulated below

The results are similar for those receiving taxane therapy (**figure 5.7**). Although the numbers receiving taxanes are small (8 AG and 24 GG), heterozygotes have a better DFS when given taxanes. Those not receiving taxanes, whilst not having a significantly worse outcome than other variations, do worse overall. There is no significance with OS (log rank p=0.9 and p=0.23 for taxane versus no taxane, respectively).

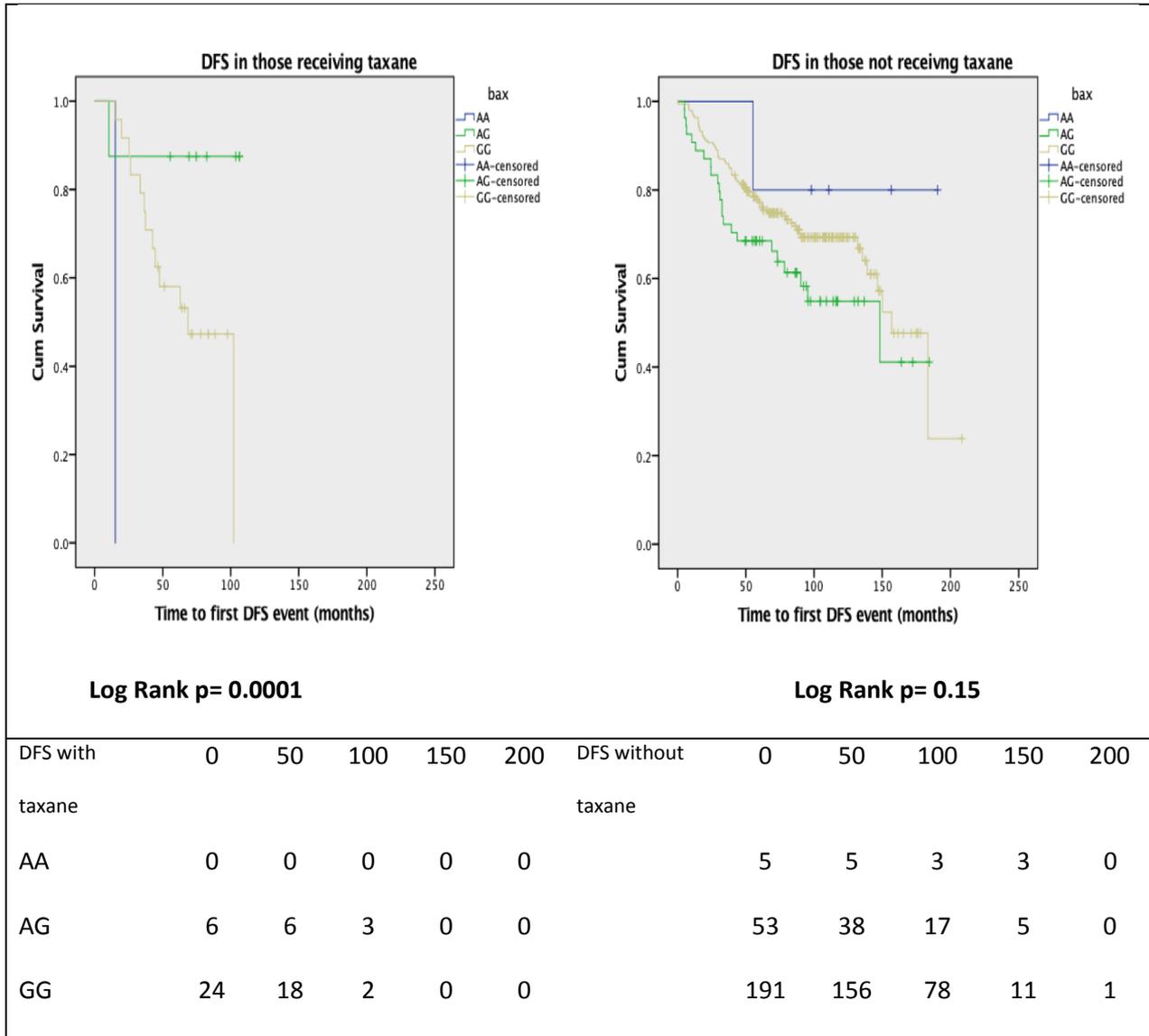


Figure 5.7 DFS for BAX -248 variants stratified according to taxane therapy with numbers at risk tabulated below

Despite being the only variant associated with poorer prognostic factors, the BAX -248 heterozygotes appear to be the most responsive to either taxane or anthracycline chemotherapy.

Genetic variation of this SNP was not associated with OS or DFS despite the clinicopathological associations. This may be because heterozygotes were more likely to receive anthracycline therapy.

This is likely to be related to the fact that they were younger and presumably fitter but it would appear fortunate, as stratifying the data according to therapy received suggests that heterozygotes may be sensitive to chemotherapy. Whilst not significant statistically, when no anthracyclines are given,

heterozygotes lie at the bottom of the KM curve. When anthracyclines are given they have better outcome than other variations. The same is true for taxanes.

The literature suggests that the GA heterozygotes and A homozygotes have a reduced level of BAX expression [290, 291]. These studies have small numbers of cases and Skogsberg *et al* [648] failed to show any association between variant and protein expression. Studies have suggested that this probable reduction in protein expression is due to the allele specific affect on c-Myb binding affecting c-Myb induced transcriptional activation. As BAX is a pro-apoptotic TP53 target gene this reduction is thought to contribute to unresponsiveness of chemotherapy [289]. There is one study on advanced breast cancer where a third of patients had reduced BAX protein levels. This was associated with decreased OS, a faster time to progression and a failure to respond to chemotherapy [269]. Perhaps the GA variant results in transcription dependent and independent factors contributing towards reducing the cellular response to p53 activation; the low BAX protein expression contributing to a failure of mitochondrial disruption.

However, we have shown that the heterozygote variant may have an improved response to chemotherapy, both anthracycline and taxane based. On the assumption that heterozygosity for this BAX SNP is associated with decreased BAX gene expression in breast cancer cells this would appear to be opposite to the published hypothesis. However, a recent study showed that BAX protein expression did not affect survival in breast cancer patients not achieving a complete pathological response to a neo-adjuvant anthracycline based chemotherapy regime [658]. It was not clear from this paper whether BAX protein levels affected the likelihood of response. An absence of the BAX protein has been shown to be associated with improved response to chemotherapy in one breast cancer study[266] whilst another showed no association between BAX protein levels and chemotherapy response [167]. There have been no published studies looking at BAX genetic variation and chemotherapy response in breast cancer.

Further information is needed on the levels of BAX gene expression in breast cancer amongst the variant groups and the affect this has on chemotherapy response, particularly those mediated by apoptosis such as anthracycline.

5.2 Cell Cycle pathway SNPs

5.2.1 Allele Frequency

The SNPs we examined as part of the cell cycle pathway were similarly analysed to the apoptotic pathway SNPs discussed above. Again, we made every effort to genotype every sample. **Table 5.5** summaries the allele frequencies for our cell cycle pathway variations. Genotype frequencies did not deviate from Hardy-Weinberg principles.

SNP	Number genotyped (%)	Allele 1 homozygote (%)	Allele 2 homozygote (%)	Heterozygote (%)	Major allele (%)	Hardy Weinberg X2 value
CDKN1A, 3'UTR (RS 1059234)	302 (99)	CC 251 (83)	TT 0 (0)	CT 51 (17)	C (92)	3.21
CDKN1A, codon 31 (RS 1801270)	289 (95)	CC 262 (90.7)	AA 1 (0.3)	CA 26 (9)	C (95)	0.04
Cyclin D1 870 G>A (RS)	300 (99)	GG 94 (31)	AA 53 (18)	GA 153 (51)	G (57)	1.25

Table 5.5 Allele Frequencies for cell cycle pathway SNPs

The CDKN1A SNPs examined both had dominant major alleles with the 3'UTR SNP having no TT homozygotes found in our sample and the codon 31 SNP only one AA homozygote. This was in keeping with other population frequency data with none of the healthy populations examined also not having any TT homozygotes (**Table 5.6**). There is no frequency data available for other breast cancer cohorts. The frequency comparisons confirm the rare presence of the A allele and AA homozygotes for CDKN1A codon 31. The only other populations that show the AA homozygote are a global healthy dbSNP cohort (with a significantly lower major allele proportion) and an Australian breast cancer cohort. Despite the paucity of the minor allele in healthy populations, the proportion of the major C allele for both the CDKN1A SNPs studied varied significantly from most of the healthy populations with the exception of a European cohort and a Caucasian cohort studied. Given the strong predominance of Caucasians in our cohort this again suggests that our results will be comparable to other Caucasian UK cohorts. The allele frequency for Cyclin D1 SNP 870 G>A is very variable throughout both healthy populations and within breast cancer study cohorts. This shows the high variability of the allele distribution in breast cancer patients. For those cohorts, both healthy and breast cancer patients, with the same major allele as us the proportion was not significantly different.

SNP	Source	Population	Cohort (N)	Major Allele (%)	P value*
CDKN1A 3'UTR		HRC panel	Healthy (94)	C (87)	0.04
	dbSNP	Global	Healthy (180)	C (87)	0.01
	dbSNP	European	Healthy (120)	C (98)	0.00
	dbSNP	European	Healthy (48)	C (98)	0.04
	dbSNP	Caucasian	Healthy (62)	C (95)	0.23
CDKN1A codon 31		HRC panel	Healthy (94)	C (78)	0.00
	dbSNP	Global	Healthy (172)	C (80)	0.00
	dbSNP	European	Healthy (48)	C (98)	0.20
	dbSNP	European	Healthy (120)	C (98)	0.06
	dbSNP	European	Healthy (224)	C (96)	0.45
	dbSNP	Caucasian	Healthy (62)	C (95)	0.93
Cyclin D1 870 G>A	Powell [308]	Australian (95% Caucasian or European descent)	Resectable breast cancers (351)	C (92)	0.03
		HRC panel	Healthy (94)	G (54)	0.51
	dbSNP	European	Healthy (224)	A (50.5)	N/A
	Ceschi [659]	Singaporean	Early and advanced breast cancer (255)	G (60)	0.31
	Krippel [660]	Austrian	Early Breast Cancer (497)	A (53)	N/A
	Onay [661]	Canadian	Breast Cancer (547)	G (62)	0.05
	Shu [662]	Chinese	Breast Cancer (1130)	A (56)	N/A
Yu [375]	Chinese	Breast Cancer (992)	G (61)	0.08	

Table 5.6 Cell Cycle pathway SNP frequency in our chemotherapy treated breast cancer cohort compared with published frequencies

*p value represents a t-test comparing with major allele of our chemotherapy cohort.

5.2.2 Association of Cell cycle pathway SNPs and Clinicopathological features

There were no associations seen between any of our cell cycle pathway SNPs and clinicopathological features in our cohort. In the past it has been demonstrated in squamous cell carcinoma of head and neck that a T allele at the 3'UTR' position increases risk by 1.5 times and an A allele at codon 31 increases risk by 1.4; and that a combination of TT and AA at the respective positions increase risk by 4 times[299]. We have not been able to demonstrate any differences; however we had only one patient of the rarer AA variant for codon 31.

5.2.3 Cell Cycle pathway SNPs and outcome

When outcome and CDKN1A variants for 3'UTR and codon 31 were assessed there was no relationship between OS or DFS and no influence of chemotherapy regime used. As previous studies have assessed the combination of genotypes at the 3'UTR site and the codon 31 site of CDKN1A we also combined the two SNPs; however we still failed to find any association, **table 5.7**.

Genotype 3 UTR Codon 31	Count	5 year DFS (%)	10 year DFS (%)	Log Rank P value (mantel cox)	Median Survival
CCCC	239	73	62	0.75	150.2
CTAA	1	-	-		-
CTCA	25	67	62		135.4
CTCC	17	86	-		-

Table 5.7 DFS of our chemotherapy treated cohort for a combination of CDKN1A 3'UTR and CDKN1A codon 31 genotypes

CDKN1A is involved in cell growth, differentiation and apoptosis. We hypothesised that variants in the 3'UTR' region and at codon 31 might influence outcome in a chemotherapy treated breast cancer cohort. However we have not been able to support this hypothesis.

In a chemotherapy trial in ovarian cancer patients, CDKN1A genotype at the 3'UTR position when combined with p53 codon 72 genotype was associated with outcome [320]. We therefore looked at

this combination, but again, we have not been able to demonstrate any associations on combination with p53 for either SNP position, **tables 5.8** and **5.9**.

Genotype 3 UTR P53	Count	5 year DFS (%)	10 year DFS (%)	Log Rank P value	Median Survival
CCCC	9	89	-	0.17	-
CCGC	69	77	72		183.4
CCGG	165	71	58		146.5
CTCC	2	-	-		
CTGC	8	100	-		
CTGG	36	67	57		135.4

Table 5.8 CDKN1A 3'UTR and p53 codon 72 genotype combinations and DFS in our chemotherapy treated cohort

Genotype Codon 31 P53	Count	5 year DFS (%)	10 year DFS (%)	Log Rank P value	Median Survival
AAGG	1	-	-	0.12	-
CAGC	6	100	-		-
CAGG	18	53	53		135.4
CCCC	11	91	-		-
CCGC	69	77	72		183.4
CCGG	174	72	58		146.5

Table 5.9 CDKN1A codon 31 and p53 codon 72 genotype combinations and DFS in our chemotherapy treated cohort

The literature is inconsistent when looking at polymorphisms and cancer risk in CDKN1A. As previously discussed the results are variable with many confounding factors making comparison difficult. There has been no prior association of either of our SNPs and breast cancer outcome or chemotherapy response. Although it is still likely that CDKN1A expression and function may influence outcome via an apoptotic pathway we have not been able to demonstrate that it does so via variation at either of these positions.

There was also no association between survival and Cyclin D1 870 G>A variation. Almost identical KM curves are present for all genotypes. However, anthracycline treatment appears to influence outcome.

Whether anthracycline was received does not separate out the survival curves for each variant combination but it is interesting to note that in those not receiving an anthracycline GG variant had the poorest survival whilst in those receiving anthracycline GG had the best survival, **figure 5.8**.

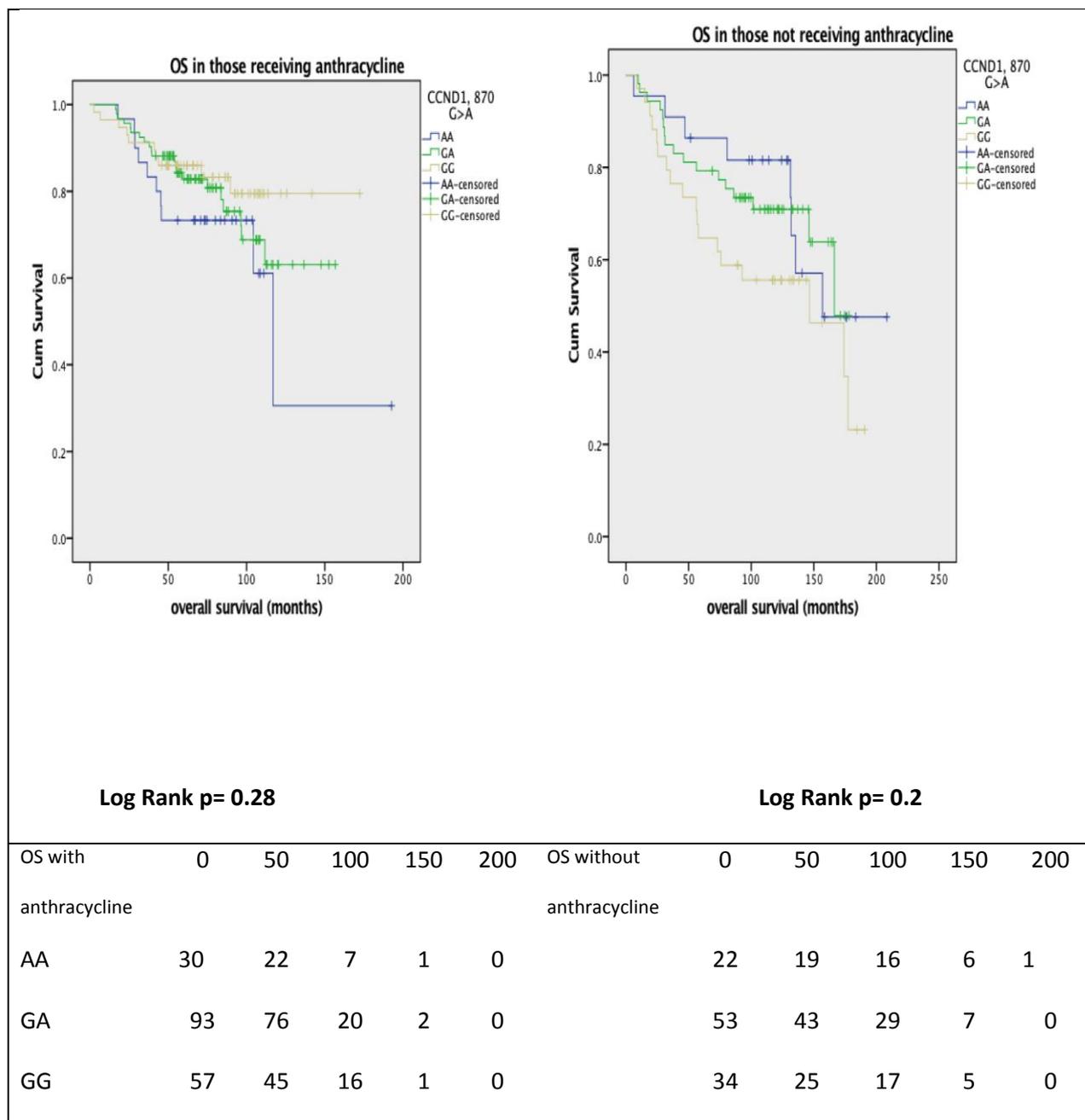


Figure 5.8 Cyclin D1 870 G>A variants and survival according to anthracycline receipt with numbers at risk tabulated below

The GG variants were therefore analysed according to whether they received anthracycline or not and compared to AA variants, **figure 5.9**

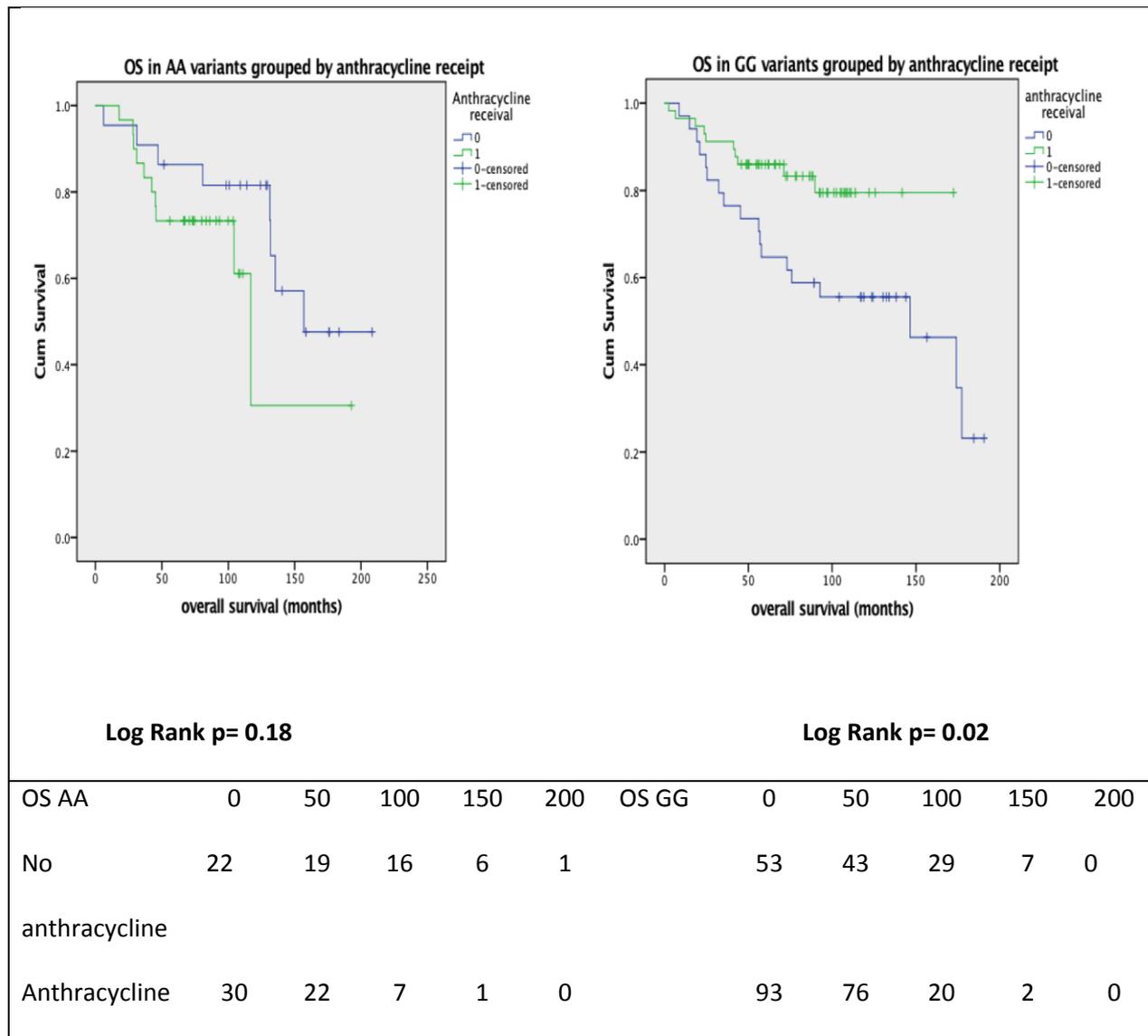


Figure 5.9 OS in Cyclin D1 870 G>A homozygotes grouped by treatment with anthracyclines with numbers at risk tabulated below

This showed that for GG variants, receipt of anthracycline was associated with a survival benefit. The same was not seen in AA variants. The findings were similar for DFS; log rank 0.01 for GG variants and 0.24 for AA variants.

There were no differences seen when data was stratified according to receipt of taxanes.

It is difficult to find evidence of cyclin D1 and anthracycline response in the literature. Perhaps because cyclin D1 is a cell cycle regulator acting on the G1 to S phase transition work has mainly focused on its role in carcinogenesis. A few studies have shown that cisplatin (a chemotherapy agent that affects the cell cycle) response in squamous cell head and neck cancer may be influenced by cyclin D1 expression levels, although both high and low levels have been found to be favourable [663, 664]. To date the evidence suggests that cyclin D1 levels are not associated with response to chemotherapy in breast cancer [598, 665, 666].

This may be in keeping with the cyclin D1 870G>A variant not being associated with expression levels but instead splice variants [667]. As a consequence of alternative splicing, cyclin D1b a truncated protein with a loss of exon 5 is produced [356]. This structural abnormality modifies the requirements for anchorage dependence and allows enhanced activity transforming fibroblastic cells [364]. It is likely that the alternative splicing is modulated by the 870G>A as the A allele is less efficient for direct splicing allowing for a possible allele-specific expression of cyclin D1b [352]. That is, the A allele favours cyclin D1b production. However, given all genotypes have cyclin D1b present it is likely to be an incomplete and complex link. Whilst both isoforms of the protein are up regulated in breast cancer, only cyclin D1b has been independently associated with adverse outcomes for recurrence and metastasis in breast cancer specific death [358].

Although we have not tested cyclin D1 protein levels in our samples, our extrapolated results are similar to other recent publications; Myklebust et al demonstrated that an increase in cyclin D1a (perhaps equivalent to our "GG" variants) had a predicted benefit from chemotherapy in a colorectal cohort [668]. The study also suggest that the increased expression of cyclin D1b, which we theorise our "AA" variants have, may have negative effects on chemotherapy response.

The reason for why a polymorphism or splice variant of cyclin D1 may affect anthracycline response particularly is not easily explained because although the exact mechanisms of the agent remain elusive it is believed to be cell cycle independent. Given that cyclin D1a is associated with increased proliferation in a way in which cyclin D1b is not [358], it is possible that the DNA damage response (DDR) pathways are involved with this finding [669]. Increased proliferation would allow for greater sensitivity to chemotherapy agents in keeping with our findings. Cyclin D1a overexpression has been associated with an enhanced DDR. It is likely that one of the mechanisms of anthracycline function is by effects on the DDR.

Future work should include the association of Cyclin D1 870 G>A polymorphism and the expression of cyclin D1a and cyclin D1b levels along with the levels of proliferation markers present in our breast cancer population.

5.3 Drug Metabolism SNPs

5.3.1 Allele Frequency

The SNPs we examined as part of the drug metabolism were treated as before. **Table 5.10** summarizes the allele frequencies for our drug metabolism variations.

SNP	Number genotyped (%)	Allele 1 homozygote (%)	Allele 2 homozygote (%)	Heterozygote (%)	Major Allele (%)	Hardy Weinberg X ² value
GSTP codon 105 (RS 1695)	299 (99)	AA (Ile/Ile) 132 (44)	GG (Val/Val) 35 (12)	GA (Val/Ile) 132 (44)	A (66)	0.06
GSTP codon 114 (RS 1138272)	299 (99)	CC 245 (82)	TT 3 (1)	TC 51 (17)	C (90)	0.2

ABCB1 (RS 1045642)	291 (96)	CC 69 (24)	TT 66 (23)	CT 156 (54)	C (50.5)	1.52
MTHFR 1298 A>C (Rs 1801131)	300 (99)	AA 140 (47)	CC 33 (11)	AC 127 (42)	A (68)	0.26
MTHFR 677 C>T (RS 1801133)	299 (99)	CC 135 (45)	TT 35 (12)	TC 129 (43)	C (67)	0.2
CYP2B6 Nr Gene 5 (RS4802101)	231 (76)	CC 82 (35)	TT 0 (0)	TC 149 (65)	C (68)	52.64
CYP2B6 Intron 3 (RS 4803419)	279 (92)	CC 152 (54)	TT 35 (13)	TC 92 (33)	C (71)	11.78
CYP2B6 Intron 5 (RS2279345)	212 (70)	CC 91 (43)	TT 35 (17)	TC 83 (39)	C (63)	3.99

Table 5.10 Allele Frequencies for drug metabolism SNPs

We also compared the proportion of the major alleles in our drug metabolism SNPs, in our breast cancer cohort to healthy and breast cancer populations (**table 5.11**). The GSTP1 SNPs studied had no variation in major allele amongst the groups looked at. The frequency of GSTP1 codon 105 major allele was similar to the HRC panel studied and a healthy global population as well as 2 large American breast cancer studies. These studies included either chemotherapy or radiotherapy treated tumours or postmenopausal patients. The characteristics are similar to our chemotherapy treated cohort; however a smaller American study of unselected breast cancers had a significantly lower proportion of the C allele. The codon 105 SNP has previously been implemented in breast cancer risk[519-521], however

given the variation in differences between populations studied here it is difficult to lend support to this theory with our data.

The ABCB1 SNP has been widely studied with regards to breast cancer risk. The summary of allele frequencies shows varying results but our breast cancer cohort is similar to most other breast cancer Caucasian/ European cohorts. MTHFR 1298 A>C and 677 C>T have also been widely studied with regards to breast cancer risk in particular with dietary and folate associations. The major allele frequencies vary but our cohort is comparable with a healthy European population, a Canadian Caucasian breast cancer, a Caucasian breast cancer and an American breast cancer population, as well as a large meta-analysis. Comparing the allele distribution for both it seems that ethnicity plays a part in variation. Our cohort had a similar allele distribution to other European/Caucasian breast cancer groups suggesting that our data is representative and not skewed by selection bias.

Although the CYP family of genes has been widely studied in the literature this is mostly in relation to pharmacokinetics and haematological malignancies. There is limited information on genetic variation and breast cancer patients receiving chemotherapy. However, with one exception, our cohort had similar genetic variation to both the HRC panel used and global dbSNP populations. Whilst the other metabolising SNPs are all in keeping with Hardy Weinberg principle, the CYP2B6 SNPs all deviate. Given that CYP2B6 is of interest to us in because of its role in the biotransformation of drugs and it is not known to be an independent risk or prognostic factor in breast cancer it is difficult to hypothesise as to why this deviation has occurred. As we are only testing at the 5% level for 1 degree of freedom (3.84) this may simply be a statistical anomaly given the large number of SNPs assessed in this study.

SNP	Source	Population	Cohort (N)	Major Allele (%)	P value*
GSTP1 codon 105		HRC panel	Healthy (94)	A (59)	0.08
	dbSNP	Global	Healthy (160)	A (60)	0.07
	dbSNP	European	Healthy (226)	A (59)	0.02
	Sweeney [513]	American	Chemo or Radiotherapy treated breast cancer (240)	A (68)	0.52
	Reding[520]	American	Post menopausal Breast cancer (891)	A (66)	0.98
	Helzlsouer [519]	American	Breast cancer (120)	A (57)	0.01
GSTP codon 114		HRC panel	Healthy (94)	C (85)	0.07
	dbSNP	European	Healthy (226)	C (81)	0.00
ABCB1		HRC panel	Healthy (94)	T (60)	0.02
	dbSNP	European	Healthy (226)	T (57)	n/a
	Vaclavikova [540]	Czech Republic	Breast cancer (90)	T (52)	0.69
	Cizmarikova [541]	Slovakian	Breast Cancer (221)	T (54)	0.26
	George [542]	Indian	LABC (86)	T (67)	0.00
	Turgut [543]	Turkey	Breast IDC (57)	T (59)	0.11
	Tatari [544]	Iranian	Breast Cancer (106)	T (58)	0.06
	Huang [547]	Taiwanese	Stage 2 and 3 breast cancer (192)	C (61)	n/a
	Kafka [545]	Caucasian	Breast Cancer (100)	T (51)	0.91
	Chang [549]	Korean	Breast Cancer (108)	C (56)	n/a
MTHFR 1298 A>C		HRC panel	Healthy (94)	A (64)	0.29
	dbSNP	European	Healthy population (226)	A (65)	0.31
	Gao[561]	Chinese	Primary breast cancer (624)	A (84)	0.00
	Ma[562]	Brazilian	Primary breast cancer (458)	A (77)	0.00
	Kotsopoulos [564]	Candian Caucasian	Primary breast cancer (941)	A (71)	0.16
	Chou [670]	Taiwanese	Primary breast cancer (142)	A (84)	0.00
	Le Marchard [671]	American (various ethnicities)	Primary breast cancer (1189)	A (77)	0.00
	Martin [568]	African American	Primary breast cancer (141)	A (84)	0.00

	Martin [568]	Caucasian	Primary breast cancer (102)	A (60)	0.03
	Shrubsole [569]	Chinese	Primary breast cancer (1045)	A (82)	0.00
MTHFR 677 C>T		HRC panel	Healthy (94)	C (71)	0.34
	dbSNP	European	Healthy population (226)	C (69)	0.50
	Gao[561]	Chinese	Primary breast cancer (624)	C (56)	0.00
	Ma[562]	Brazilian	Primary breast cancer (458)	C (70)	0.23
	Kotsopoulos [564]	Canadian Caucasian	Primary breast cancer (944)	C (63)	0.07
	Huang[547]	Taiwanese	Primary breast cancer (192)	C (75)	0.01
	Campbell[672]	Caucasian UK	Primary breast cancer (335)	C (66)	0.68
	Lee[673]	Korean	Primary breast cancer (186)	C (57)	0.00
	Martin[568]	African American	Primary breast cancer (141)	C (90)	0.00
	Martin[568]	Caucasian	Primary breast cancer (102)	C (70)	0.42
	Shrubsole[569]	Chinese	Primary breast cancer (1038)	C (56)	0.00
	Chou[670]	Taiwanese	Primary breast cancer (142)	C (59)	0.02
	Le Marchand[671]	American (various ethnicities)	Primary breast cancer (1189)	C (68)	0.56
	Zintzaras[565]	Meta-analysis	Primary breast cancer (5467)	C (64)	0.13
CYP2B6 Nr Gene 5		HRC panel	Healthy (94)	C (71)	0.49
	dbSNP	Caucasian	Healthy (120)	C (66)	0.57
CYP2B6 Intron 3		HRC panel	Healthy (94)	C (69)	0.95
	dbSNP	Caucasian	Healthy (120)	C (66)	0.15
CYP2B6 Intron 5		HRC panel	Healthy (94)	C (60)	0.50
	dbSNP	Caucasian	Healthy (118)	C (71)	0.03

Table 5.11 Drug Metabolism SNP frequency in our chemotherapy treated breast cancer cohort compared with published frequencies

*p value represents a t-test comparing with major allele of our chemotherapy cohort.

LABC - Locally advanced breast cancer

IDC - Invasive ductal Carcinoma

5.3.2 Drug Metabolising SNPs and clinicopathological associations.

Each of the SNPs previously mentioned in our drug metabolizing groups were assessed for associations with the clinicopathological features of our chemotherapy treated breast cancer cohort. As the primary reason for analysing this group of SNPs was to assess tumour response to treatment it was not expected that variant type would predict pathology so any associations would be incidental but still relevant. The statistically significant findings are summarised in table 5.11. GSTP1 codon 114 SNP was significantly associated with lymph node status in this cohort. Heterozygotes were more likely to be associated with positive node status. GSTP1 codon 105 genotype was not associated with any clinicopathological features. Interestingly when analysed in combination codon 105 SNP only those heterozygote for both alleles remained at increased chance of positive lymph node status. 6 of the 9 possible allele combinations were present in our population. All of the codon 105 AA genotypes were homozygote for the C allele in codon 114. The three codon 114 TT variants were all codon 105 GG. When clinicopathological factors were examined the finding that the Lymph node status was significantly associated persisted ($p=0.04$). There were 2 combinations with the TC SNP, GATC (number = 40) or GGTT (number= 11). Only the GATC appeared to remain significant. Those with GG codon 105 genotype were not at increased likelihood of having involved nodes. Given this, it is likely that another SNP within the haplotype is responsible. Of note, previous work looking at GSTP protein analysis in breast cancer has shown an association with higher expression being linked with lymph node positivity[674].

The ABCB1 SNP was significantly associated with patient age and menopausal status with CC variants tending to be older and peri or post menopausal and TT variants being younger and pre-menopausal. As previously discussed, ABCB1 is a member of the ATP binding cassette transporters which code for P-glycoprotein (P-gp). It has been shown that over expression of P-gp can convey resistance to a number of cytotoxic agents. The T allele of the 3435 SNP we have studied has been associated with decreased protein expression in breast cancer[540] and the TT allele is associated with an increased

risk of breast cancer in some populations, perhaps due to the accumulation of carcinogenic toxins [542, 543, 675]. Although we could not demonstrate that the TT variant is associated with an increased risk of breast cancer, as we had no control group, we did demonstrate that this genotype was linked to younger, pre-menopausal women in our cohort. This perhaps supports the theory of toxin accumulation leading to breast cancer.

There were no associations between the MTHFR SNPs and clinicopathological features. When MTHFR 1298 A>C and MTHFR 677 C>T genotypes are combined all variations were present with the expectation of the double homozygote for both lesser alleles (CCTT) and there remained no clinicopathological associations. There have not been any reports in the literature of MTHFR genotype being associated with any particular clinicopathological features and our study would support this. The literature does however suggest that genotype plays a part in MTHFR efficacy [553, 554]. The 677 location SNP T allele has reduced enzyme activity for homozygotes (35% of CC) and heterozygotes (65% of CC)[553]. At the 1298 position the lesser allele C results in a glutamate to alanine substitution removing an MboII recognition site (MboII is a restriction enzyme) and reducing enzyme activity, with homozygotes being more pronounced than heterozygotes [554]. As MTHFR is the main controller for 5,10, methylenetetrahydrofolate and increased levels of this allows for optimal TS inhibition by 5-FU, then theoretically lower enzyme activity should lead to an accumulation of 5,10 methylenetetrahydrofolate and therefore improved sensitivity[566].

While CYP2B6 Nr gene 5 genotype was not associated with any clinicopathological findings CYP2B6 Intron 3 was associated with patient age and method of detection of cancer (screening or symptomatic) and CYP2B Intron 5 variation was associated with patient age and presence of lymphovascular invasion (**table 5.12**). CC variants were more likely to be over 50 and have LVI whilst those who were TT were younger with less LVI. Although the CYP family of genes has been widely studied in the literature this is mostly in relation to pharmacokinetics and haematological malignancies. There is limited information on genetic variation and breast cancer patients receiving chemotherapy.

CYP2B6 has been shown to be overexpressed in tissue from ER positive breast cancers suggesting a role in tumourigenesis [676]. This may at least partially explain why our two intronic SNPs both showed associated with age and genotype. However, no population risk studies have been reported to ascertain this link further. CYP2B6 is classified as a class II CYP gene metabolising drugs and not pre-carcinogens perhaps explaining why this gene has not been assessed in this way [677]

SNP	Variable		Genotype count (%)			P
			CC	TC	TT	
GSTP1 codon 114						
	Lymph node Involvement	No	90 (91)	9 (9)	0 (0)	0.01
		Yes	155 (78)	42 (21)	3 (2)	
ABCB1			CC	CT	TT	
	Age	<50	44 (29)	80 (53)	26 (17)	0.02
		>50	25 (18)	76 (54)	40 (28)	
	Menopausal Status	Peri-menopausal	4 (50)	3 (38)	1 (13)	0.01
		Post-menopausal	27 (19)	75 (52)	42 (29)	
		Pre-menopausal	32 (28)	66 (58)	16 (14)	
CYP2B6 Intron 3			CC	TC	TT	
	Age	<50	73 (51)	56 (39)	13 (9)	0.03
		>50	79 (58)	36 (26)	22 (16)	
	Method of detection	Screening	6 (35)	5 (29)	6(35)	0.006

		Symptomatic	85 (54)	52 (33)	19 (12)	
CYP2B6 Intron 5			CC	TC	TT	
	Age	<50	34 (35)	41 (43)	21 (22)	0.04
		>50	60 (52)	42 (36)	14 (12)	
	Lymphovascular invasion	Absent	51 (52)	36 (37)	11 (11)	0.04
		Present	39 (36)	46 (43)	23 (21)	

Table 5.12 Drug metabolism SNPs and associations with clinicopathological features in a chemotherapy treated breast cancer cohort

5.3.3 Drug Metabolising SNPs and outcome.

We were unable to demonstrate any association between the two GTSP SNPs studied and outcome.

However, when grouped according to whether taxane therapy was received there is a non-significant trend suggesting that cases with the AA genotype fare worse if not given a taxane (**Figure 5.10**).

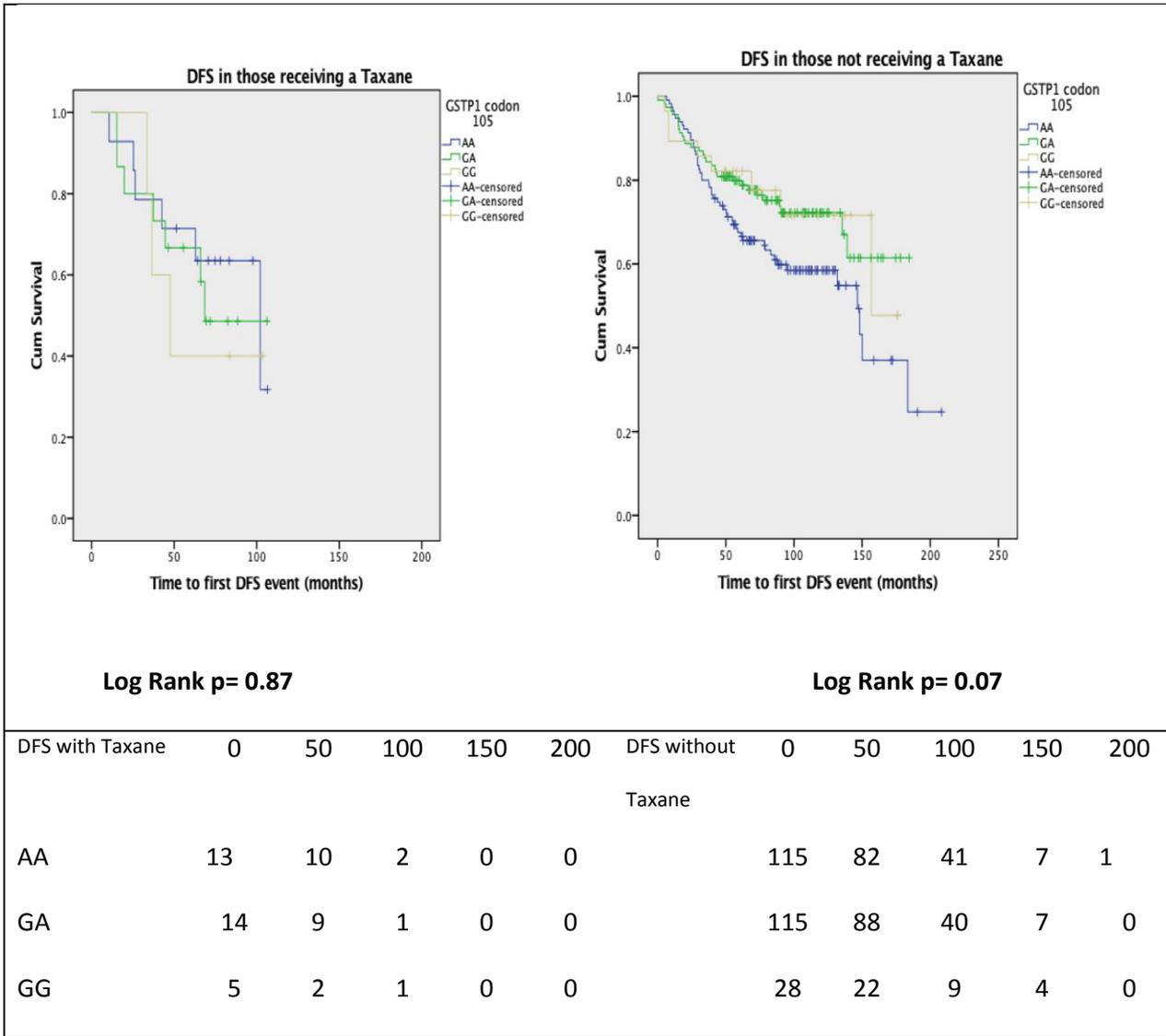


Figure 5.10 DFS for GSTP1 codon 105 variant and taxane receipt with at risk numbers tabulated below

The codon 105 genotype has previously been associated with outcome in a cyclophosphamide treated breast cancer cohort[513]. In this study Val/Val (GG) genotype had an increased hazard ratio for death compared to other variants and also had a shorter time to relapse. As the majority of our cohort received cyclophosphamide we may have expected similar results but we did not see this. The hypothesis was that cyclophosphamide acts through alkylating intermediates and therefore it was possible that genotype affected the GSH conjugation of reactive metabolites.

We did however note a possible sensitivity of the codon 105 AA variant to taxanes. When no taxanes are received the AA variant appears to have a poorer outcome although not reaching statistical significance ($p=0.07$), 68% 5yr DFS compared to 80% of the same number of GA variants. When taxanes are given there is no difference in outcome. Caution needs to be taken when interpreting these results, as the absolute number of people, 32, receiving taxane in our study is small.

The data on GSTP1 and chemotherapy response is conflicting. While pre-clinical studies have suggested that increased GSTP1 expression is associated with chemo-resistance [678] this has not been confirmed clinically. There certainly appears to be no association between protein expression and anthracycline-based regimes [180, 679-681]. Again the data on protein expression levels and survival prediction are conflicting [682]. A recent study suggested that high GSTP1 protein expression was associated with improved DFS in early breast cancer treated with chemotherapy. This was more evident when those receiving taxanes were analysed as a sub group but the numbers are too small to draw any definite conclusions. The authors do recommend that further study into GSTP1 and taxane response would be helpful [682].

Previously the two SNPs we have studied which are situated in the catalytic site of GSTP1 have been shown to cause a reduction of substrate affinity [683]. The effect of expression levels and catalytic activity has been related to docetaxel treatment *in vitro* [684] and GST can characterise non-responders to docetaxel in breast cancer [506]. The exact mechanism is unclear but in one paper that reported results for taxane induced peripheral neuropathy, showed that the Ile/Ile (AA) genotype was an independent risk factor for developing this side effect, the authors hypothesised that it is due to the inhibitory role GSTP1 has in the JNK pathway [682]. They postulate that Ile/Ile enhances neurotoxicity through the inhibition of JNK whereas Val allows for a higher activity of JNK inducing the expression of genes involved in cellular defence. This may also compliment our finding of Ile/Ile perhaps having increased sensitivity to taxanes. Certainly, there is building evidence on the role of GSTP1 and taxane response and the influence of genetic variation of the gene should be further investigated.

We were particularly interested in the ABCB1 SNP as it has previously been linked to both anthracycline and taxane chemotherapy response in breast cancer. This was especially true of the TT variant. However we were unable to demonstrate any association with clinical outcome or chemotherapy response. With regards to taxane therapy this may be due to the small number of people in our population who receive taxane. The evidence for anthracycline response in the literature is varied with some studies not reaching statistical significance and some contradicting the favourable allele. Study numbers are often small, for example Kafka et al found that the T allele was associated with a better response to anthracyclines in 68 patients with locally advanced breast cancer[545]. It seems that to assess this SNP reliably, DNA obtained from a large prospective randomised trial is necessary.

Individually, neither of the MTHFR SNPs studied influenced outcome or specific chemotherapy response. However, combination of the two, shows that although there is no significant difference in the survival curves (log rank 0.12), there is a separation between the best (AACC) and poorest (ACCC) performing patients stratified according to their genotypes (**figure 5.11**). Results presented here have the 1298 SNP followed by the 677. When AACC is compared to ACCC then there suggests a possible tendency for AACC to have improved OS (Log rank = 0.06) however this is seen when AACC vs. non – AACC is compared.

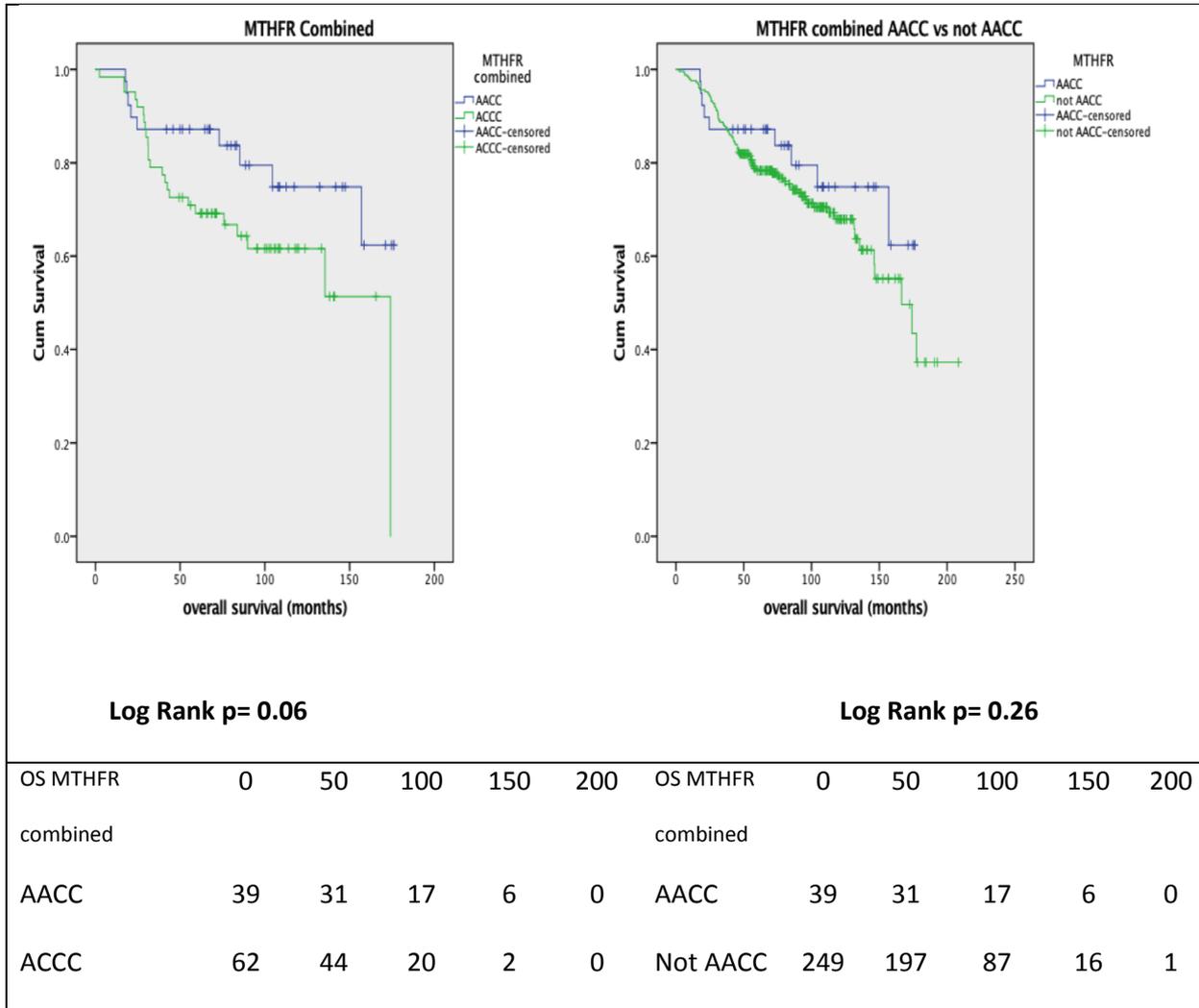


Figure 5.11 Kaplan Meier curves for a) separation of the best and poorest SNP combinations outcome of MTHFR 1298 and MTHFR -677 and b) MTHFR combined AACC genotype vs. non-AACC with numbers at risk tabulated below

As discussed above, we were studying MTHFR given its theoretical role in lowering enzyme activity and allowing accumulation of 5,10 methylenetetrahydrofolate and therefore improved sensitivity to 5-fU. *In vitro* studies have supported this with the 1298 CC and AC variants showing increased 5-fU sensitivity in colorectal and breast cancer cell lines[566]. In this study 677 variant did not affect sensitivity. However a further study showed that the T allele 677 SNP (which decreases enzyme activity) was associated with increased 5-fU sensitivity [567]. Our study has been unable to verify these cell line findings with no difference being seen in outcome for individual SNPs. No cell line studies have assessed the combination of SNPs. Theoretically the double homozygotes for the lesser alleles (CCTT)

should perform best but as we do not have any of this variant in our cohort it is impossible to say. Our results are difficult to interpret as the patients with the 1298 AA variant in combination with 677 CC perform best but the 677 CC variant patients with 1298 AC have the worst outcome. It would be interesting to look at enzyme activity in association with the combination of SNPs.

The 677 genotype has been studied in breast cancer patients receiving chemotherapy with mixed results. The CC variant has been associated with earlier relapse in those receiving FEC regimes [547] while the TT variant is associated with increased death in a larger study of early breast cancer where chemotherapy regime information was not collected[569]. There is certainly promise that pharmacogenetics can play a major role in individualised patient treatment plans and the MTHFR SNPs look hopeful to playing a role in this. We can support this with our study but further larger, controlled population studies are necessary.

Once again whilst none of the CYP2B6 SNPs studied had any influence on outcome individually there was some difference depending on whether a taxane had been used and CYP2B6 Near-gene 5 genotype (**table 5.13**)

KM Log Rank	Taxane given	No Taxane given
Overall Survival	0.03	0.79
Disease Free Survival	0.53	0.79

Table 5.13 Log Ranks for CYP2B6 Nr gene 5 according to taxane receipt

Closer analysis of this shows that very little separates the 2 variations when no taxane is given but upon receipt of a taxane heterozygotes' OS improves (**Fig 5.12.**). This was not seen with DFS.

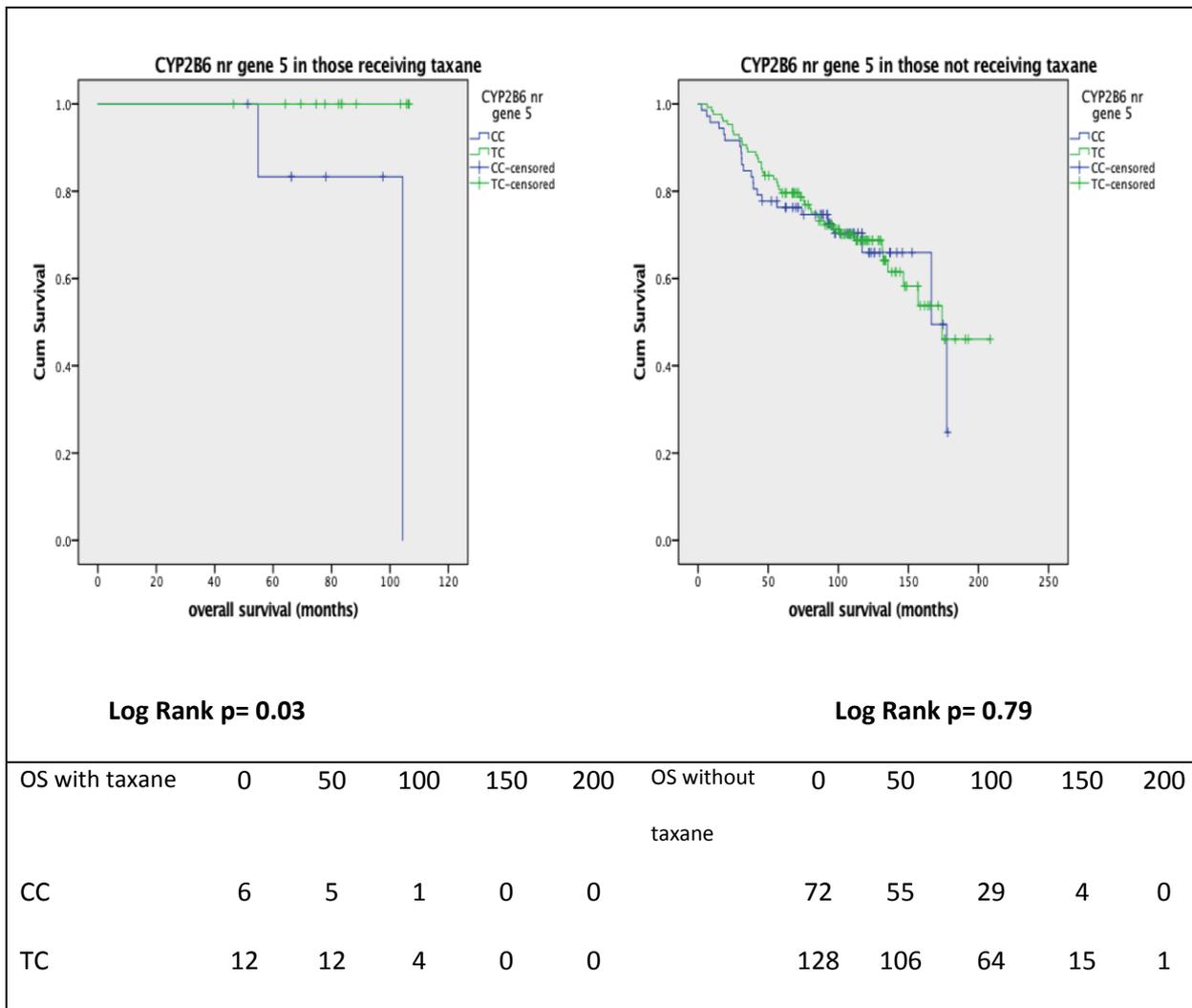


Figure 5.12 Survival in CYP2B6 variants when stratified to taxane receipt with numbers at risk tabulated below

CYP2B6 variation was of particular interest to us as the majority of our cohort received cyclophosphamide as part of their chemotherapy regime. Cyclophosphamide is an inactive prodrug which undergoes metabolic activation to 4 hydroxycyclophosphamide by hepatic cytochromes with CYP2B6 having the highest 4-hydroxylase activity[685]. CYP2B6 genotypes have been associated with altered protein expression and activity [686] Pharmacokinetic studies in Japanese cancer patients demonstrated that SNPs in CYP2B6 reduced 4-hydroxylation [482] but this has not been repeated in studies with Caucasian patients[685, 687].

It is known that CYP2B6 is one of the most polymorphic CYP genes and is mainly expressed in the liver where it constitutes 3-5% of the total microsomal pool[677]. However, polymorphisms have been variable across ethnicities and studies have been unable to produce consistent results.

Overall, we were not able to demonstrate any difference in long-term outcome in breast cancer patients receiving chemotherapy according to CYP2B6 genotype. Combination of polymorphisms for the 3 SNPs studied also failed to demonstrate any difference in outcome (data not shown). Further clarification on the effect on pharmacokinetics of this gene in Caucasian patients is therefore needed.

Perhaps surprisingly our data hints at an effect of CYP2B6 variation and taxane response. When no taxane is given there is no difference in survival in the Nr gene 5 SNPs. However, when taxane therapy is given, heterozygotes do better than the CC variant. Although marginally statistically significant with a Log rank of 0.0269 the numbers involved are small. Receiving a taxane there were 7 CC patients, 2 of whom died and 13 TC patients who all survived. Further work is warranted to explore this on a larger cohort of taxane treated patients.

The effect is difficult to explain biologically as CYP2B6 is not known to be involved in the metabolism of taxanes. Docetaxel is predominately metabolised by CYP3A4/5 and paclitaxel by CYP2C8 and CYP3A4. Polymorphisms of these genes have been shown to impair intrinsic clearance *in vitro* but not *in vivo* [688]. Perhaps the CYP2B6 Nr gene 5 SNP is in linkage disequilibrium with CYP3A4/5 SNPs as simultaneous polymorphisms in these genes have demonstrated a 64% higher docetaxel clearance[689].

5.4 Predictive Factor SNPs

5.4.1 Allele Frequency

The HER 2 SNPs we examined were analysed as described for the previous SNPs. **Table 5.14** summaries the allele frequencies for our drug metabolism variations.

SNP	Number genotyped (%)	Allele 1 homozygote (%)	Allele 2 homozygote (%)	Heterozygote (%)	Major allele (%)	Hardy Weinberg X ² value
HER 2 codon 655 G>A (RS 1136201)	296 (98)	AA 24 (8)	GG 180 (61)	AG 92 (31)	G (76)	5.73
HER 2 - 1170 G>C (Rs 1058808)	300 (99)	CC 40 (13)	GG 153 (51)	CG 107 (36)	G (69)	7.17

Table 5.14 Allele Frequencies for predictive factor SNPs

The HER2 codon 655 SNP has been widely studied in the literature. This has mostly been looking at breast cancer risk compared to control populations. The SNP is highly variable among different ethnic groups. Our data supports this. We have a similar genotype distribution to other Caucasian groups studied. The HER2 -1170 has not been studied in a breast cancer population. When compared with dbSNP data our population is comparable with other European groups but not a global population. However the populations also differ from each other suggesting that ethnicity has an influence in this particular SNP variation (**table 5.15**).

SNP	Source	Population	Cohort (N)	Major Allele (%)	P value*
HER 2 codon 655 G>A		HRC panel	Healthy (94)	G (74)	0.56
	Xie[615]	Chinese	Breast Cancer (339)	G (85)	0.00
	Mutluhan[616]	Turkish	Breast Cancer (166)	G (88)	0.00
	Asisik[617]	Turkish	Breast Cancer (121)	G (90)	0.00
	Kamali-Sarvestani[618]	Iranian	Breast Cancer (204)	G (85)	0.00
	Millikan[619]	African American	Breast Cancer (754)	G (93)	0.00
	Millikan[619]	Caucasian American	Breast Cancer (1261)	G (77)	0.61
	Baxter [690]	British	Breast Cancer (315)	G (78)	0.42
HER 2-1170		HRC panel	Healthy (94)	G (64)	0.19
	dbSNP	European	Healthy (48)	G (65)	0.39
	dbSNP	European	Healthy (120)	G (71)	0.60
	dbSNP	Global	Healthy (170)	G (51)	0.00

Table 5.15 Predictive factor SNP frequency in our chemotherapy treated breast cancer cohort compared with published frequencies

*p value represents a t-test comparing with major allele of our chemotherapy cohort.

HER2 *neu* is a member of the tyrosine kinase erb-B receptor family and has an intrinsic role in regulation of cell differentiation, proliferation and motility. Despite its importance in breast cancer prognosis and its role as a predictive factor for targeted monoclonal treatments, information on polymorphisms within this gene is limited. One SNP that has been widely studied with regards to breast cancer risk is the non-synonymous codon 655 Val to Ile substitution. Due to large inter-ethnic variation the studies have mixed results but a recent meta-analysis concluded that it was weakly associated with risk but may be a more useful biomarker for susceptibility in Asian women or those under 45 [624]. The Val (minor) allele is associated with increased risk.

The mechanisms for this are unclear as the functional characteristics of the Val variant are not well characterised. The substitution does occur in the transmembrane region of the receptor and this is where mutations are observed in the rat *neu* homologue[619]. It has been suggested that the presence of Val stabilises the formation of an active dimer of the protein thereby predisposing to auto activity of the receptor[614]. Given that protein expression is a known prognostic factor and as our cohort have all received chemotherapy, biasing the selection, this may go some way as to explaining the deviance from Hardy Weinberg equilibrium seen.

5.4.2 Predictive factor SNPs and clinicopathological associations.

Both the HER2 SNPs were significantly associated with ER status (**table 5.16**). Minor allele homozygotes of each variant were more likely to be associated with an ER negative tumour. This was the only clinicopathological variation noted. While some studies have tried to link HER2 codon 655 variant status with clinicopathological factors of the breast cancer many were unsuccessful [616-618]. Millikan found that the SNP was linked with cancer stage with the Val allele being predominant in cases of DCIS only [619]and Xie found that the val allele was associated with amplification of the HER2 receptor[615]. We did not find this but it is important to note that our cohort did not include any cases of pure DCIS, as they do not usually require chemotherapy and a large proportion of our cohort did not have information on their HER2 *neu* status available.

We did find however that both SNPs analysed were associated with ER receptor status with the codon 655 AA variant and the -1170 CC variant predisposing to ER negativity. This has not been previously reported in the literature and a possible mechanism is not clear. ER negative tumours have higher proliferation rates which may be influenced by the HER2 *neu* receptor[691]. Genomic profiling has shown that amongst the HER2 over amplified tumours 2 distinct groups can be described depending on ER status [692]. Further work is needed to assess the influence of HER2 polymorphism on gene amplification, tumour proliferation and ER status.

SNP	Variable		Genotype count (%)			P
HER2 codon 655	ER Status		AA	GA	GG	
		Negative	14 (13)	28 (26)	66 (61)	0.01
		Positive	7 (4)	63 (37)	101 (59)	
HER2 codon - 1170 G>C	ER Status		CC	GC	GG	
		Negative	22 (20)	35 (32)	54 (49)	0.04
		Positive	16 (9)	66 (38)	90(52)	

Table 5.16 Predictive factor SNPs and clinicopathological variations

5.3.4 Predictive Factor SNPs and outcome

HER 2 Codon 655 SNP was not associated with outcome, however there were trends with the -1170 variant. This SNP either codes for Ala or Proline peptide and our data shows that the presence of both alleles appears to be associated with improved outcome (**figure 5.13**). This become statistically significant when heterozygotes are compared with all homozygotes as a group. There was no differentiation between types of chemotherapy given. When the variants for both SNPs were combined there was no difference in survival.

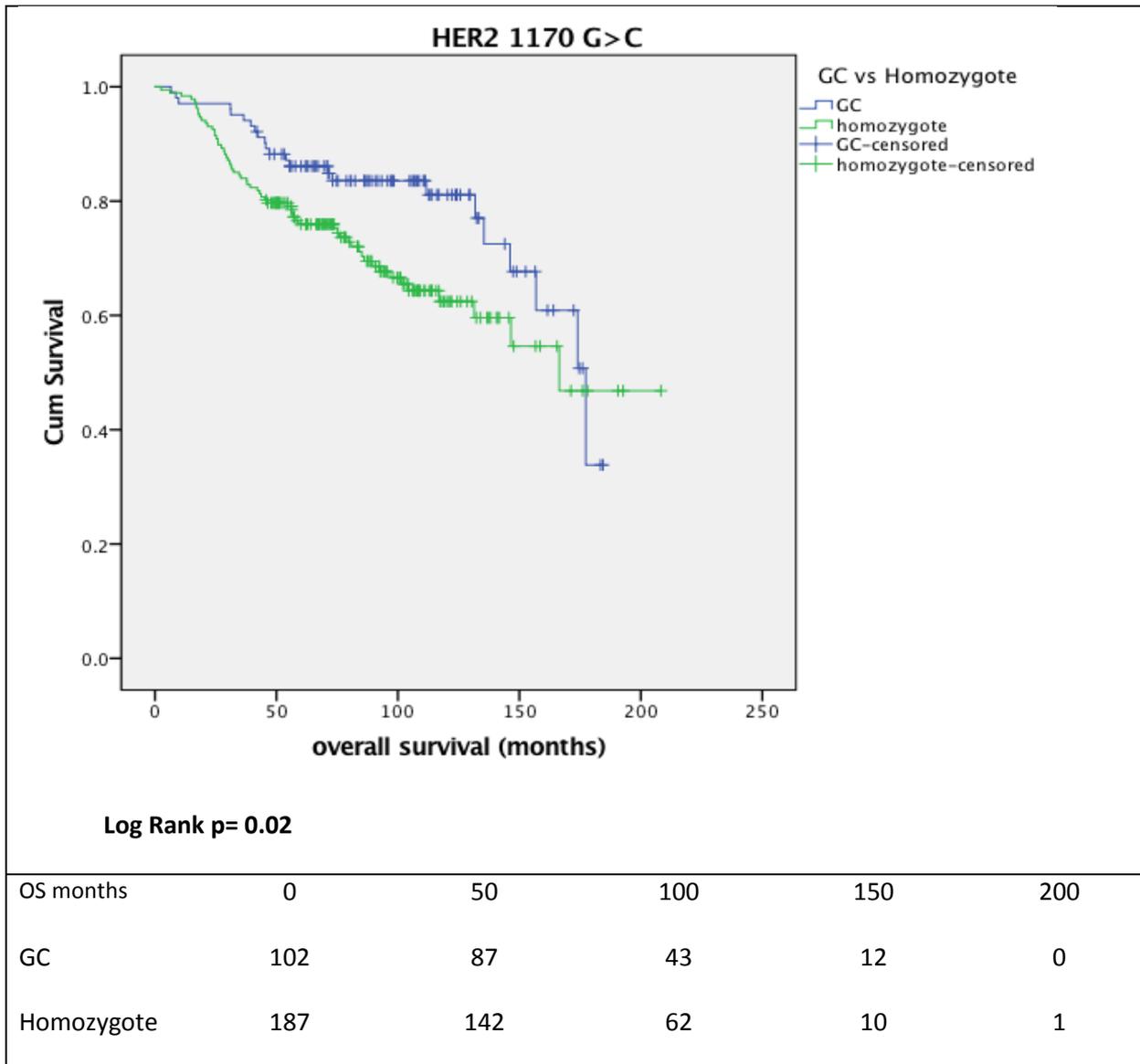


Figure 5.13 OS for HER2 1170G>C homozygote compared to heterozygote with numbers at risk tabulated below

This finding of -1170 G>C has not been previously reported. There is nothing published in the literature comparing HER2 polymorphism and breast cancer outcome or chemotherapy response in solid cancers. Very little work has been performed comparing the -1170 G>C SNP so nothing is known on its effect on the receptor, amplification rates, proliferation rates or why the presence of both alleles may confer a survival advantage.

Of note, we were hoping to analyse HER2 SNPs in combination with Topoisomerase II α polymorphisms. As it is difficult to find data in the literature on breast cancer and topoisomerase II α genotype associations one SNP was chosen based on reasonable minor allele frequencies in dbSNP. However, our cohort had only the major allele present so further analysis could not be performed.

5.5 Metastor SNPs

5.5.1 Metastor gene SNPs allele frequency

Using the methods previously described it was possible to call genotypes for 298 of 303 (98%) patient's DNA analysed. Homozygotes for each allele were clearly identified (**table 5.17**). There are no published breast cancer cohorts to compare our allele distributions. Hardy-Weinberg equilibrium was met. Our cohort did not differ significantly from our HRC panels or a healthy Caucasian cohort for the SPP1 SNPs. XBP1-66 G>C had a similar distribution to our HRC panel but significantly lower major allele frequency when compared to a European dbSNP population. (**table 5.18**)

SNP	Number genotyped (%)	Allele 1 homozygote (%)	Allele 2 homozygote (%)	Heterozygote (%)	Major allele (%)	Hardy Weinberg X ² value
SPP1 -443 C>T (Rs 11730582)	298 (98)	TT 80 (27)	CC 67 (22)	TC 151 (51)	T (52)	0.1
SPP1 -66 T>G (28357094)	298 (98)	GG 18 (6)	TT 166 (56)	GT 114 (38)	T (75)	0.11
XBP1 -166 G>C (Rs	297 (98)	CC 37 (13)	GG 140 (47)	GC 120 (40)	G (67)	2.07

Table 5.17 Allele Frequencies for metastor gene SNPs

SNP	Source	Population	Cohort (N)	Major Allele (%)	P value*
SPP1 -443 C>T		HRC panel	Healthy (94)	C (50.5)	0.54
	dbSNP	Caucasian	Healthy (92)	T (56)	0.35
SPP1 -66 T>G (Rs 28357094)		HRC panel	Healthy (94)	T (76)	0.77
XBP1 -166 G>C (Rs		HRC panel	Healthy (94)	G (71)	0.34
	dbSNP	European	Healthy (120)	G (85)	0.00

Table 5.18 Metastor gene SNP frequency in our chemotherapy treated breast cancer cohort compared with published frequencies

*p value represents a t-test comparing with major allele of our chemotherapy cohort.

5.5.2 Metastor gene SNPs and clinicopathological associations

SPP1, -443 C>T genotype is associated with ER status in this cohort (non-significant trend, $p=0.07$). A higher than expected proportion of the CC variant were ER negative. No previous studies have linked variation in this polymorphism with ER status. There were no associations between SPP1 -66 T>G variants and clinicopathological features studied.

Breast cancers have been demonstrated to over express SPP1 [436, 437] and expression is especially high in metastatic tumours [438, 439]. SPP1 levels have also been shown to be independently associated with prognosis in breast cancer[441]. The metastatic ability of breast cancer cells can be significantly reduced by SPP1 gene knockdown [447, 448].

The SPP1 -66 G>T did not reveal any association with either clinicopathological features or outcome; there was also no associations when the two SNPs were combined.

There was a trend for XBP1 -166 G>C variation to be associated with the HER 2 *neu* status of the tumour ($p= 0.07$). The minor allele homozygote, CC variant is more likely to be HER2 negative.

5.5.3 Metastor Gene SNPs and outcome

5.5.3.1 SPP1 Variants and outcome

There was no association between SPP1 -66 T>G polymorphism and either OS or DFS and the presence of an anthracycline or a taxane in the chemotherapy regime did not alter overall survival. There was also no association when the two SPP1 SNPs were combined. The discussion will therefore centre on the SPP1 -443 C>T polymorphism. As there was a trend for the SPP1 -443 C>T TT variant to be related to a poorer outcome the genotypes were re-analysed comparing this variant with CC and CT combined (**figure 5.14**). This demonstrated that the TT variant had a worse OS when compared to other variants ($p=0.02$) and a trend towards a poorer DFS ($p=0.06$).

However, when stratified as to whether patients received an anthracycline containing chemotherapy regime, this difference was only present in those receiving an anthracycline (**figure 5.15**). As there was no difference in survival in those not receiving anthracyclines, this suggests that the TT variant may be less sensitive to anthracycline based chemotherapy regimes. The poorer OS in the TT variant disappears when taxane based chemotherapy is given (**Figure 5.16**). Although the numbers receiving taxane-based chemotherapy are small,32, this suggests that the TT genotype may be particularly sensitive to taxanes.

The SPP1 -443 C>T polymorphism is located within the 500bp upstream of the transcription start site [449]. It is a Tag SNP that has been shown to influence gene regulation in melanoma cells, with the CC genotype increasing mRNA levels [451]. It was demonstrated that the preceding sequence, AAGTT[C/T] is similar to the c-Myb core-binding motif, CAGTT. C-Myb binds to this region in an allele specific manner with the C allele enhancing transcription.

There are no prior published series looking at SPP1 polymorphisms and outcome in a chemotherapy treated breast cancer cohort. We have demonstrated a trend towards the SNP being associated with ER status, although there were no other clinic pathological associations. As the CC genotype has been linked to increased expression of SPP1, which is in turn associated with a poorer prognosis, one could hypothesize that the CC variant would be associated with poorer outcome in our cohort. In fact, we have found that the TT variant was significantly associated with worse overall survival ($p=0.02$) and had a tendency to be associated with poorer DFS ($p=0.06$).

In addition to this we have also found that response to specific chemotherapy regimens may be influenced by this variant. In patients who did not receive anthracycline therapy, genotype did not significantly influence overall survival ($p=0.16$). However, if an anthracycline was given CT and CC variants had a superior outcome compared to TT ($p=0.04$), suggesting that the TT genotype may be less sensitive to anthracycline therapy.

Conversely, if a taxane agent was included in the regime then the differences between the variants disappear. If no taxane is given, then the TT variant does significantly worse (OS $p=0.02$, DFS $p=0.03$) but there is no difference in the survival curves when a taxane is given (OS $p=0.98$, DFS $p=0.75$). Although the number of patients receiving a taxane is small (34 as compared to 264 not receiving) there is suggestion that TT genotypes are particularly sensitive to this treatment and further investigation may be warranted.

In summary, we have demonstrated that the TT variant is associated with poorer outcome in a chemotherapy treated breast cancer cohort and that this may be agent specific. This SNP has not previously been published in detail in breast cancer. As this is a tag SNP it is possible that another sequence in the haplotype is responsible for these findings. However, it is known that the SPP1 gene is regulated by TP53 (127) and recent work (currently unpublished – Rudland / Sibson – personal communication) from the University of Liverpool has validated this. Using recombinant cell lines where the polymorphisms were substituted, they have shown difference in SPP1 protein expression *in vitro* and *in vivo* according to TP53 status and the SNP variants of each gene present.

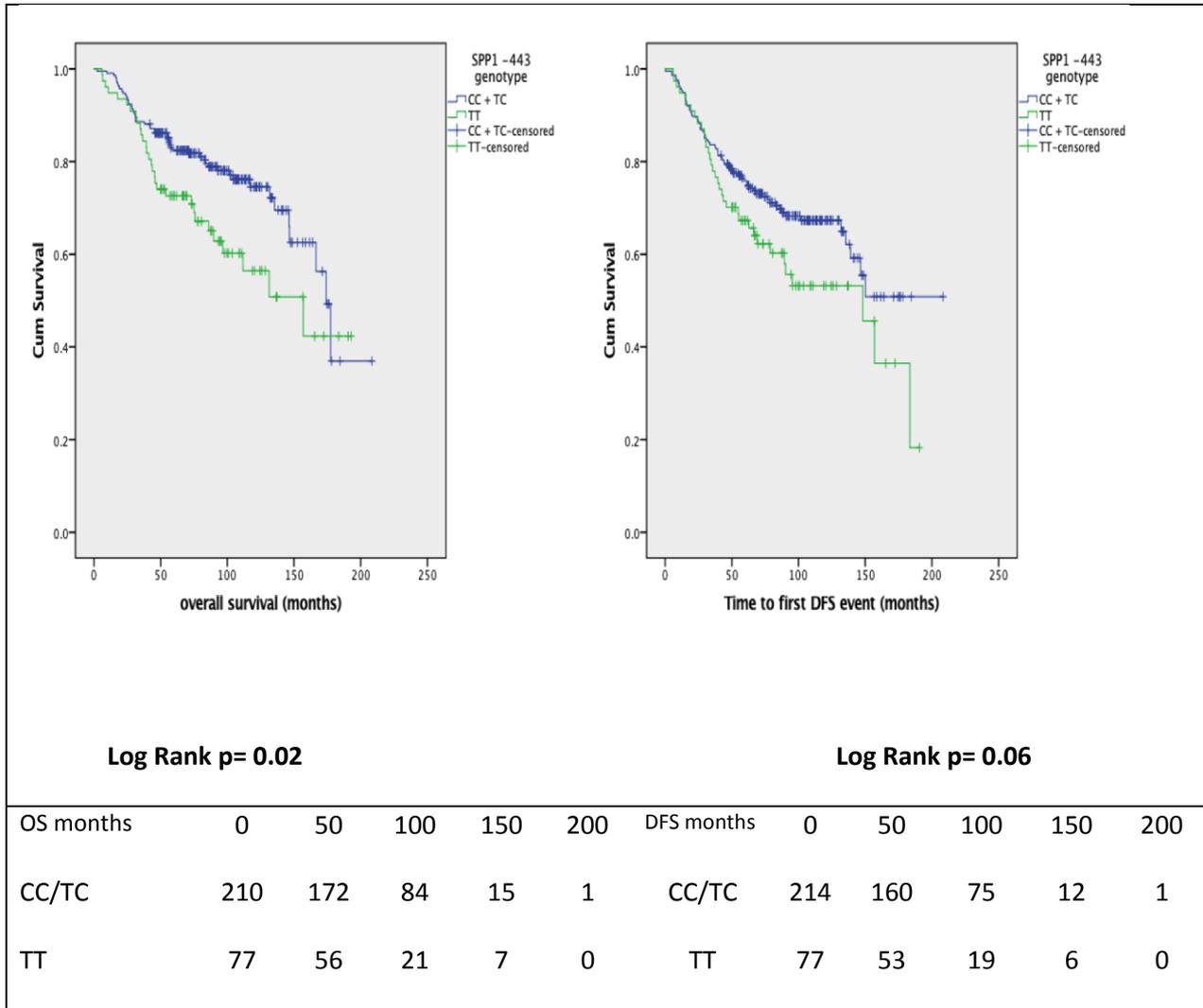


Figure 5.14 SPP1 -443 homozygote for TT compared to other variants and OS and DFS with numbers at risk tabulated below

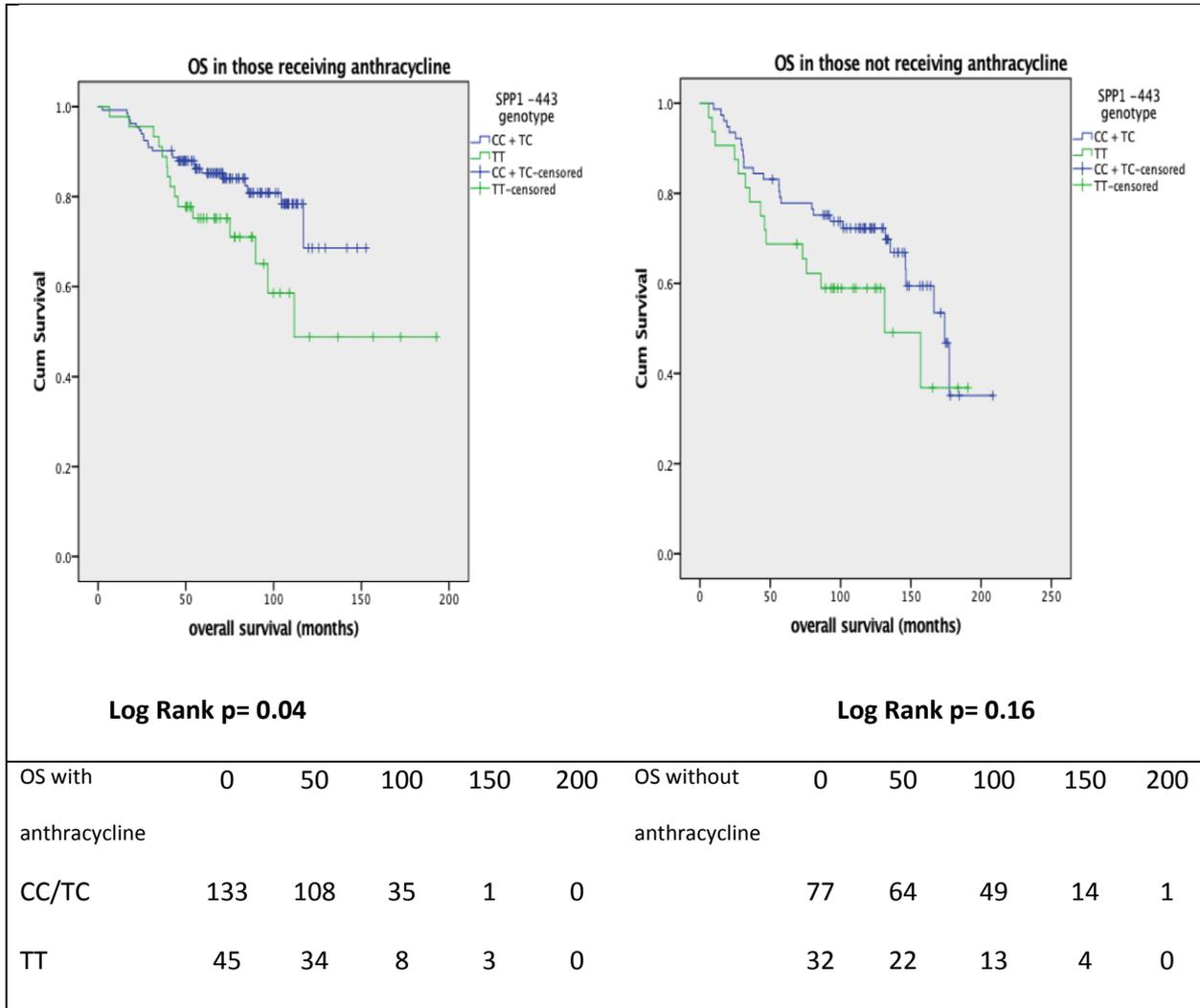


Figure 5.15 OS in SPP1 -443 variants stratified according to receipt of anthracyclines with numbers at risk tabulated below

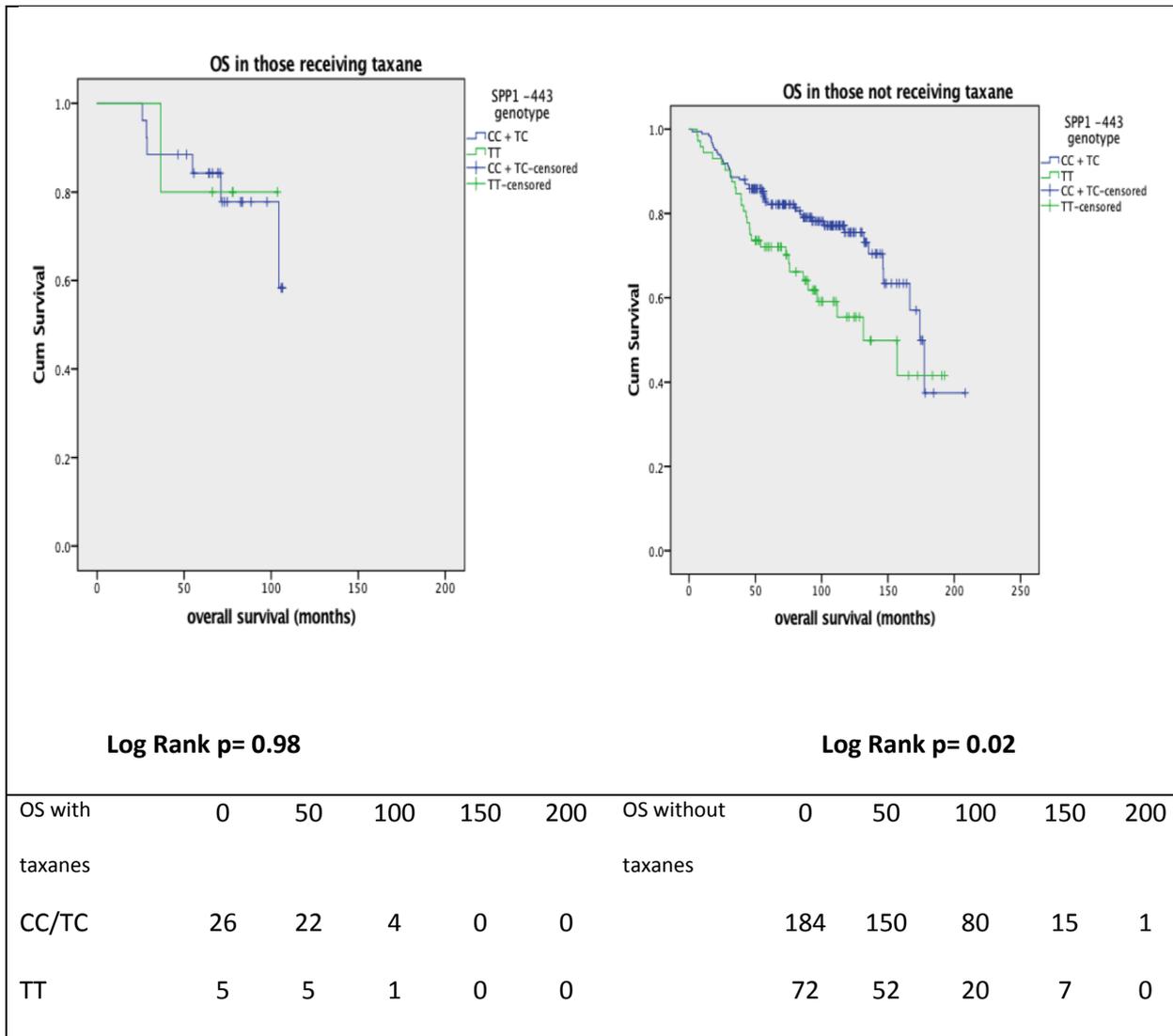


Figure 5.16 OS in SPP1 -443 variants stratified according to receipt of taxanes with numbers at risk tabulated below

5.5.3.2 XBP1- 166 G>C and outcome

There were no associations between XBP1 -66 G>C polymorphism and either OS or DFS. Although there is no statistical difference between genotypes when anthracyclines are given there is a noticeable trend. **Figure 5.17** shows the survival curves for each genotype stratified according to whether they received an anthracycline. The curves appear to come together more when anthracyclines are administered. This is particularly evident in the CC genotype. **Figure 5.18** shows only the CC variant and although not significant (log rank = 0.07) there is a definite trend towards improved survival amongst those receiving anthracycline and of CC genotype. The 5-year

survival improves from 53% to 95%. However the numbers are small; 17 received no anthracycline and 19 received it.

There is no alteration in OS or DFS when stratified according to taxane therapy.

XBP1 has been studied in relation to ER in breast cancer previously [458]. Upregulation of XBP1 protein was demonstrated in tamoxifen resistant breast cancer cells. Cells that over-expressed XBP1 no longer required oestrogen for cell growth. The c allele of XBP1 has previously been linked to overexpression of the protein.

This study also showed that the overexpression of XBP1 promoted cell survival by affecting the activity of the intrinsic apoptotic pathway affecting cell survival and the cell cycle distribution. Several genes were shown to be linked with XBP1 upregulation (**figure 5.19**).

Of note this includes the upregulation of BCL2 in overexpressing cells. This may provide some insight into our other finding of CC genotypes having a trend towards increased anthracycline sensitivity. Although overall there was no association between variant and outcome when the cohort was stratified according to whether they had received an anthracycline or not there was a pattern evident showing CC to change the shape of its survival curve.

Although non-significant, presumably due to the small numbers involved, when those carrying the CC SNP were given anthracycline the OS improved from 53% at 5 years to 95%. As previously discussed the exact mechanism of anthracycline remains elusive and is likely to be multi-factorial. Assuming that our CC variants correspond to increased XBP1 expression as previously demonstrated then we could hypothesize that the affect on the apoptotic pathway in these tumours is favourable to the action of anthracycline.

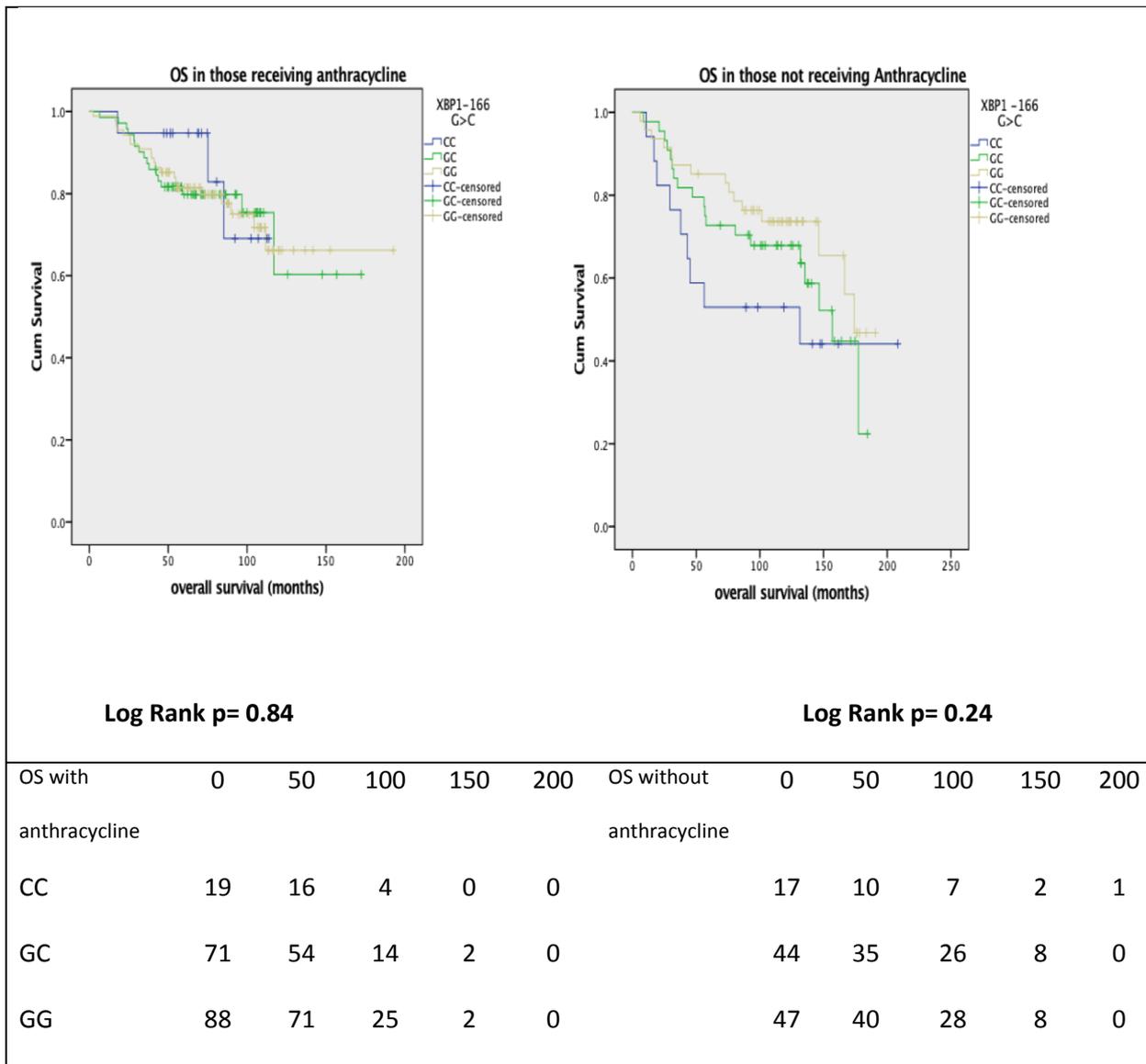


Figure 5.17 Survival curves for XBP1 -166 G>C when stratified to receipt of anthracyclines with numbers at risk tabulated below

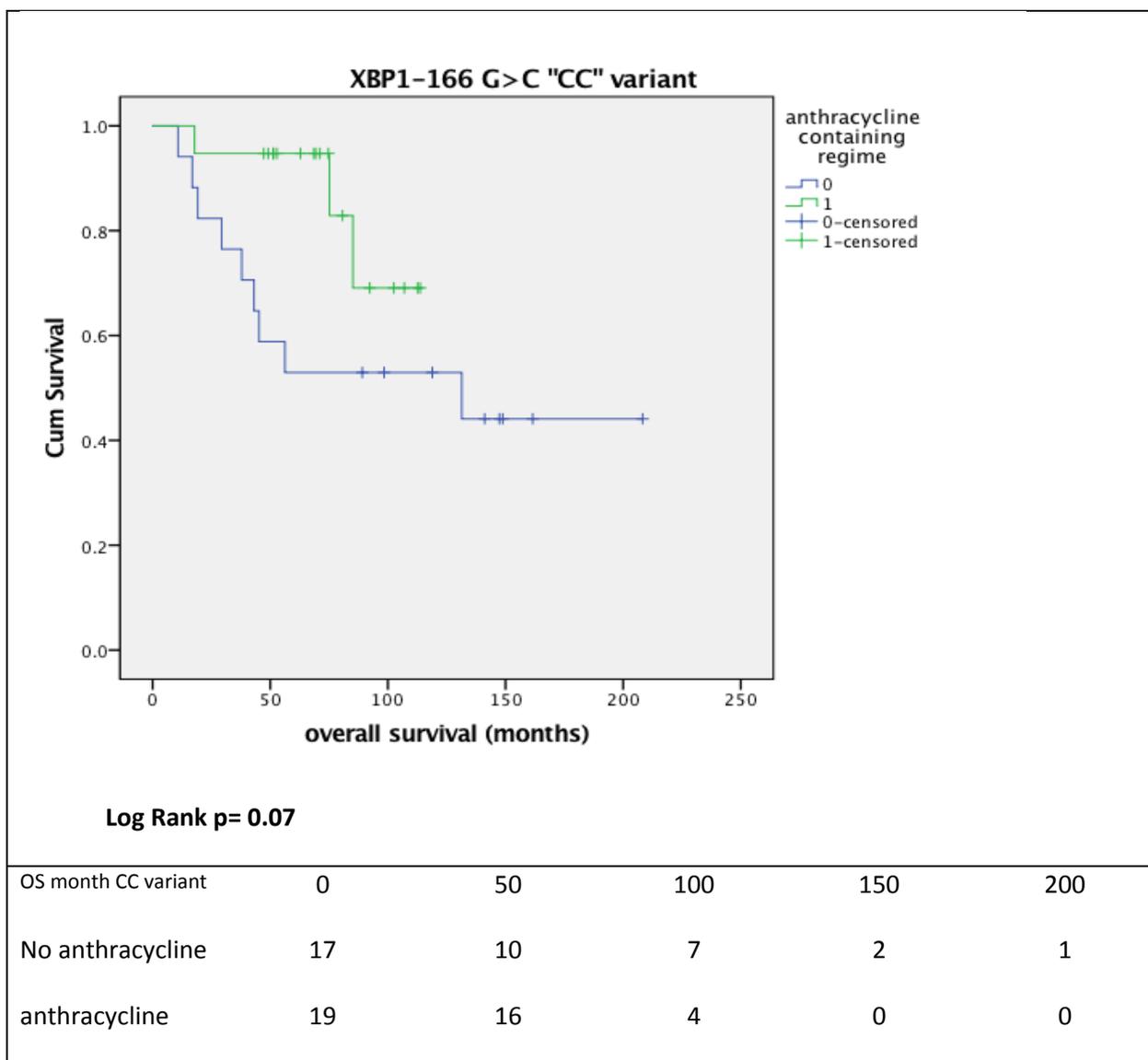


Figure 5.18 OS in XBP1 -66 CC variants according to receipt of anthracycline with numbers at risk tabulated below

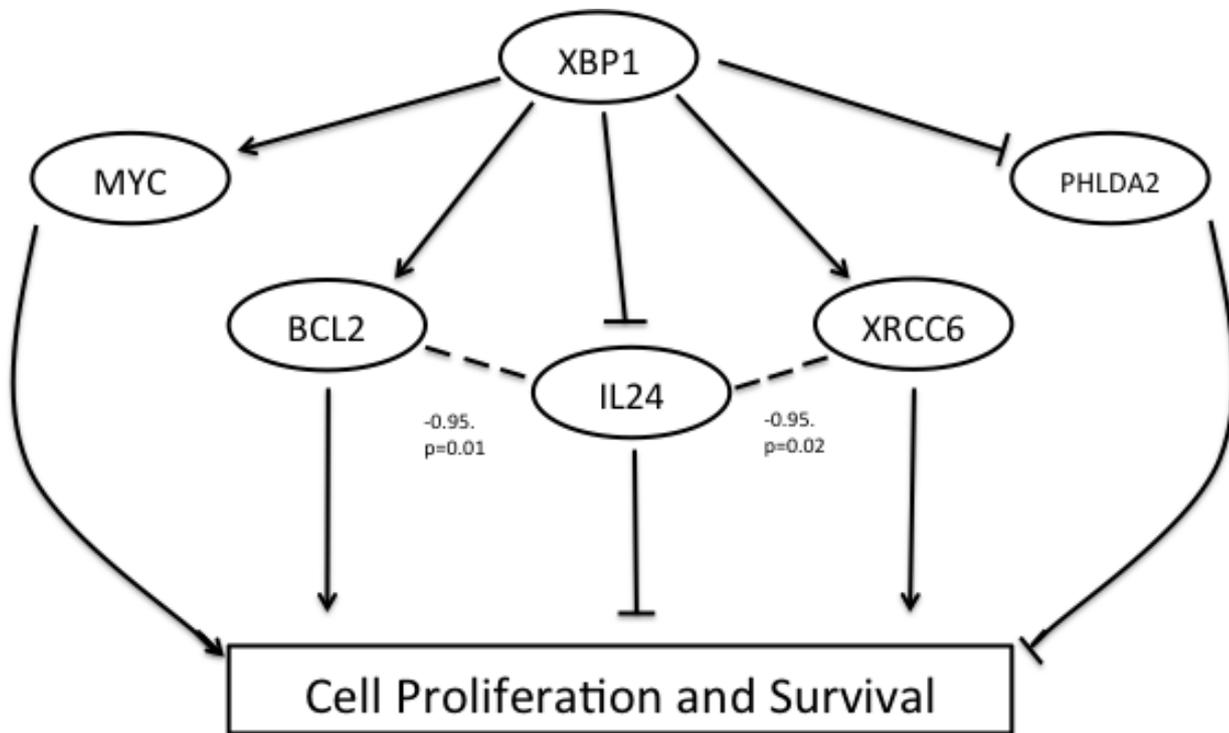


Figure 5.19 Model of XBP1-regulated gene expression in breast cancer cell proliferation and survival

Dashed lines depict relationships between molecules whose expression is significantly correlated in the microarray data; pair-wise correlation coefficients and *P* values are shown. Modified from Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines [458]

6. Chapter 6 - Discussion

The ultimate ideal in cancer therapy is to treat patients on a personalised basis, maximising clinical effectiveness and ultimately survival whilst minimising unwanted and harmful side effects. Indeed in a web-based survey among leading breast cancer specialists in 2007, the identification of molecular signatures for better selection of chemotherapy treatment benefit was voted as the most important priority for breast cancer research [693]. Genetic biomarkers provide a theoretical basis for this to occur and much research has been targeted towards identification of useful markers. This body of work has shown promise in the use of SNPs as simple biomarkers. We have demonstrated with our work with cyclin D1 genotype in particular, tendencies of patient genotypes to influence response to specific chemotherapy regimes. A poorly performing genotype had significant improvement when anthracyclines were administered. A molecular fingerprint such as this, can be used as a phenotypic indicator of early disease detection, disease progression, prognosis and prediction of therapy response. Biomarkers of treatment response can assist in rationale clinical decision-making.

Multi-gene signatures such as Mammaprint and Oncotype Dx have been shown to be useful in prognostic settings with survival associations. They are now being tested in trials, MINDACT (microarray in node negative disease may avoid chemotherapy) and TAILORx (trial assigning individualised options for treatment (Rx)) as predictive indicators in node negative patients[694-696]. The aim is to reduce the unnecessary chemotherapy given in this often difficult to manage group of patients. This is a promising start in personalised chemotherapy decisions

Although not yet used in routine clinical practice, individualised cancer therapies are not as elusive as they once were. Pilot sites in Australia are currently recruiting for the Individualised Molecular Pancreatic Cancer Therapy (IMPACT) trial. This is a randomised, open label, phase II trial comparing standard treatment (gemcitabine) with targeted treatments using genomic sequencing and protein expression. Based on work done by Jones et al [697] on core signalling pathways in pancreatic cancer, 3

subgroups have been identified. The HER2 *neu* over expression group where the treatment group will receive trastuzumab in addition to gemcitabine; the homologous recombinant defect (BRCA1/BRCA2, PALB2) mutation group where the treatment arm will receive fluorocil plus mitomycin C; and the anti EGFR responsive subgroup (KRAS wild type or KRAS 13 mutation) where erlotinib will be given alongside the gemcitabine. In pre-clinical studies null mice were grafted with individual patients tumours and treated with the above drugs according to mutation identified. The first two subgroups are of obvious interest to a breast cancer situation.

Malignancies can occur when mechanisms in various well-documented pathways become altered or behave according to an inbuilt deviant. We have discussed how various cancer pathways may work earlier in the introduction. Polymorphisms within these genetic profiles are one factor in cancer development. The IMPaCT study is a perfect example of how understanding the biology of cancer can lead to personalised medicine.

Our search for biomarkers has focused on SNPs as they are reliable, relatively easy to detect and can be detected with a simple blood test. SNP array chips can measure multiple genomic variations allowing whole genome or candidate gene phenotype association studies to be performed.

Many polymorphism and drug metabolism studies have demonstrated interethnic variation in drug response. An early example is that of variation in the N-acetyltransferase-2 gene causing interethnic differences in drug clearance, when studied in relation to amonafide; a site specific intercalating agent and topoisomerase II inhibitor which had shown activity in advanced breast cancer[688]. Interethnic differences are also widely documented in the use of warfarin, a commonly prescribed anti-coagulant [698].

We have not been able to comment on interethnic variation effect that may be exhibited in our population. Clinical data revealed that ethnicity was documented for less than half of our population. All were recorded as white. Although it is possible to determine ethnicity by running various SNP

panels, resource limitations (including DNA available, time and monetary) prevented us from doing so. Given that we know the population treated is predominately white and given our sample size, it is entirely probable that we would not have been able to make any significant conclusions. Of course, any further work repeated on larger subsets should include this information.

Table 6.1 summarises the SNP findings. In a hypothesis driven manner we have attempted to demonstrate that there is a suggestion that pathway based SNPs may play some role in predicting chemotherapy response. Our study has strengths in the moderate sample size and long follow up. We have clinically relevant endpoints of OS and DFS. We were blinded to clinical information whilst performing genetic analysis. There are of course limitations; clinical data was updated in a retrospective fashion. However we have tried to compensate for this by using clinical records from two hospitals to improve accuracy. We have not performed statistical correction for the large number of tests performed. When contemplating using the Bonferroni correction we thought it too stringent as when all clinicopathological features, genotypes and chemotherapy regimes were considered, over 300 tests had been performed. This would give a p value of 0.0001 to be significant. We felt that, as Bonferroni assumes all tests are independent of each other (which is not the case for this study) and as this was a hypothesis driven study, this inherent increase in type 2 errors resulting from Bonferroni adjustment did not guarantee a prudent interpretation of results. It has been argued in the literature that this type of correction shouldn't be used when assessing evidence about specific hypothesis [699]. It is possible that some of the trends we have reported may be false positives but, appropriately powered follow up studies to this pilot study, would be helpful in demonstrating this without the risk of ignoring potentially important clinical associations.

SNPs were derived from tumour samples rather than germline DNA. There simply was a not blood sample available for the majority of patients. We know from previous work done within the group and information given to us by the tissue bank that most specimens are a maximum of 70% cellularity. By

developing assays with 90-95% sensitivity we hope to have accounted for any loss of heterozygosity.

Prospective studies would always be better performed using germline DNA.

We have also tried to demonstrate a difference in response to particular chemotherapy regimes given.

We have shown particular interest in 2 of the commoner drug classes given in today's practice that have particularly toxic side effects- anthracyclines and taxanes. Being that this is a historical data set only a small proportion received taxanes. Despite this we have still shown some interesting trends with some SNPs (Rs 1042522, Rs 2279115, Rs 4645878, Rs 1695, Rs 4802101, Rs 11730582) although not enough to draw any definitive conclusions. An ideal next step would be to interrogate these SNPs using blood samples from patients recruited into a taxane based trial meaning larger datasets and more meaningful statistics.

SNP	Clinical association	Outcome trends	Regime specific trends
P53 codon 72	NPI group	GG had worse OS and DFS	GG may be more sensitive to taxanes
MDM2 intron -309	None	None	None
CDKN1A 3 UTR	None	None	None
CDKN1A codon 31	None	None	None
BCL2 -938	None	AA had worse OS	CC may be sensitive to taxanes
BAX -248	Age, menopausal status	None	Heterozygotes may have improved outcome when either anthracyclines or taxanes given

Cyclin D1 870	None	None	GG had better survival if given an anthracycline.
GSTP1 codon 105	None	None	AA had better survival if given a taxane
GSTP1 codon 114	Lymph node status	None	None
ABCB1 677	Age and menopausal status	None	None
MTHFR 1298	None	None	None
MTHFR 677	None	None	None
CYP2B6 Nr gene5	None	None	Heterozygotes trended towards improved survival if given a taxane
CYP2B6 Intron 3	Age	None	None
CYP2B6 Intron 5	Age and LVI status	None	None
HER2 codon 655	ER status	None	None
HER2 -1170	ER status	Heterozygotes had improved OS	None
SPP1 -443	None	TT had poorer survival	TT appeared less sensitive to anthracyclines and more sensitive to taxanes
SPP1 -66	None	None	None

XBP1 -166	None	None	CC had improved outcome when given anthracycline
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Table 6.1 Summary table of all SNPs analysed and the clinicopathological and outcome associations

Despite earlier diagnosis through breast screening, improved oncological surgical practices, a better understanding of hormone receptors, advances in cytological agents and targeted therapies breast cancer still remains one of the biggest causes of cancer death in women. Clearly, breast cancer treatment is not a one size fits all program. Prognostic factors such as tumour size, grade, nodal status go some way into treatment planning and along with patient factors such as age and co-morbidities are the mainstay of clinical decision making in current practice. Given the multiple subtypes of breast cancer, individualised patient treatment plans are likely to hold the key to improved survival and biomarkers are the ideal blueprint on which to base decisions

Phenotypic differences are not entirely based upon genetic makeup but are influenced by external factors such as the metabolic state, disease and immune system of an individual as well as environmental factors. It seems likely therefore that rather than one biomarker, a combination of multiple biomarkers will be necessary. Combinations of biomarkers are becoming part of clinical practice for prognostication of tumours, for example Oncotype Dx previously discussed. Whilst the search for biomarkers has undoubtedly increased our knowledge and understanding of tumour biology with the exception of HER2 *neu*, a reliable marker of tumour response to cytotoxic agents in everyday clinical use is lacking.

Future work based on our findings would be to explore further SPP1-443, BCL2-938 and Cyclin D1 splice variants in particular. In this hypothesis testing study these markers have shown some promise , albeit in a selected retrospective cohort that they may be useful in starting to select out responders to chemotherapy. There has already been some work performed by a Liverpool group that has shown

that SNP variation in SPP1 -443 is associated with protein expression. Prior to this work very little information was available on this SNP.

Point of care testing is fast becoming a reality with bed side “mini- laboratories” which may be able to provide genomic or proteomic information in a quick, cost effective manner [700]. To some extent, for personalised medicine, the future looks likely to assess many prognostic and predictive biomarkers to assess need and response to adjuvant or neo-adjuvant therapies. It seems likely that SNPs will have a role to play.

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