Predictive markers for chemotherapy response in breast cancer

Maryvonne Steenkamer
(3282384)

Master thesis for Cancer Genomics and Developmental Biology, Utrecht University, the Netherlands

Under the supervision of Sven Rottenberg, PhD. The Netherlands Cancer Institute, Amsterdam, the Netherlands

August 2012

cancer genomics & developmental biology

The Master's & The PhD Programme

Universiteit Utrecht

NKI-AVL

Table of Contents

Abstract **3**

1.0 Introduction **4**

1.1 Prognosis v.s. predictive markers 4

2.0 Background on breast cancer **5**

2.1 Epidemiologic features 5

2.2 Pathological features 6

2.3 Clinical presentation, diagnosis & conventional treatment 7

3.0 Chemotherapy **7**

3.1 Various types of chemotherapy 8

4.0 Which approaches have been used to identify predictive markers for chemotherapy **8**

4.1 Cell lines 9

4.2 Human tumor material 10

4.2.1 In situ analyses (Immunohistochemistry and in situ hybridizations) *10*

4.2.2 Gene expression profiling *11*

4.2.3 Whole-genome sequencing *13*

4.3 *In vivo* models 14

4.3.1 Xenotransplants of human cancer cell lines and human biopsies *14*

4.3.2 Genetically engineered mice *15*

5.0 Why is it difficult to discover predictive markers for chemotherapy **17**

6.0 What may improve the identification of predictive markers for chemotherapy response **18**

7.0 How can we apply preclinical data to the clinic? **19**

7.1 Monitor the patient during treatment 19

7.2 Combination Therapy 20

7.3 Improvement of the design of clinical trials 21

8.0 Discussion **21**

9.0 References **24**

Abstract

More than 13,000 women are diagnosed with breast cancer in the Netherlands each year. 86% of breast cancer patients survive 5 years after diagnosis, however, many patients still succumb to this disease eventually. In addition to surgery, radiation, or targeted therapy (*e.g.* hormone or growth receptor antagonism), patients with an unfavorable prognosis are usually treated with chemotherapy. To identify the therapy of which an individual patient has most benefit, it is a major goal of molecular oncology to find markers that predict therapy response. Such predictive markers are already common in decisions to prescribe targeted therapy to breast cancer patients. In contrast, markers that predict the tumor response to chemotherapy are elusive. In this study I review different techniques and approaches used to discover markers that predict breast cancer response to different chemotherapy treatments. The advantages and disadvantages of these techniques are discussed and suggestions are made about what could be improved in the future. The hope is that predictive biomarkers can contribute to personalized chemotherapy treatment in breast cancer.

1.0 Introduction

Breast cancer therapy is a major burden for patients, because therapy often comes with a range of side effects. As a consequence of these side effects, women who receive chemotherapy as breast cancer treatment are more frequently hospitalized[1]. This would be acceptable if all patients benefited from the therapy. However, several breast cancer patients treated with toxic cytostatic drugs may experience only the side effects of the chemotherapy and not the desired tumor-shrinking effect. It is of importance to identify the right therapy at an early stage to kill off as many tumor cells as possible and thereby avoid or delay the development of drug resistance. Unfortunately, at present there are no reliable approaches routinely used in the clinic to identify the poor responders before chemotherapy is given.

We know that breast cancers that look alike histologically, have substantial molecular differences which probably contribute to the variable responses to anti-cancer therapies. In fact, breast cancer is a very heterogeneous type of cancer[2, 3]. On the one hand this heterogeneity is clearly a complication to find the optimal treatment. On the other hand, it offers the opportunity to discovery molecular patterns that may be causally linked to therapy response.

To avoid extensive side effects, unnecessary suffering and high costs of anti-cancer chemotherapy, it is of interest for the patient, physicians and insurance companies to choose the right chemotherapy for the breast cancer patient from the start.

1.1 Prognostic versus predictive biomarkers

There are two types of clinically relevant biomarkers, and there is a fundamental difference between them[4]. Prognostic biomarkers are features of the tumor which predict the general outcome for the patient (fig. 1A)[5] . Prognostic gene expression profiles are used to assess the chance of tumor recurrence or metastasis[4]. In contrast, predictive markers detect features of the tumor, which can predict the response to a specific therapy before treatment (fig. 1B). Traditionally, the choice of therapy is based on the estimated prognosis. The prognosis is determined from anatomical and pathological features of the cancer; tumor size, grade and lymph node status [6]. If the patient is relatively fit, an adjuvant chemotherapy is offered. Despite the overall benefit that patients with a poor prognosis have from chemotherapy, many patients are treated and only suffer from the side effects. Unfortunately, the anatomical and pathological features provide only a weak indication for chemotherapy sensitivity[6].

Some markers can be used as both predictive and prognostic marker. For example, breast cancers that express the estrogen receptor (ER) have a better clinical outcome than triple-negative breast cancers. In addition the presence of ER expression is a predictive marker for receiving hormonal therapy. However, even if a patient has ER-positive tumor cells, response to hormone therapy (*e.g.* with tamoxifen) is not guaranteed[4]: about 75% of the women with ER-positive breast cancer have a benefit from the treatment. Intriguingly, about 19% of the patients with ER-negative breast cancers still benefit from this targeted hormonal therapy[7].

Over the last years various prognostic gene expression signatures have been developed (comprising genes that regulate cell cycle, invasion, metastasis and angiogenesis[8]) to distinguish patients who have a high chance of metastatic disease from patients with little chance of metastatic disease[9]. One of these gene expression platforms (Mammaprint®) was validated to be of more clinical utility than the traditional prognosis for certain breast cancer subtypes[10]. For patients in the gene signature high-risk group, 10-year overall survival was 69%; for patients in the low-risk group, the 10-year survival rate were 89%[10, 11]. Good prognostic profiles are mostly those with low proliferative rates. It is therefore often assumed that a breast cancer with a good prognosis signature may be less

sensitive to chemotherapy than tumors with poor prognosis signature. In a study of Straver *et a.l*[11], 32% of the poor prognostic signature tumors had a pathological complete remission after chemotherapy in contrast to 9% of the good prognostic signature (P = 0.023)[5, 6, 12-14]. This makes this prognostic gene expression signature also a predictive biomarker for chemotherapy to some extent.

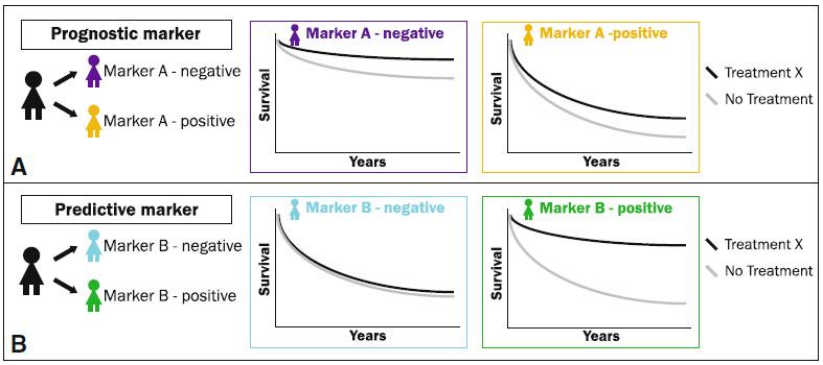

Figure 1 A; prognostic markers indicate the survival of the patients independent of treatment. In this figure, having marker A is a poor prognosis. B; The predictive marker predicts a better patient outcome in the presence of a specific therapy; survival will increase with the specific therapy. If the tumor is negative for marker B, there is no difference in survival between treatment X or no treatment. However, if the predictive marker for treatment X is present, patient survival increases significantly (Figure derived from[15]).

In contrast to prognostic markers, predictive markers with a high positive and negative predictive value are still elusive. In this literature study, different approaches that are used in clinical research to discover predictive markers for chemotherapy treatment are discussed. The positive and negative aspects are weighed and suggestions about how to approach and improve research to these predictive markers are made.

2.0 Background on breast cancer

The female breast is made up of milk producing glands (the lobules) and the tubes that carry the milk from the lobules to the nipple (ducts). The surrounding stroma comprises fat, connective tissue, blood and lymphatic vessels[16]. Breast cancer is caused by the malignant growth of the epithelial cells of the breast (carcinomas). It is the most frequently occurring cancer in women in the Netherlands (33% of all tumors in females in 2008)[17]. The world health organization classified tumors of the breast since 2003 in two categories: benign- and invasive tumors. In invasive tumors, the cells have crossed the epithelial lining of the breast tissue and are able to metastasize to lymph nodes and other organs.

2.1 Epidemiologic features

In 2010, 13257 females were diagnosed with breast cancer in the Netherlands[17]. In the same year 3213 females died from the disease (24.2%). 1 in 8 (12.08%) women develop breast cancer in their lives (from 0 to 85 years), but the chance to develop breast cancer before 30 years is small (0.08%). The incidence of breast cancer also rises due to yearly screening programs[18]. Breast tissue of males can also give rise to cancer: the incidence of breast cancer in males was 94 cases in 2010 (0.7% of the total breast cancer incidence)[17]. For breast cancer the survival at 5 years after diagnosis is 82% measured in the period of 1989-2008. Taking only the numbers into account from 2004-2008 the five year survival rate has increased to 86%. Hence, mortality numbers follow a downward trend over the years. It has been shown that this decrease is most likely not caused by the introduction of mammography screening[19]. Mortality decreases between countries were similar even if there was a

time difference of about 10-15 years between the implementation of mammography screening[20]. However, mortality in the Netherlands compared to other west European countries is still one of the highest with 20.5% of breast cancer patients dying in 2008 (Fig. 2). Other high mortalities are Ireland (21.8%), Belgium (21%) and Denmark (20.8%). The countries with the lowest breast cancer mortality rate are: Spain (12.9%), Portugal (13.5%) and Luxemburg (14.2%)[21].

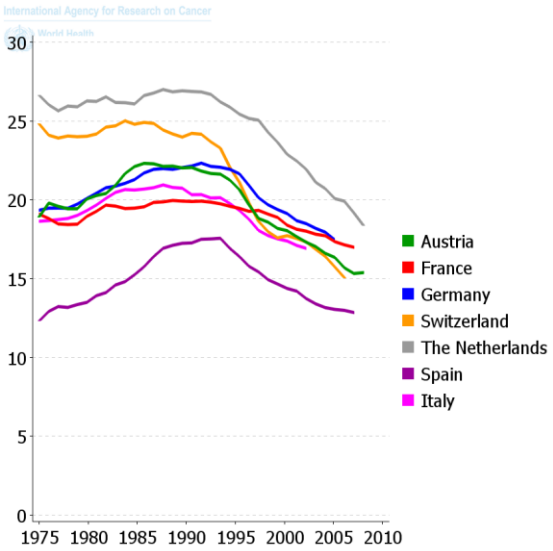

Figure 2 Trends in mortality from breast cancer in selected countries: age-standardized rate per 100.000 cases [21].

2.2 Pathological Features

Many breast cancers begin with an increase in normal cells (hyperplasia) that then transform to atypical breast cells (atypical hyperplasia) followed by carcinoma *in situ* (non invasive), carcinoma with micro-invasion to invasive breast cancer. However the speed of progress differs between individuals and some cancers may never proceed beyond the *in situ* disease. In a histological classification, breast cancers are divided in ductal or lobular breast cancer. 11% of breast cancer patients have ductal carcinoma in situ (DCIS), which is a non-invasive breast tumor. The incidence of DCIS has increased remarkably in the Netherlands as an effect of mammography screening. Despite the fact that DCIS is a benign disease, it is considered a precursor state of invasive ductal carcinoma (IDC)[18]. IDC is the most common invasive breast cancer[16]. Lobular carcinoma in situ (LCIS) is a non-invasive carcinoma from the lobes in the breast. LCIS is less common and it may progress into Invasive Lobular Carcinoma (ILC)[16]. There are more less common types of breast cancer: Inflammatory breast cancer, which accounts for 1 – 3 % of all breast cancers. Paget disease of the nipple, which starts in the breast ducts and spread to the skin of the nipple and Phyllodes tumor, which develops in the stroma of the breast[16].

Over the past decade this morphologic classification has been challenged and complemented by the molecular classification of breast cancer[2,8,9,22,23]. In particular the group of Perou investigated the variation in gene expression patterns that may account for the biological diversity by using cDNA microarrays. Four main molecular classes of breast cancer have been distinguished by these gene expression profiling. **The basal-like breast cancers** largely overlap with the group of "**triple-negative" breast cancer**[14]. These tumors are estrogens receptor (ER) negative, progesterone receptor (PR) negative, and human epidermal growth factor receptor 2 (HER2) negative. **Luminal-A tumors** are mostly ER positive and proliferate rather slowly, whereas **Luminal-B tumors** are ER-positive and have a high gene expression of proliferation markers. **HER2-positive** cancers are tumors which show amplification and overexpression of the *HER2* gene[2].

2.3 Clinical presentation, diagnosis and conventional treatment

The earliest sign of breast cancer is usually a new lump or a mass within the breast, but it can also include a swelling of (a part of) the breast, skin irritation, pain, nipple retraction, redness or nipple discharge[24]. Due to regular screening of the breast using mammography (which is a X-ray of the breast) the number of breast cancers that had no clinical symptoms yet has increased. If results of this physical examination suggest breast cancer, additional tests will be performed. Visualization can be done by mammograms, MRI, ultrasound or a ductogram (where a contrast medium is injected in the nipple). If the results of these test show some abnormalities, a more invasive strategy is applied by taking a sample from the suspicious area. This is called a biopsy and it is the only way to tell if cancer is really present. The types of biopsy range from fine needle biopsy to a surgical open biopsy[24]. A pathologist can determine morphologically from the specimen whether it is cancer and which type. Also the grade of the tumor, the differentiation of the cells and the mitotic count are determined[24].

There are several techniques to treat cancer and they can be roughly divided in local or systemic therapy. Local therapy is intended to treat the tumor site without affecting the rest of the body. Surgery and radiation therapy are examples of such local therapy. In contrast, systemic therapy intends to reach tumor cells that may be spread throughout the body. For this purpose, a drug is administrated which will be distributed through the whole body. It is of importance that the effective dose of a systemic drug gives more desired (*e.g.* eradication of cancer cells) than adverse effects (*e.g.* toxicity of healthy tissue). This is called the therapeutic window of the drug. Chemotherapy, hormone therapy and targeted therapy are systemic therapies. Local and systematic therapies are given sequentially to patients. If a drug is injected before the main treatment (most often surgery), it is called neo-adjuvant treatment. The goal of this approach is to reduce the tumor volume before surgery to be able to perform a breast-sparing operation. If a systemic therapy is given after tumor excision to eliminate residual local or disseminated cancer cells, it is called adjuvant treatment. Chemotherapy agents target individual proteins that are required for cell division, like microtubules or topoisomerases. However, these proteins are also important in non-cancer cells, resulting in side effects due to toxicity. Hormonal therapy is given to block hormone receptors on tumor which still contain this feature from the original hormone depended glands/tissue. Targeted therapy tries to selectively target proteins which are activated in tumor cells and drive tumor growth (like the oncogene BRAF in melanoma, overexpressed kinases in growth pathways or *HER2* amplification in breast cancer). However, proteins targeted in tumor cells are also present in normal tissues, and therefore also small inhibitory molecules or antibodies have side effects. Moreover, although being called targeted, small inhibitors are not necessarily specific and also inhibit related enzymes, resulting in side effects. In contrast to hormone receptor or HER2-positive breast cancers, there is currently no validated targeted therapy which is routinely used in the clinic to treat triple-negative breast cancer. New therapeutic approaches, such as Poly (ADP-ribose polymerase (PARP) inhibitors inhibitors to target DNA repair deficiencies, are being tested. At present, however, chemotherapy is the therapy of choice for many patients who face a poor prognosis[25].

3.0 Chemotherapy

Chemotherapy compounds (administered as single drugs or in combination) can be derived based on their origins, for example: chemicals, antibiotics or plant-derivatives. The first drug used for cancer chemotherapy was not originally intended to work as anti-cancer drug. Mustard gas was used as a chemical warfare agent during world war 1. During world war 2 a group soldiers were accidentally exposed to (a dilution) of the gas and were later found to have very low white blood cell

counts. The link was made between the highly proliferating white blood cells in patients with advanced lymphomas and these soldiers. In the clinic small doses of mustard gas were then given to these patients to decrease their white blood cell count. Since then, several cytotoxic agents with different mechanisms of action have been identified and further developed.

3.1 Various types of chemotherapy

There are several kinds of chemotherapy. The most frequently used chemotherapies for breast cancer are listed in table 1. Cyclophosphamine (previously called nitrogen mustard gas) is an alkylating agent which acts through the covalent binding of alkyl groups to cellular molecules. Other alkylating agents are cisplatin, which has an platinum group. There are also anti-metabolites (*e.g.* Methotrexaat; MTX), which, when incooperated into DNA, cause miscoding during DNA replication[26]. Another example are 5- fluoropyrimidines (like gemcitibine and 5-fluorouracil) which incorporate fluorouridine nucleotides into DNA and thereby triggers DNA breaks. Anthracycline-based chemotherapy (*e.g.* doxorubicin and epirubicin) inhibits topoisomerase 2 keeping the DNA in a supercoiled state causing double stranded breaks (DSBs) in the DNA[27,28]. As a cell enters mitosis, the presence of the stabilized double-strand breaks is thought o trigger either mitotic catastrophe or apoptosis[29]. For chemotherapy that stops mitosis or inhibits cell cycle by stabilizing the microtubule taxanes (*e.g.* docetaxel, paclitaxel) and vinorelbine are frequently used[26].

To achieve a broad attack on breast tumor cells and avoid resistance to a single drug calss, different types of chemotherapy are combined (in series or parallel or with a targeted therapy). A downside of this approach is that the toxicity can accumulate; hence, a lower dose of each compound within the chemotherapy cocktail is given that the dose of the agent if it was administered as single agent. This may substantially reduce the effectiveness of the individual drug within the combination.

Table 1 Overview of different types of chemotherapy agents, their effect and trade names commonly used for anti-breast cancer therapy.

Chemotherapy type	Mechanism of action	Trade name
5-fluoropyrimidines, nucleoside analogs.	Cooperates Fluorouridine nucleotide into DNA, causing DNA breaks	gemcitibine, 5-fluorouracil
Alkylating agents	Covalent binding of akyl or platinum groups to cellular molecules	cyclophoshamine, cisplatin
Anthracyclines	Inhibition of topoisomerase 2 which causes double stranded breaks[27,28].	doxorubicin, epirubicin
Antimetabolites	Cooperation of metabolites into DNA causing miscoding during DNA replication[26].	methotrexaat; MTX
Anti-mitotic agent	Inhibits cell cycle progression by stabilizing microtubules	docetaxel, paclitaxel, vinorelbine

4.0 Which approaches have been used to identify predictive markers for chemotherapy

To achieve the holy grail of getting the right drug to the right patients from the start of treatment, extensive research has been done to discover predictive markers for sensitivity to a certain chemotherapy. Several approaches have been taken to identify predictive markers for chemotherapy. The first one is the use of cell lines derived from human tumors. Such cell lines are mostly used for drug screening. Second are analyses of human tumor material like micro-arrays, immunohistochemistry and sequencing. A third approach is to test *in vivo* models, comprising

genetically engineered mice, xenotranplantation of patient-derived tumor material, or xenotransplantation of human cancer cell lines.

4.1 Cell lines

In 2006, Potti *et al.* implemented a well-recognized study to predict sensitivity to individual chemotherapeutic drugs. Their approach was to combine drug response data, together with microarray gene expression data from an *in vitro* panel containing 60 human cancerous cell lines (NCI-60, US National Cancer Institute). They identified cell lines that were most resistant or sensitive to a cytotoxic drug, and looked at which genes correlated with the drug sensitivity or resistance. From these data they developed models that could potentially predict response to single and combinations of cytotoxic chemotherapeutic regimes[30]. Clinical trials had already started when Dr. Baggerly and Dr. Coombes found out that the study was a listing of flaws and corrupt data. Other research groups that tried to reproduce the results also failed and the article of Potti *et al.* was retracted in the beginning of 2011. This incidence is very unfortunate for this field of research.

Nevertheless, research to predict anti-cancer drug sensitivity using cell lines is continuously identifying new markers that may indeed be useful to predict therapy outcome in patients. Recently, the Cancer Cell Line Encyclopedia (CCLE) compiled gene expression, chromosomal copy number and massive parallel sequencing data from hundreds of human cancer cell lines together with the pharmacological profiles of 24 anticancer drugs to predict drug sensitivity[31].This study showed that the expression of *SLFN11* correlates with sensitivity to two TOP1 inhibitors (irinotecan and topotecan) in Ewing's sarcoma cell lines[31]. Studies are not only based on single cell lines and are often combined with other techniques. Garnett *et al.*[32] combined screening data of 368 cell lines for 13 different kinds of chemotherapeutics together with sequencing the commonly mutated genes, genome-wide analysis of copy number gains and microarrays. They found that the genomic associations they identified for the 13 clinically approved cytotoxic chemotherapeutics used in the panel, were less significant than for targeted drugs. This indicates that their chosen single gene biomarkers are less informative for drugs with broad action in cancers[32]. This study is ongoing (http://www.cancerrxgene.org) and new results suggest that cell lines expressing the EWS_FLI1 translocation gene are more sensitive to cisplatin treatment with high significance ($p < 0.001$) and cell lines with *BRCA1* expression have a increased resistance for docetaxel. In a pre-clinical study of Geutjes *et al*.[33] a correlation was found between the expression of deoxycytidine kinase (DCK), a rate limiting enzyme for activation of deoxyribonucleoside prodrugs (nucleoside analogs), and clinical outcome. DCK is one of the genes in the Mammaprint® gene expression array and a high expression was correlated with a poor prognoses for the breast cancer patient. Thus, patients that have a poor prognosis may be susceptible to treatment with nucleoside analogs (*e.g.* gemcitibine). To support this finding a breast cancer cell line screening was conducted and a relationship between DCK levels and sensitivity to nucleoside analogs was found. The data imply that it may be helpful to exploit DCK expression in breast cancer to select patients which are likely to respond to nucleoside analog chemotherapy[33].

Although cell lines are unavoidable to investigate basic mechanisms, there are also concerns about their use. One criticism is that cell lines can only be derived from some cancers, and we do not really understand the mechanisms underlying this selection. In addition, those cell lines that do grow *in vitro* differ genetically from the primary tumor they originate from[34]. This may be due to selecting the fastest growing cells which accumulating new mutations as they adapt to artificial culturing conditions[35,36]. Because of this, one cannot conclude that cell lines fully reflect the original tumor of

the patient. In a petri dish the cells grow into a two-dimensional culture in high oxygen with less interaction with other cancer cells. Moreover, stromal cells and angiogenesis are missing. There are also patient-associated factors like drug metabolism and the tumor micro-environment (immune response, interstitial pressure and vascular leakiness) that cannot be mimicked[37]. All of these complex items may cause the difference in gene expression that is found when cell lines are compared to their original tumor.

An important reason for the use of cell lines is the possibility of high throughput screening with potential chemicals which is not possible *in vivo.* Careful selection and characterization of cell lines is needed to determine how close the cell line resembles the conditions in which you want to find the predictive biomarker; then they can be useful to evaluate the potential of new therapeutic approaches[4].

4.2 Human tumor material

4.2.1. In situ analyses (immunohistochemistry and in situ hybridizations)

Immunohistochemistry (IHC) is a technique that visualizes proteins in the cell by using antibodies with a fluorescently or enzymatically labeled tag that binds to specific epitopes in the tissue. This technique is popular because it is cheap and easy to do when a specific antibody for the protein of interest is present. A disadvantage is that one can only visualize a few protein at a time, making it very elaborative if a range of proteins needs to be detected. This technique is already routinely used to determine the estrogen- and progesterone receptor status and for the presence of HER2 amplifications. In the search for predictive biomarkers, researchers have used immunohistochemical analyses for diagnostics and localization of biomarkers /differentially expressed proteins in tumor biopsies/specimen.

In a study of Tewari *et al.*[38] breast cancer pathologists interpreted the pre-chemotherapy specimen of patients with invasive breast cancer (no metastasizes). Immunohistochemistry staining was performed using antibodies for markers like estrogen receptor, progesterone receptor, HER2 receptor, P53 protein and two anti-apoptotic proteins (Bcl-2 and BAX). After biopsy, the patients received 2-6 round of chemotherapy (combination of cyclophosphamide, adriamycin, 5-fluorouracil and epirubacin). After therapy, the researchers tried to find a correlation between the markers and clinical outcome of the patient. The tumor reduction rate per chemotherapy cycle was significant higher in BAX-positive and Bcl-2-negative tumors[38]. This implies that BAX-positive and Bcl-2 negative tumors are more sensitive to this combination of chemotherapy. They did not find ER, PR and HER2 as a predictive marker because the majority of their patients (64%) had triple negative breast cancer, which is already known to response better to chemotherapy[14,39]. Although they found that apoptosis-related genes seemed to influence the response to neoadjuvant chemotherapy, they stated that their patient number (n=50) was not enough to simultaneously analyze the predictive markers they had chosen[38].

Hypoxia, a pathological feature of many solid tumors, is also an important factor for chemotherapy response. In an epirubicin-based clinical trial, researchers found that a higher hypoxia inducible factor-1 (*HIF-1α*) expression is associated with a significantly shorter disease-free survival in patients with ER-positive breast cancer but not in ER-negative breast cancer[40]. A factor that is correlated with the expression of *HIF-1α* is hepatocyte growth factor activator inhibitor type 2 (*HAI-1*). High levels of HAI-1 a predicter for poor clinical outcome to pre-operative anthracycline (epirubicin)[40]. The same conclusion was found in an immunohistochemistry study to carbonic anhydrase 9, which is an important marker of hypoxia in breast tumors. Patients received

doxorubicin (anthracycline) as adjuvant therapy and were followed more than 10 years. They demonstrated that *CA9* expression is correlated with worse progression free and overall survival rates for breast cancer patients treated with doxorubicin (independent of HER2 status). This study provides evidence that using immunohistochemistry to detect *CA9* in excised breast tumors may be of clinical use to not use doxorubicin and choose for another more appropriate chemotherapy regimen[41].

Miyake *et al.*[42] discovered that breast tumor that express glutathione S-transferase P1 (GSTP1), which is a metabolic enzyme that detoxification toxic substances and anticancer drugs by conjugating them with glutathione, are more likely to be ER negative tumors. They administrated P-FEC (5-fluorouracil, epirubicin, cyclophosphamide) in a neoadjuvant setting and determined the reduction rate in tumor size using MRI. They found that the pathological complete response was significantly higher in GSTP1-negative tumors then GSTP1-positive tumor among ER- negative tumors but not ER-positive tumors. Given the conclusion that P-FEC regime can be used in GSTP1-negative, ER-negative breast cancers. These studies are preliminary and need to be confirmed in future studies with larger number of patients[42].

The transcription factor c-Myc, which regulates the gene expression of growth factor and hormone genes, has been put forward as a predictive marker for chemotherapy in metastatic breast cancer[43]. Tumors from metastatic breast cancer patients were investigated for the amplification of *c-Myc* with chromogenic *in situ* hybridization. The amplification of this gene is already frequently related and observed in different tumors. However, it is not yet established as a predictive marker due to inconsistent results of c-Myc in different molecular breast cancer classes. A correlation was found between ER positivity and c-Myc amplification and between c-Myc and the existence of distant metastasis. This correlations makes a high c-Myc expression a poor-outcome cancer signature (c-Myc also regulates 13 genes that are being used in prognostic gene expression profiles). It is interesting that patients with high c-Myc amplification had a better response to chemotherapy (anthracycline based) than patients without c-Myc amplification. Patients who had c-Myc amplifications and were treated with anthracycline chemotherapy had a longer survival then patients with c-Myc amplification that were not treated with anthracycline chemotherapy. However, the overall survival of patients with c-Myc amplifications and chemotherapy was as long as patients who had metastatic disease without the c-Myc amplification. This means that c-Myc is a poor prognosis marker but this poor prognosis can be compensated for with the right chemotherapy. Despite the fact that these findings still need to be confirmed in larger studies, *c-Myc* amplification could be a clinically useful predictive parameter in metastatic breast cancer[43].

A downside of in situ technologies is that the results are dependent on the experience level of the executor and quality/quantity often is an estimation of the observant. This even clearly differs between (experienced) pathologists[38]. The error due to interpretation on the amount of protein can be negated by proteomics through biomolecular mass spectrometry . Proteomics aims at detecting (almost) all the proteins in a (cancer) cell and is not yet conventional in predictive biomarker research. It is a powerful tool to compare samples and can be used to generate protein profile from breast cancer biopsies before and after a cycle of chemotherapy. This can be useful to find new proteins that may be used in IHC to help predicting therapeutic outcome[44].

4.2.2. Gene expression profiling

The applications of gene expression data analyses in the field of oncology are manifold. They include early cancer detection, monitoring of disease progression, and estimation of risk for metastasis or recurrence (hence prognostic information). Moreover, it is the main tool used to

identify predictive markers for treatment response. Nowadays, high-throughput sequencing of RNA transcripts (RNASeq) is the method of choice to quantify and characterize transcripts. Since this technique is relatively new, I will first focus on the data analysis using ordered arrays to identify predictive signatures. As described above, a prognostic signature like the Mammaprint® gene expression array has also some predictive power for chemotherapy [5, 14]. However, the predictive power is not good enough for clinical decision making on choosing a specific type of chemotherapy [11] The aim is that a predictive signature indicates with high accuracy whether a patient is likely to respond to a specific type of chemotherapy.

Hannemann *et al.*[45] tried to identify a gene expression pattern for neoadjuvant chemotherapy (doxorubicin and cyclophosphamide or doxorubicin and docetaxel) in primary breast carcinomas. After the therapy was given, a comparison was made between gene expression patterns of biopsies obtained before treatment from patients which showed a (near) pathological complete remission (pCR) versus patients with stable or progressive disease. No gene expression profile was identified to predict response to neoadjuvant chemotherapy[45].

In a study of Tordai *et al.*[46] anthracycline sensitivity was measured in ER-positive and ER-negative breast cancer tumors. Within the group of ER-negative tumors, the cell cycle progression transcription factor E2F3 was correlated with higher neoadjuvant anthracycline sensitivity. ER positive breast cancer were resistant to neoadjuvant anthracyclinetherapy. However, these tumors had a higher expression of mutant p53 which was not seen in the ER negative tumors. Mutant p53 may have a different consequence on chemotherapy sensitivity depending on hormonal-status of the tumor. This can explain the conflicting results of mutant p53 being sensitive or resistant to anthracycline chemotherapy[46]. However, this study lacks a follow up study, suggesting the predictive power is not sufficient for clinical decision making.

A gene expression profile study from Hallet *et al.*[47] showed an increased response to combination chemotherapy (paclitaxel, 5-FU, doxorubicin and cyclophosphamide) if the tumor had high topomerase 2 alpha (*TOP2A*) transcription levels (*TOP2A* is the main cellular target of anthracycline-based chemotherapy). *TOP2A* levels were also higher in patients who showed a complete pathological response then those who had residual disease after treatment[47]. This was consistent with previous studies[29]. Moreover, their study showed a correlation of beta-tubulin expression to the response of microtubules stabilizing agents (taxanes) which suggests that target expression is associated with response to their chemotherapeutic agent counterparts[47]. Their opinion was that the data remains predictive even after adjusting to clinical parameters. Nevertheless, a clinical trial to confirm this is still required.

Micro-arrays are robust platforms to give a prognostic diagnosis for the course of the breast cancer, because the prognosis of development is a result of many genes and gene groups, while response to therapy can change with a few subtle changes in gene expression. Most of the platforms are not sensitive enough to give a signal for many relevant genes with low expression[48]. In addition a breast tumor is a molecularly heterogeneous disease, meaning that cells in the same tumor can differ from each other in gene expression, averaging the outcome on the micro-array. A powerful and sensitive technique that can illustrate the transcriptome at a very high resolution is called massive parallel RNA sequencing (RNASeq). This technique covers the total transcript expressions of a cancer and is different from the preselected microarray approach, which relies on specific probes that hybridize with the cDNA. In a study of Eswaren *et al.*[49] transcriptional and post-transcriptional profiling of different molecular subtypes of breast cancer (triple negative, non-triple negative and *HER2* amplification breast cancers) has been conducted. When they compared data with RNA

sequencing and with different microarray platforms; results showed an overlap with their gene expressions. However, this still needs a prospective study to confirm the results. The emerging mRNA sequencing technology is able to detect transcriptional and post-transcriptional elements of breast cancer at a high resolution (75.000 transcripts per sample, a number which is increasing). The hope is that this technology may be helpful to find new predictive markers in the future and thus raise new therapeutic possibilities.

4.2.3. Whole-genome DNA sequencing

DNA sequencing is used to determine the order of the nucleotide bases. This technique allows the detection of various genetic abnormalities which includes small mutations in the sequence, structural variation (such as deletions, insertions or translocation), and copy-number variants[50]. In 1989 the Human Genome Organisation (HUGO) was founded. This is the organization involved in mapping the human genome (Human Genome Project). Researchers from all over the world collaborated to sequence the whole human genome. It was a long and expensive (almost 3 billion dollar over a decade) project. However, sequencing techniques are getting faster and prizes are exponentially decreasing each year, making it feasible nowadays to sequence a whole cancer genome. Sequencing is not (yet) routine for identifying predictive markers in individual patients, but promising because tumor-specific somatic chromosomal rearrangement have the potential to serve as a sensitive biomarker for tumor detection[51]. Personalized analysis of rearranged ends (PARE) can be used to detect tumor DNA in blood plasma to monitor the patients response to therapy, tumor progression and identification of residual disease[51]. First results of sequencing cancer genomes (Shah *et al.*[52]) show that mutations and abnormalities vary between and within triple-negative breast cancer. Only one-third of the mutated genes were transcribed into RNA, which suggest that the mutations may be unrelated to tumorgenesis or the mutations were involved in tumor-suppressor genes[52]. In a recent retrospective study Ellis *et al.*[53] sequenced 77 ER positive breast cancers and correlated the data to response to hormonal therapy (aromatize inhibitors). They found 18 significantly mutated genes. Of these, mutant GATA3 correlated with suppression of proliferation upon aromatase inhibitor treatment. Unfortunately, the data of this study failed to pinpoint a solid predictive biomarker for (hormone) therapy[53]. Although this study is focused on hormonal therapy, the same study to define the landscape of a breast cancer can be done for chemotherapy response.

DNA sequencing does not provide information about the expression level of genes. In some instances transcriptional features shows correlation with drug sensitivity that are equal or stronger than those observed with gene mutation[32]. An example is P-glycoprotein (P-gp; ABCB1; MDR1), a ABC transporter which effluxes a broad range of drugs from the cell, resulting in an lower accumulation of the drug in the cell. A moderate increase at the mRNA level of the *Abcb1a/b* genes encoding P-gp makes it possible for cancer cell in mice to become completely resistant to some cytotoxic drugs[54,4]. This is not caused by an alteration of the DNA copy number and would therefore be missed by DNA sequencing. Another complication with genome sequencing is the interpretation of the data. Sequencing is a very sensitive technique, which generate a lot of data. To filter these data, thresholds are being used to distinguish a hit from background noise. However, relevant mutations, which are in the noise level may not be detected if they are only present in a subpopulation of the tumor. Hence, intra-tumoral heterogeneity in mechanisms of resistance may further complicate the data analysis. If one tumor subclone is homozygous for specific mutation, but another subclone has a different homozygous mutation, both mutations may be interpreted as heterozygous when a mixed tumor population is analyzed. It is also difficult to distinguish driver mutations (mutations that contribute to tumorigenesis and tumor progression) from passenger mutations about which little is known. The

hope is that by sequencing more and more tumors, a better understanding of the tumor biology will be achieved which may facilitate the development of new therapeutic approaches[50].

4.3 In vivo models

4.3.1. Xenotransplants of human cancer cell lines and human biopsies

The classical *in vivo* model used for predictive markers research is to graft cancer cell lines or human biopsies into immunodeficient mice. The advantage of using this model is that such mice are readily available, and there is no need to generate a new genetically engineered mouse strain. Results are often available within a few weeks and multiple therapies can be tested on a single tumor or cell line graft. Immunodeficient mice have defects in their adaptive immune response, but their innate immune system is still intact. There is even a compensatory increase in both natural killer cell activity and macrophages in these mice[55]. In such models engrafted tumors often do not reach drug resistance, because the remaining immune system helps to clear away the surviving debulked (foreign) tumor cells after therapy.

Injecting cancer cell lines into mice gives a bias because these cell lines are a selected subpopulation due to cell culture conditions and do not reflect the breast cancer heterogeneity[56]. The advantage of injecting cell lines is the reproducibility of the experiment, but these tumors show a monomorphic, poorly differentiated histology and lack of tissue organization. Hence, the resulting tumors in the mice are poor surrogate of the original tumor they were derived from[57,58]. Many agents that showed consistent and potential anti-cancer activity in a cell line xenograft model, prove to be of limited use in clinical decision making[59].

To enhance the xenotransplantation technique, transplantation of primary human tumors directly into the immunodeficient mice without any *in vitro* culturing avoids the cell culture artifacts may make the xenograft studies more predictive for the behavior of human tumors. The xenografts feature the complexity of genetic and epigenetic abnormalities of the human tumor population. Such models are called patient-derived xenotransplantation models (PDX), and this approach may be suitable for some cancers which are difficult to get into culture[59]. The advantage of these models is that a primary tumor can be transplanted in multiple mice, making it possible to monitor how the transplanted tumor respond to different chemotherapeutics[57, 60]. Marangoni *et al.* [58] generated a panel of breast cancer PDX models in nude mice. Intriguingly, the majority of tumors that grew out were triple-negative cancers. A difficulty is the efficacy of the technique; breast tumors have one of the lowest tumor take rates (as low as 4% for ER positive tumors, and about 10-20 for the triple-negative[58]). For some tumors serial transplantation was needed to increase the tumor take rate. For the mice that did take the tumor has to grow several months to establish into a full blown tumor. Nevertheless, those xenografts that were established in mice resemble the original tumor pretty well[58]. Such a panel of tumors may be a useful resource to try to discover predictive biomarker response. It can be used to detect the difference in genetics and transcriptome between tumors that responded under controlled conditions to chemotherapy versus tumors that did not.

Recently Oakes *et al.*[61] revealed that inhibiting BCL-2 (with ABT-737) in breast cancer synergizes with the effect of docetaxel, resulting in an enhanced effect in triple-negative breast cancer with elevated BCL-2 levels. Xenotransplants with very low levels of BCL-2 showed no elevated response to the chemotherapy. Mentioned was that ABT-737 showed single-agent efficiency in cancer cell lines but was not effective as a single agent for any of the primary breast tumor xenografts models. ABT-737 binds to the BCL-2 protein in the mitochondrion membrane, which frees

an pro-apoptotic marker (BIM). This resulted in specific sensitization of cells dependent on the binding of pro-apoptotic signals (BH3 only proteins) to the BCL-2 proteins to docetaxel chemotherapy. Thus, elevated BCL-2 expression is not the predictive marker for the use of taxane therapy, but for the use of an inhibitor of BCL-2 together with docetaxel[61].

Xenotransplantation studies are still a crucial part of cancer drug development. The argument is that compounds that fail in xenotransplants are likely to fail in humans as well. Another argument is the difficulty in prioritizing promising novel therapies due to shortage of patient in which to test these therapies[59].

4.3.2. Genetically engineered mice

Another approach to detect predictive markers for chemotherapy is the use of conditional mouse cancer models, in which somatic mutations can be induced in a tissue specific and time controlled fashion. This results in spontaneously developing cancers that resemble human cancer. The mouse is one of the best model systems for cancer studies due to its small size, short generation time, entirely sequenced genome and physiological and molecular similarities to humans. At the Netherlands Cancer Institute the lab of Jos Jonkers generated genetically engineered mouse models (GEMMs) for breast cancer by a recombinase-mediated gene mutation strategy (*e.g.* Cre-loxP or FLP-FRT system)[62]. The targeted gene is flanked by recombinase recognition sites (LoxP or FRT), that are placed in an orientation which does not compromise gene function when the recombinase (Cre or FLP) is absent. In the presence of the recombinase, which catalyzing the recombination between the recognition sites, the intermediate DNA segment is deleted[62]. GEMMs that are used for breast cancer research are mouse strains with multiple gene replacements, guided by the knowledge of human cancer genetics. These models better mimic the natural history of tumorigenesis seen in breast cancer patients[57, 62].

An example is the *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* mouse model for BRCA1-deficient breast cancer. The resulting GEMMs develop mammary tumors which are p53 and *Brca1*-deficient and shows key features of the human breast cancer disease, such as the expression of basal markers, lack or expression of *ER*, *PR* or *HER2* expression, and genomic instability[63]. BRCA1 plays a major role in the error-free repair of DNA double strand breaks by homologous recombination. Its dysfunction to repair damaged DNA is exploited by the labs of Jos Jonkers and Piet Borst by using drugs that directly or indirectly induce these double strand breaks[57,64]. In this mouse models primary sensitivity to doxorubicin (Topoisomerase 2 inhibitor[60]), olaparib (PARP inhibitor)[65], topotecan (Topoisomerase 1 inhibitor)[66], and cisplatin (DNA cross-linker[60]) was found. This is not unexpected given the function of well-defined tumor-specific targets (*e.g.* defects in BRCA1 or BRCA2 in homology-directed DNA repair). Despite the initial high sensitivity, tumors are usually not eradicated and eventually acquire resistance. Hence, this model is useful to study mechanisms of acquired resistance, and also to investigate why it is so difficult to eradicate tumors by chemotherapy.

Intriguingly, mice with *BRCA$^{-/-}$/p53$^{-/-}$* mammary tumors showed differential responses to the microtube-targeting drug docetaxel[48]. The response measured by the shrinkage of tumor volume was then used to supervise the gene expression profiles of the tumors before treatment. Based on treatment outcome, there was clearly a separation between tumors that did not shrink below their original size and tumors that shrunk to 50% and relapsed after docetaxel treatment. However, using unbiased gene expression profiling, no differentially expressed gene was found between these two groups. When comparing RNA samples from the same tumor before treatment and after they acquired resistance to docetaxel, an increased gene expression of the *Abcb1a* and *Abcb1b* genes was

observed. The genes encode the mouse P-glycoprotein (P-gp) that, is responsible for pumping toxins out of the cell. Docetaxel is a good substrates for P-glycoprotein and it also explains some of the primary resistance to this anti-mitotic agent[48]. Nevertheless, it is still unclear what other mechanisms may be responsible for the poor upfront response of several of the tumors.

These GEMMs were also used to predict sensitivity to the DNA crosslinking agent cisplatin. Although all tumors were sensitive, there were differences in time until relapse between tumors[48]. A supervised gene expression profile analysis of these groups showed a correlation between low *Xist* expression and cisplatin hypersensitivity (the major reason of low *XIST* expression was loss of the Xi gene). To test whether *XIST* expression could serve as a biomarker in human breast cancer, 60 samples of stage 3 (HER2 negative) breast cancer were tested for *XIST* expression. Results showed that patients with a low *XIST* expression significantly benefited from intensive platinum-based therapy compared to conventional chemotherapy, thereby increasing the recurrence-free survival from 37% to 75%. Patients with high *XIST* gene expression had no significant survival benefits observed for platinum-based chemotherapy[48].

Recently a co-clinical trial with GEMMs for lung cancer (*Kras, Kras/P53* and *Kras/Lkb1* mutation) was performed. These trials are performed at the same time as the human clinical trial; providing several insights and prediction that affect the interpretation of the concurrent human clinical trial and how the data are analyzed[67]. The study of Chen *et al.*[67] showed that high metabolism visualized by Fluorodeoxyglucose (FDG) avidity with positron emission tomography (PET) scan may predict poor response to therapy. GEMMs that harbor the loss of *Kras/LKB1* showed an increased FDG avidity compared with the other genotypes and lower response rate to the combination therapy a MEK inhibitor (selumetinib) and docetaxel. The mice with *Kras* and *Kras/P53* treated with this combination showed an increased response to the combination therapy compared to the monotherapy[67].

The shortcoming of studying mouse tumors is that genes in the mouse may be differently regulated than the human genes. An altered gene in a breast tumor in the mouse model does not mean this gene is also altered in human breast cancers. Again, an instructive example is the P-gp drug export transporter, which is shown to have a role in acquired chemotherapy drug resistant in the preclinical models. However, the evidence for a role of these transporters in resistance of human cancer is largely negative. Effective inhibitors of P-gp have shown limited effect in clinical trials[54]. One plausible explanation for this difference is that the mouse *Abcb1a/b* genes are more readily induced than the human *ABCB1* gene. This is supported by the observation that mouse tumors already have a basal level of gene expression whereas the human breast cancers usually do not. To drive ABCB1 gene expression in human tumors, more complex genetic rearrangements appear to be required[54].

Another complication with drug intervention studies in mice is that pharmacodynamics and pharmacokinetics may differ between mice and man. The maximum tolerable dose of a drug given to a mouse may not be the same as the maximum tolerable dose in a human[68]. An advantage of the use of GEMMs is that the chemotherapy response of mammary tumors which resemble breast cancer in humans can be investigated under controlled conditions. There are never enough human patients to test new drugs and different combinations or schedules. In addition, clinical trials are more expensive and time-consuming then analyzing genetic engineered mouse models. A lot of (cost) savings can be achieved by optimizing the clinical trial by first doing experiments in GEMMs[68]. Because these mice develop breast cancer spontaneously, there is the heterogeneity, interaction of the microenvironment, immune system and individual variation, which also applies to human breast cancer.

5.0 Why is it difficult to discover predictive markers for chemotherapy

As with any model, the approaches discussed above have their advantages and disadvantages. Individual studies show predictive markers for chemotherapy response, however, the predictive power is often not sufficient for clinical decision making. Reproducing the data reported in an original publication is an important validation in science. This process involves multiple steps and several critical decision points which can alter results[4]. Therefore it is important that the original research group provides sufficient information about the data generation and analysis. Complex analytical methods to suit a pre-existing theory should be avoided[69].

An important reason why it is difficult to identify predictive signatures by comparing responding and non-responding cases is that the line between response to therapy and resistance is thin. An alteration in a single gene can change the tumor from being sensitive to chemotherapy to completely resistant. This signal may not be noticeable in the complex transcriptome of the tumor, with the standard gene expression techniques[6]. In addition, the individual techniques available today to predict chemotherapy response are on their own not overarching. A mutation in an oncogene/ tumor suppressor gene can be picked up by DNA or RNA sequencing, but not if a method is used that depends on hybridization of a probe. Moreover, genes that are not changed at the transcript level may be altered by post-translational mechanisms that have an influence on the activity of the translated enzyme. There are different mechanisms that may operated at different molecular layers in which the response to chemotherapy can be masked and resistance can be explained[6]. The problem with the standard algorithms used for identifying differentially expressed genes is that it relies on the hypothesis that drug-resistant mechanisms are shared most of the tumors. For example, if there are poor responding tumors which do not have a specific change in gene expression that causes resistance in other tumors, the gene may not be picked up as significant[4, 48]. A standard algorithm is only usable to identify predictive markers if the tumors all use the same drug escape mechanism[4]. If this is not the case a special algorithm is needed, which is designed to specifically detect differential gene expression that only occurs in a subgroup of tumors within the nonresponding group[48].

Several mechanisms have been identified that cause drug resistance. These include low concentration of a cytotoxic agent in the cell (*e.g.* due to an overexpression of efflux transporters[57]), increased drug metabolism[68], downregulation of the drug's cellular target, decreased influx of the drug, poor accessibility to the drug or lack of tumor proliferation[70]. The complication of intertumoral heterogeneity that may also affect heterogeneity in mechanisms of resistance was already mentioned in the introduction. Another complication is intra-tumoral heterogeneity. Although a tumor usually develops from a single cell, several subclones eventually evolve. Hence, heterogenic tumors are composed of mosaic clonal subpopulations harboring numerous individual genetic aberrations[6]. This mechanism was demonstrated in a recent deep sequencing study using 21 breast cancers[71]. Bioinformatic algorithms to infer the order of mutations based on their prevalence among sequencing reads were used with the reasoning that genetic alterations present in a high fraction of reads represent early events in tumor evolution. This analysis showed the presence of multiple discrete subclones. This heterogeneity is obviously advantageous for cancers to cope with a range of stressing conditions. In term of chemotherapy the majority of cells in the primary breast cancer may be killed by this treatment. However if a small subclone in the same tumor acquires second-site mutations (or has already acquired such a mutation), which confers drug resistance to the chosen therapy, it will be spared and selected out during treatment[72,73]. Analyzing of the bulk tumor RNA can only reflect the composition of the most prevalent type of tumor cells and multiple biopsies will be

required to fully characterize all the subclones in order to make a reliable therapeutic decision[74]. It may therefore be very challenging to find a predictive marker, which predicts chemotherapy sensitivity to the whole arsenal of subclones in the same primary breast cancer.

6.0 What may improve the identification of predictive markers for chemotherapy response

To find predictive markers it is of great importance that research is done in a professional setting with good study/clinical trial design. In addition, all the derived information and methodology needs to be documented correctly to ensure transparency and reproducibility of the published data[69]. Since techniques for the identification of predictive biomarkers all have their pros and cons, their combination may reach a higher level of confidence. A combination of *in vitro* and *in vivo* techniques may be helpful to obtain more reliable preclinical response data that may help optimizing clinical trials.

Techniques such as micro-arrays have to be optimized to detect smaller changes in RNA content. Here, the emerging technology of high-throughput RNA (cDNA) sequencing is promising. It provides information about the RNA content of a cancer cell at high coverage and base-level resolution. It also provides more information about differential expressed genes and splice variants[49].

It is likely that predictive markers for chemotherapy lie in pathways that are involved in the repair of damage caused by chemotherapy. An example is the BRCA1/2 deficiency in breast cancer being a predictive marker for double strand inducing chemotherapy, because the BRCA-deficient tumor cannot repair these double stranded breaks due to impaired homologous recombination. This has been demonstrated by the high sensitivity of BRCA1- or BRCA2-deficient mouse mammary tumors to various DNA-targeting anti-cancer drugs[64]. Still, the translation of such finding to the clinic is not trivial. In mouse models it was found that different pathogenic BRCA1 mutations result in highly similar genomic profiles but have different consequences on the response to DNA damage-inducing therapy[75]. Tumors with a N-terminal BRCA1 mutation still showed the presence of RAD51 foci, suggesting that they are still capable of homology-directed DNA repair. By using a BRCA1-like aCGH signature Volleberg *et al.*[76] found that this profile predicts sensitivity of high risk breast cancer patients to DNA double strand break-inducing chemotherapy[76]. There was no evidence for this correlation in patients without the BRCA1-like aCGH profile. Within the tumors that show a BRCA1-like aCGH profile, there are several tumors for which no genetic alteration of the BRCA1 gene have been identified. It is therefore possible that the CGH signature measures a specific functional defect in the DNA repair pathway, which involves BRCA1. However, a prospective study in a randomised clinical trial still needs to follow to confirm these results and introduce BRCA1-like aCGH profiles as a predictive marker for the use of DSB-inducing regimes in the clinic[76].

A functional assay to predict the outcome of chemotherapy response is still desirable. An example of a functional assay to identify BRCA1 or BRCA2-deficieny is the identification of RAD51 foci, which is a surrogate marker for homologous recombination. In a recent study it was shown that low scores of RAD51 foci, assessed 24h after the first chemotherapy cycle, may help to find patients with breast cancers that are defective in DNA repair by HR[77]. It is therefore important to select patients for platinum drug not only on their genomic profile, but also according to functional assay[75]. Future clinical trials will show whether the predictive value of RAD51 scores is sufficient to identify patients who may benefit from DNA repair-targeting chemotherapy.

Another functional assay is the reverse-phase protein assay. This array allows measurement of protein expression levels in a large number of biological samples simultaneously and quantitative with antibodies on a platform. This assay can detect modified or phosphorylated proteins which can

be used for the identification of activated pathways in a cancer cell. In a study of Stemke-Hale *et al.*[56], this reverse-phase protein assay to was used to find the mutational frequency of phosphatidylinositol 3-kinase (P13K) pathway members in breast cancers. Dysregulation of the PI3K signaling pathway occurs frequently in human cancer. *PTEN* tumor suppressor or *PIK3CA* oncogene mutations both direct PI3K-dependent carcinogenesis largely through activation of the AKT/PKB survival pathway[56]. This creates the expectation that this pathway can be a target for effective therapeutic approach in breast cancers[56]. This functional assay can be useful to identify activation of such strong survival pathway can contribute to chemotherapy resistance. It is of advantage to use pathway-targeted inhibitors together with chemotherapy to enhance chemotherapy response/sensitivity[78]. However, if an activated pathway of a carcinogenic subclone of the breast cancer is only present in part of the tumor, problems with significance can occur due to dilution of cancer cells without this hyper activated pathway.

A functional screen to find individual alterations in the mosaic of genetically heterogeneous clonal subpopulations uses gain-of-function or loss-of-function of (single) genes in cell lines. This explores which of the genes are able to modify chemotherapy response. This unbiased search has the potential to deepen the understanding of functions of (unknown) genes and can be used to identify novel targets for therapy[79].

7.0 How can we apply preclinical data to the clinic?

In the war against cancer, various approaches have been used preclinically to eradicate malignant cells. How can we use this information and build predictive markers to improve chemotherapy treatment in the clinic?

7.1 Monitor the patient during treatment

A predictive biomarker is always about primary response or resistance, but due to the adaptive nature of breast cancers it seems unlikely that a predictive marker for chemotherapy applies to the whole tumor or the whole duration of the treatment. Because of this, it is important to predict the chemotherapy response before treatment, but also to monitor the patient throughout therapy to measure whether predictive markers have changed after a few rounds of chemotherapy. The initial predictive marker can be followed, and also markers which predict progressive disease. In patients with advanced malignancy, a transient increase of tumor cell necrosis or apoptosis factors can be applied to monitor breast tumor regression[80]. This information can be used to adjust therapy quickly if needed. With the increasing information gained from high-throughput sequencing, tumor-specific mutations can be identified. Based on this information, conventional PCR strategies can be designed to detect tumor-specific DNA. With this approach, cell-free tumor DNA from the blood of patients can be measured. Alterations that are tumor specific are not present in normal human plasma or non-tumor tissue. This personalized analysis of rearranged ends (PARE) in the DNA can be used for accurate identification of surgical margins and the analysis of regional lymp nodes as the measurement of circulating tumor DNA after surgery, radiation or chemotherapy (a sign for tumor regression). However, this approach is not without limitations. A somatic alteration can be lost during tumor progression and the current cost are quiet expensive for general clinical use (but are decreasing fast)[51].

7.2 Combination Therapy

Although giving a combination of different kind of chemotherapy may limit the escape

possibilities of the breast cancer, it usually requires lowering of the doses of the individual drugs to avoid accumulating toxicity. The identification of therapeutic combinations that synergize is therefore crucial. The hope is that predictive markers for chemotherapy response also provide new insight into the biology of breast cancers, particularly the transcriptional programs that facilitates therapy sensitivity. This may provide a new angle to identify novel drug regimes, where a signaling pathway inhibitor or activator is combined with the chemotherapy regime to increase treatment efficacy[47].

An example are BCL-2 inhibitors which promote apoptosis of tumor cells. When tested in mouse xenotransplantion models, these inhibitors boosted the effects of chemotherapy[61]. Another successful targeted therapy which has shown great promise in preclinical models is the use of PARP inhibitors to induce synthetic lethality in *BRCA1/2*-deficient tumors. PARP facilitates DNA repair by binding to DNA breaks and attracting DNA repair proteins to repairs single strand breaks in the DNA. When PARP is inhibited, cells cannot repair single strand breaks and these will result over time in double stranded breaks. These double stranded breaks are more toxic to a cell than single stranded breaks. If a functional homologous recombination system is present it can repair these double stranded breaks. Cancer cells which are BRCA 1 or 2 deficient cannot repair double strand breaks in an error-free manner by homology-directed DNA repair. Due to this defect, PARP inhibition results in the accumulation of toxic DNA breaks which specifically kill the BRCA1/2 tumor cells, whereas the BRCA-proficient cells can still repair the DNA breaks[81, 82]. Also for PARP inhibitors the hope is that the combination with chemotherapy my increase its efficacy [77,76,81, 82]. In preclinical models and first clinical trials such combinations were shown to also enhance the toxicity of normal chemotherapy[65]. Nevertheless, PARP inhibitors may still be useful for maintenance after debulking chemotherapy. Since PARP inhibitors are relatively innocuous, sequential treatment of patients after chemotherapy may help to avoid or delay tumor relapse.

Experiments using the *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* mouse model for BRCA1-deficient breast cancer showed that drug resistance was frequently due to elevated drug extrusion by ABC transporters[57]. In this model the use of the P-gp inhibitor (tariquidar) reversed doxorubicin or olaparib-induced resistance[65,83]. Such combination treatment does not make a clear distinction between tumor cells and normal cells. Hence, chemotherapy uptake is also increased in normal cells resulting in more intoxicating effects on the patient. Moreover, the evidence for a role of these transporters in resistance of human cancer is largely negative, and effective inhibitors of P-gp have shown limited effect in clinical trials[54].

In the lung cancer GEMMs of Wong *et al.*[67], which harbor a *Kras/p53* mutation like many human non-small cell lung carcinomas, the effect of a combination therapy was proved. When these mice were treated with docetaxel, 30% of *Kras* and 5% of the *Kras/p53* mice achieving a partial response. If docetaxel therapy was combined with a MEK inhibitor (selumetinib), the overall response rate increased to 92% for *Kras* and 61% in *Kras/p53* mice. This combination therapy resulted in increased apoptosis, reduced proliferation and longer progression-free survival[67]. These results are encouraging and suggest that inhibitions of key regulatory pathway for self-renewal can enhance the effects of conventional chemotherapy and improve clinical outcome.

In recent years, the inhibition of the BRAF(V600E) oncoprotein by the small-molecule drugs such as (vemurafenib) was found to be highly effective to treat melanoma. Unfortunately, the therapeutic success is limited by the development of drug resistance. Using functional RNAi screens Prahallad *et al*.[84] recently found that there is a synergistic effect of the BRAF(V600E) inhibitor with EGFR inhibition. When BRAF(V600E) is blocked, EGFR signaling is induced and sustains proliferation[84].

In particular for colon cancers that harbor the BRAF(V600E) but do not respond to PLX4032 such combination may be an option.

7.3 Improvement of the design of clinical trials

In clinical trials new drugs, drug combinations or a different mode of administration are tested. Most clinical trials with anti-cancer drugs follow the same development path and are divided in three phases. Clinical trial phase 1 is used to look at safety and toxicological properties with late-stage cancer patients who often do not have any option for treatment anymore. If the drug is approved, it continuous to phase 2 to test whether the compound is working against a specific cancer (exploratory phase). In the third phase (confirmatory phase) the compound is tested against a large group of cancer patients randomized in (often) two arms; one with standard care or the best cancer therapy against their cancer and one group that is receiving the new drug or drug combination. Here it will be tested whether the new drug is better compared to the best available drug at that moment.

Before the biomarkers era, patients that were enrolled in clinical trials were based on their tumor grade and patients were randomly assigned to treatment groups (random clinical trial; RCT). However, research showed that success of chemotherapy treatment is based on the molecular subtypes and having specific predictive markers for sensitivity[38,41,46,56,73,76,77].

In case data from a retrospective study is available, it may be helpful to show that there is compelling evidence for the potential benefit of a new therapy for the biomarker-positive subgroup. Then it would be the most efficient way to evaluate the new therapy in an enriched design in which the biomarker is verified in all patients but the randomization is restricted to the biomarker-positive patients. This way, the efficiency of patients that respond to the therapy can be increased[85]. However, if a biomarker separates patients into an biomarker-positive (sensitive) and biomarker-negative (nonsensitive) subgroups it is of importance to keep in mind that the benefit of the new therapy can also apply to the biomarker-negative group due to other unknown factors. An efficient approach for this is a biomarker-stratified design, where all patients are randomized regardless of biomarker status, but the analysis is done with the knowledge of which patients have the biomarker.

Clinical trials are a long and slow process that cost pharmaceutical companies many resources. Failed compounds investment need to be earned back with drugs that do succeed, indirectly raising costs of clinical trials. Remarkably, the failure rate of 90% for a drug to make it from phase 1 to phase 3 is still as high as it was 25 years ago[86]. Maybe the use of biomarker-adaptive clinical trial design can decrease this failure rate. This study also promotes clinical trials in smaller patient groups who have a high change to respond to therapy, making it possible to easier and cheaper test new drug (combinations) and making a step forward in personalized medicine treatment.

8.0 Discussion

Breast cancer is the tenth cause of death in high-income countries in 2008, reaching the number of 170.000 mortalities of breast cancer in that year[21]. Despite the good facilities that are often correlated with high-income countries, many breast cancer patients still die due to the malignant growth. Trying to eradicate cancer is like playing a strategy game. If a weak spot is identified and therapy is given to target that weak spot, the cancer cells often find a way to deal with the therapy stresses, resulting in relapses or even metastasis. To date, research has focused on a cancer cell-specific response to therapy. It is important to have as much information about the biology of the breast cancer in the patient to select a treatment which could be beneficial for the patient. Too often therapy turns out to be beneficial for only a fraction of the breast cancer patients.

To optimize anti-cancer therapy it would be greatly beneficial to be able to select an effective treatment beforehand rather than after trial and error in order.

There are many factors to take into account in the search for a predictive marker for chemotherapy (Fig. 4). The past has shown it is tough to identify a solid predictive marker for chemotherapy response, which applies to a whole group of breast cancer patients. In the search to find predictive markers for chemotherapy, a range of different approaches has been used. At first, research was done in breast cancer cell lines, because these cell lines were easy to maintain. However, breast cancer cell lines adapt to live under culturing conditions[56]. In parallel, analyses using patient material were explored, in particular gene expression profiling. Predictive markers were mostly discovered in retrospective studies and most of them are still awaiting validation in a prospective study and validation through other research groups. To see the tumor development interacting with its microenviroment, genetically engineered mouse models (GEMMs) were developed which resemble human breast cancer[87]. Such models are suitable to look at the development of breast cancer and the influences of the tumor environment on tumor growth and resistance. GEMMs are useful to investigate chemotherapy response in a prospective study under controlled conditions and improve the clinical success rate for chemotherapy in humans[62].

Some of the discovered predictive markers are specific for a molecular subtype of breast cancer. The search for prognostic markers already showed that breast cancers with a poor prognosis (which are mostly triple negative breast cancer and breast cancers with a high proliferating rate) have a better response to chemotherapy[5]. It is therefore important to select the patients on their molecular subtype in clinical trials to improve clinical success rate and preventing rejection of the chemotherapy drug or combination. Nevertheless, within the group of triple-negative breast cancers, there are still many tumors that do not respond well to chemotherapy.

The discovered markers that potentially have the most impact on chemotherapy sensitivity are the cellular targets of chemotherapy. It is important to find predictive markers that lie in the pathway of action of the chemotherapy, such as beta-tubulin for treatment with taxanes or TOP2A for anthracyclines[47]. If the biology of the tumor and the target of action of the chemotherapeutic agent are known, it is easier to select a more applicable therapy. This biological information and the knowledge about the ways a breast tumor becomes resistant to therapy can be used to treat the patient with combination therapy. An example is the use of EGFR inhibitors in combination with BRAF(V600E) oncoprotein inhibitors in colon cancers[84] or the combination of docetaxel and MEK inhibitors in the GEMMs for human lung cancer[67], where the combination achieves an greater anti-tumor effect the monotherapy alone.

If a predictive marker is validated for a certain chemotherapy, it is still important to monitor the patient during treatment. Breast cancer cells have several ways of becoming resistant to therapy and such a mechanism needs to be detected as early as possible. Then there may be an alternative therapy, that can reverse resistance. In the pool of anti-cancer drugs which are available today and which may not be beneficial for the large population of breast cancer patients, it is likely that some are helpful for individual patients at a specific time. Classical clinical trials will have to be adapted on prospective information provided by predictive marker to increase the success rate of many drugs now being dismissed because of their low success rate[85].

The development of more sensitive and faster technologies makes it possible to discover new predictive markers and deepen the knowledge of existing ones. Predictive markers for chemotherapy response in breast cancer could result in clinically important applications which may lower the distress of patients, result in a lower mortality due to quick and correct administration of the most

potent drug, improve the clinical success rate for chemotherapy in humans, and improve the search for better therapies. The hope is that such customized treatment plans will result in large benefit for individual breast cancer patients.

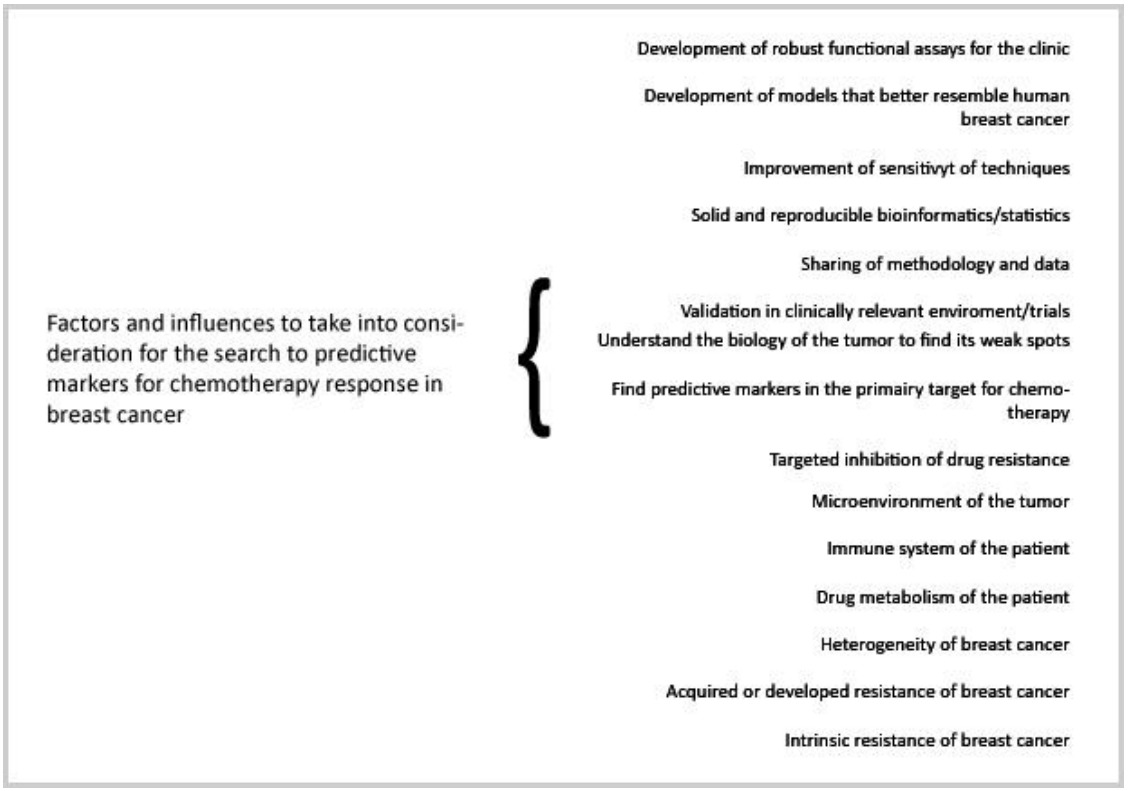

Figure 4 Summary of factors, which should be taken into account for the development of robust predictive markers for chemotherapy in breast cancer patients.

References

1. Hassett, M. J., O'Malley, A. J., Pakes, J. R., Newhouse, J. P. & Earle, C. C. Frequency and cost of chemotherapy-related serious adverse effects in a population sample of women with breast cancer. *J. Natl. Cancer Inst.* **98**, 1108-1117 (2006).

2. Perou, C. M. *et al*. Molecular portraits of human breast tumours. *Nature* **406**, 747-752 (2000).

3. Lavasani, M. A. & Moinfar, F. Molecular classification of breast carcinomas with particular emphasis on "basal-like" carcinoma: a critical review. *J. Biophotonics* **5**, 345-366 (2012).

4. Borst, P. & Wessels, L. Do predictive signatures really predict response to cancer chemotherapy? *Cell. Cycle* **9**, 4836-4840 (2010).

5. Bernards, R. It's diagnostics, stupid. *Cell* **141**, 13-17 (2010).

6. Weigelt, B., Pusztai, L., Ashworth, A. & Reis-Filho, J. S. Challenges translating breast cancer gene signatures into the clinic. *Nat. Rev. Clin. Oncol.* **9**, 58-64 (2011).

7. Margreiter, R. The antiestrogen tamoxifen in advanced breast cancer (author's transl). *Langenbecks Arch. Chir.* **351**, 249-262 (1980).

8. Reis-Filho, J. S., Weigelt, B., Fumagalli, D. & Sotiriou, C. Molecular profiling: moving away from tumor philately. *Sci. Transl. Med.* **2**, 47ps43 (2010).

9. van 't Veer, L. J. *et al*. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530-536 (2002).

10. Buyse, M. *et al*. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. *J. Natl. Cancer Inst.* **98**, 1183-1192 (2006).

11. Straver, M. E. *et al*. The 70-gene signature as a response predictor for neoadjuvant chemotherapy in breast cancer. *Breast Cancer Res. Treat.* **119**, 551-558 (2010).

12. Carey, L. A. *et al*. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin. Cancer Res.* **13**, 2329-2334 (2007).

13. Lee, J. K. *et al*. Prospective comparison of clinical and genomic multivariate predictors of response to neoadjuvant chemotherapy in breast cancer. *Clin. Cancer Res.* **16**, 711-718 (2010).

14. Perou, C. M. Molecular stratification of triple-negative breast cancers. *Oncologist* **16 Suppl 1**, 61-70 (2011).

15. Vollebergh, M. A., Jonkers, J. & Linn, S. C. Genomic instability in breast and ovarian cancers: translation into clinical predictive biomarkers. *Cell Mol. Life Sci.* **69**, 223-245 (2012).

16. http://www.cancer.org/Cancer/BreastCancer/DetailedGuide/breast-cancer-what-is-breast-cancer?rnav=cri.

17. http://www.cijfersoverkanker.nl.

18. van Steenbergen, L. N. *et al*. Screening caused rising incidence rates of ductal carcinoma in situ of the breast. *Breast Cancer Res. Treat.* **115**, 181-183 (2009).

19. Olsen, A. H. *et al*. Breast cancer mortality in Norway after the introduction of mammography screening. *Int. J. Cancer* (2012).

20. Autier, P., Boniol, M., Gavin, A. & Vatten, L. J. Breast cancer mortality in neighbouring European countries with different levels of screening but similar access to treatment: trend analysis of WHO mortality database. *BMJ* **343**, d4411 (2011).

21. Ferlay, J. *et al*. GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase
Lyon, France: International Agency for Research on Cancer; *2010* **10**.

22. Reis-Filho, J. S. & Lakhani, S. R. Breast cancer special types: why bother? *J. Pathol.* **216**, 394-398 (2008).

23. Weigelt, B., Baehner, F. L. & Reis-Filho, J. S. The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade. *J. Pathol.* **220**, 263-280 (2010).

24. http://www.cancer.org/Cancer/BreastCancer/DetailedGuide/breast-cancer-diagnosis.

25. Grob, T. J. *et al*. Rare oncogenic mutations of predictive markers for targeted therapy in triple-negative breast cancer. *Breast Cancer Res. Treat.* (2012).

26. Chabner, B. A. & Longo, D. L. in *Cancer Chemotherapy and Biotherapy: Principles and Practice* (Lippincott Williams & Wilkins, 2011).

27. Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. & Gianni, L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56**, 185-229 (2004).

28. Larsen, A. K., Escargueil, A. E. & Skladanowski, A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther.* **99**, 167-181 (2003).

29. Munro, A. F., Cameron, D. A. & Bartlett, J. M. Targeting anthracyclines in early breast cancer: new candidate predictive biomarkers emerge. *Oncogene* **29**, 5231-5240 (2010).

30. Potti, A. *et al*. Retraction: Genomic signatures to guide the use of chemotherapeutics. *Nat. Med.* **17**, 135 (2011).

31. Barretina, J. *et al*. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603-607 (2012).

32. Garnett, M. J. *et al*. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* **483**, 570-575 (2012).

33. Geutjes, E. J., Tian, S., Roepman, P. & Bernards, R. Deoxycytidine kinase is overexpressed in poor outcome breast

cancer and determines responsiveness to nucleoside analogs. *Breast Cancer Res. Treat.* **131**, 809-818 (2012).

34. Gillet, J. P. *et al*. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 18708-18713 (2011).

35. Borrell, B. How accurate are cancer cell lines? *Nature* **463**, 858 (2010).

36. Osborne, C. K., Hobbs, K. & Trent, J. M. Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Res. Treat.* **9**, 111-121 (1987).

37. Burdall, S. E., Hanby, A. M., Lansdown, M. R. & Speirs, V. Breast cancer cell lines: friend or foe? *Breast Cancer Res.* **5**, 89-95 (2003).

38. Tewari, M., Pradhan, S., Singh, U., Singh, T. B. & Shukla, H. S. Assessment of predictive markers of response to neoadjuvant chemotherapy in breast cancer. *Asian J. Surg.* **33**, 157-167 (2010).

39. Fountzilas, G. *et al*. Differential Response of Immunohistochemically Defined Breast Cancer Subtypes to Anthracycline-Based Adjuvant Chemotherapy with or without Paclitaxel. *PLoS One* **7**, e37946 (2012).

40. Generali, D., Symmans, W. F., Berruti, A. & Fox, S. B. Predictive immunohistochemical biomarkers in the context of neoadjuvant therapy for breast cancer. *J. Natl. Cancer. Inst. Monogr.* **2011**, 99-102 (2011).

41. Betof, A. S. *et al*. Carbonic anhydrase IX is a predictive marker of doxorubicin resistance in early-stage breast cancer independent of HER2 and TOP2A amplification. *Br. J. Cancer* **106**, 916-922 (2012).

42. Miyake, T. *et al*. GSTP1 expression predicts poor pathological complete response to neoadjuvant chemotherapy in ER-negative breast cancer. *Cancer. Sci.* **103**, 913-920 (2012).

43. Todorovic-Rakovic, N., Neskovic-Konstantinovic, Z. & Nikolic-Vukosavljevic, D. C-myc as a predictive marker for chemotherapy in metastatic breast cancer. *Clin. Exp. Med.* (2011).

44. Chakravarthy, B. & Pietenpol, J. A. Combined modality management of breast cancer: development of predictive markers through proteomics. *Semin. Oncol.* **30**, 23-36 (2003).

45. Hannemann, J. *et al*. Changes in gene expression associated with response to neoadjuvant chemotherapy in breast cancer. *J. Clin. Oncol.* **23**, 3331-3342 (2005).

46. Tordai, A. *et al*. Evaluation of biological pathways involved in chemotherapy response in breast cancer. *Breast Cancer Res.* **10**, R37 (2008).

47. Hallett, R. M., Pond, G. & Hassell, J. A. A target based approach identifies genomic predictors of breast cancer patient response to chemotherapy. *BMC Med. Genomics* **5**, 16 (2012).

48. Rottenberg, S. *et al*. Impact of Intertumoral Heterogeneity on Predicting Chemotherapy Response of BRCA1-Deficient Mammary Tumors. *Cancer Res.* **72**, 2350-2361 (2012).

49. Eswaran, J. *et al*. Transcriptomic landscape of breast cancers through mRNA sequencing. *Sci. Rep.* **2**, 264 (2012).

50. Gray, J. & Druker, B. Genomics: the breast cancer landscape. *Nature* **486**, 328-329 (2012).

51. Leary, R. J. *et al*. Development of personalized tumor biomarkers using massively parallel sequencing. *Sci. Transl. Med.* **2**, 20ra14 (2010).

52. Shah, S. P. *et al*. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature* **486**, 395-399 (2012).

53. Ellis, M. J. *et al*. Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature* **486**, 353-360 (2012).

54. Borst, P. Cancer drug pan-resistance: pumps, cancer stem cells, quiescence, epithelial to mesenchymal transition, blocked cell death pathways, persisters or what? *Open Biol.* **2**, 120066 (2012).

55. Richmond, A. & Su, Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis. Model. Mech.* **1**, 78-82 (2008).

56. Stemke-Hale, K. *et al*. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res.* **68**, 6084-6091 (2008).

57. Rottenberg, S. & Borst, P. Drug resistance in the mouse cancer clinic. *Drug Resist Updat* **15**, 81-89 (2012).

58. Marangoni, E. *et al*. A new model of patient tumor-derived breast cancer xenografts for preclinical assays. *Clin. Cancer Res.* **13**, 3989-3998 (2007).

59. Sharpless, N. E. & Depinho, R. A. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat. Rev. Drug Discov.* **5**, 741-754 (2006).

60. Rottenberg, S. *et al*. Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 12117-12122 (2007).

61. Oakes, S. R. *et al*. Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2766-2771 (2012).

62. Jonkers, J. & Berns, A. Conditional mouse models of sporadic cancer. *Nat. Rev. Cancer.* **2**, 251-265 (2002).

63. Liu, X. *et al*. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 12111-12116 (2007).

64. Michalak, E. M. & Jonkers, J. Studying therapy response and resistance in mouse models for BRCA1-deficient breast cancer. *J. Mammary Gland Biol. Neoplasia* **16**, 41-50 (2011).

65. Rottenberg, S. *et al*. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 17079-17084 (2008).

66. Zander, S. A. *et al*. Sensitivity and acquired resistance of BRCA1;p53-deficient mouse mammary tumors to the topoisomerase I inhibitor topotecan. *Cancer Res.* **70**, 1700-1710 (2010).

67. Chen, Z. *et al*. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature* **483**, 613-617 (2012).

68. Rottenberg, S. & Jonkers, J. Modeling therapy resistance in genetically engineered mouse cancer models. *Drug Resist Updat* **11**, 51-60 (2008).

69. Shi, L. *et al*. The MicroArray Quality Control (MAQC)-II study of common practices for the development and validation of microarray-based predictive models. *Nat. Biotechnol.* **28**, 827-838 (2010).

70. Gillet, J. P. & Gottesman, M. M. Mechanisms of multidrug resistance in cancer. *Methods Mol. Biol.* **596**, 47-76 (2010).

71. Nik-Zainal, S. *et al*. The life history of 21 breast cancers. *Cell* **149**, 994-1007 (2012).

72. Haber, D. A., Gray, N. S. & Baselga, J. The evolving war on cancer. *Cell* **145**, 19-24 (2011).

73. Li, X. *et al*. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J. Natl. Cancer Inst.* **100**, 672-679 (2008).

74. Caldas, C. Cancer sequencing unravels clonal evolution. *Nat. Biotechnol.* **30**, 408-410 (2012).

75. Drost, R. *et al*. BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance. *Cancer. Cell.* **20**, 797-809 (2011).

76. Vollebergh, M. A. *et al*. An aCGH classifier derived from BRCA1-mutated breast cancer and benefit of high-dose platinum-based chemotherapy in HER2-negative breast cancer patients. *Ann. Oncol.* **22**, 1561-1570 (2011).

77. Graeser, M. *et al*. A marker of homologous recombination predicts pathologic complete response to neoadjuvant chemotherapy in primary breast cancer. *Clin. Cancer Res.* **16**, 6159-6168 (2010).

78. Wang, K., Zhuang, Y., Liu, C. & Li, Y. Inhibition of c-Met Activation Sensitizes Osteosarcoma Cells to Cisplatin via Suppression of the PI3K-Akt Signaling. *Arch. Biochem. Biophys.* (2012).

79. Ashworth, A. & Bernards, R. Using functional genetics to understand breast cancer biology. *Cold Spring Harb Perspect. Biol.* **2**, a003327 (2010).

80. Duffy, M. J. Tumor Markers in Clinical Practice: A Review Focusing on Common Solid Cancers. *Med. Princ Pract.* (2012).

81. Bryant, H. E. *et al*. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913-917 (2005).

82. Farmer, H. *et al*. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**, 917-921 (2005).

83. Pajic, M. *et al*. Moderate increase in Mdr1a/1b expression causes in vivo resistance to doxorubicin in a mouse model for hereditary breast cancer. *Cancer Res.* **69**, 6396-6404 (2009).

84. Prahallad, A. *et al*. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* **483**, 100-103 (2012).

85. Freidlin, B. & Korn, E. L. Biomarker-adaptive clinical trial designs. *Pharmacogenomics* **11**, 1679-1682 (2010).

86. Blair, E. D. Molecular diagnostics and personalized medicine: value-assessed opportunities for multiple stakeholders. *Personalized Medicine* **7**, 143-161 (2010).

87. van Miltenburg, M. H. & Jonkers, J. Using genetically engineered mouse models to validate candidate cancer genes and test new therapeutic approaches. *Curr. Opin. Genet. Dev.* **22**, 21-27 (2012).