IDENTIFICATION OF PREDICTIVE MARKERS FOR SYSTEMIC TREATMENT IN BREAST AND LUNG CANCER PATIENTS

Personalizing medicine using molecular aberrations

Marieke Anne Vollebergh

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IDENTIFICATION OF PREDICTIVE MARKERS FOR SYSTEMIC TREATMENT IN BREAST AND LUNG CANCER PATIENTS

Personalizing medicine using molecular aberrations

IDENTIFICATIE VAN PREDICTIEVE MARKERS VOOR SYSTEMISCHE THERAPIE VAN BORST- EN LONGKANKER PATIËNTEN

Individualiseren van behandeling door het gebruik van moleculaire aberraties

(met een samenvatting in het Nederlands)

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"Medicine is a riddle, and the key to a riddle is another riddle."

Adapted from Ralph Waldo Emerson

Voor twee bijzondere vrouwen:
Nicola Vollebergh-Bekkers die moedig de strijd verloor
en Berthe Vollebergh-Gallez, als u toch eens had kunnen studeren!

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LIST OF ABBREVIATIONS:

8-OHdG	8-Hydroxy-2'-deoxyguanosine
ABC	ATP-Binding Cassette
aCGH	array Comparative Genomic Hybridization
AC	Adriamycin - Cyclophosphamide
AR	Androgen Receptor
ARG	Amphiregulin
AT	Doxorubicin – Docetaxel
BAC	Bacterial Artificial Chromosome
BCSS	Breast Cancer Specific Survival
BER	Base Excision Repair
BLBC	Basal-like Breast Cancer
CEA	Carcinoembryonic Antigen
CGH	Comparative Genomic Hybridization
CIN	Chromosomal Instability
CK	Cytokeratin
CNA	Copy Number Aberration
CS	Cockayne syndrome
CMF	Cyclophosphamide – Methotrexate – 5-Fluourouracil
CONV	Conventional chemotherapy ($FE_{90}C$)
CT-scan	Computed Tomography scan
CTC	Cyclophosphamide – Thiotepa - Carboplatin
DFS	Disease-Free Survival
DSB	Double-Strand Break
DSS	Disease Specific Survival
E2	Estrogen
EGFR	Epidermal Growth Factor Receptor
EGFR-TKI	Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMT	Epithelial-Mesenchymal Transition
ERE	Estrogen Responsive Element
ER	Estrogen Receptor
FA	Fanconi Anemia
FAC	5-Fluorouracil – Adriamycin – Cyclophosphamide
FDR	False Discovery Rate
FEC ($FE_{90}C$)	5-Fluorouracil – Epirubicin – Cyclophosphamide
FFPE	Formalin-Fixed Paraffin-Embedded
GEMM	Genetically Engineered Mouse Model
HD	High Dose chemotherapy (CTC)
HD-PB	High-Dose Platinum-Based
H&E	Haemtoxylin and Eosine
HER2 (ERBB2)	Human epidermal growth factor receptor-2
HR	Homologous Recombination (chapter 2 & 6 only)
HR	Hazard Ratio (other chapters)
HRc	Hormone-receptor

HRT	Hormone Replacement Therapy
ICL	Interstrand Crosslinks
IGF1	Insulin-like Growth Factor-1
IGFBP	Insulin-like Growth Factor Binding Protein
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
IPBC	Intensified Platinum Based Chemotherapy
LBD	Ligand Binding Domain
LN	Lymph Node
MAPK	Mitogen-Activated Protein Kinase
MBC	Metastatic Breast Cancer
MEEBO	Mouse Exonic Evidence Based Oligonucleotide
MMR	Mismatch Repair
MRN-complex	MRE11–RAD50–NBS1 complex
MTD	Maximum Tolerated Dose
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NSCLC	Non Small Cell Lung Cancer
PARP	Poly(ADP-ribose) polymerase
PARPi	PARP inhibitors
pCR	pathological Complete Remission
PD	Progressive Disease
PFS	Progression-Free Survival
p-GP	p-Glycoproteine
PgR	Progesterone Receptor
PI3K-AKT	Phosphatidylinositol-3 Kinase - AKT
PRE	Progesterone Binding Element
OS	Overall Survival
RCT	Randomized Controlled Trial
RECIST	Response Evaluation Criteria in Solid Tumors
RT-MLPA	Reverse Transcription-Multiplexligation-dependent probe amplification
RFS	Recurrence-Free Survival
SAM	Significance Analysis of Microarrays
sEGFR	soluble Epidermal Growth Factor Receptor
SSB	Single-Strand Break
TGFa	Transforming Growth Factor Alpha
TLDA	TaqMan low density arrays (TLDA)
TTD	Trichothiodystropy
TN	Triple-Negative
TNBC	Triple-Negative Breast Cancer
XP	Xeroderma Pigmentosum

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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

BREAST CANCER AND LUNG CANCER

Breast and lung cancer comprise approximately 10% and 13% of all cancers worldwide. Both account for the most common cause of cancer-related deaths in women and men, respectively [1,2]. Every year, more than 1.3 million women are diagnosed with breast cancer and over 458,000 women die of this malignancy worldwide [1,2]. Similarly, over one million men are diagnosed with lung cancer every year with almost 949,000 men dying of this disease yearly. Lung cancer can be divided into two main subtypes: small cell lung cancer (~20% of all lung cancers) and non-small cell lung cancer (NSCLC, ~80% of all lung cancers). Lung cancer is also frequently found in women and is the second most common cause of cancer-related deaths. In both breast and lung cancer it is not usually local tumor growth that causes cancer death but dissemination of tumor cells to distant sites, *i.e.* metastasis formation. Treatment for both cancer types generally consists of surgery followed by radiotherapy and, or systemic therapy. Especially the choice of the latter, adjuvant systemic therapy, is essential as its primary goal consists of eradicating potentially metastasized tumor cells. Decisions regarding systemic therapy are based on prognostic and predictive markers. It is therefore crucial to understand the difference between the two, as both guide treatment choice in different ways.

PROGNOSTIC AND PREDICTIVE MARKERS

Prognostic markers refer to the prognosis of a disease, *i.e.* the natural outcome of a disease without interference of any treatment. In other words, taking into account the fact that metastasis formation is the most common cause of breast and lung cancer death, prognostic markers most often predict the likelihood of patients developing metastases and dying of the disease (Figure 1). Prognostic markers therefore tell us *whom to treat,* and try to prevent such occurrence. Examples of prognostic markers used to guide adjuvant systemic treatment in breast cancer patients are age, the presence of lymph node metastasis, histological grade and tumor size; in lung cancer patients, examples also consist of tumor size and presence of lymph node metastases. In contrast, predictive markers are ideally only related to response of the tumor to a specific therapy and not related to natural outcome of disease. These markers predict which tumors will be sensitive to a specific therapy and which will be resistant (Figure 1). They therefore tell us *how to treat* or *with what to treat* patients and guide treatment choice [3].

Although the last decade has seen an increased interest in predictive markers for systemic therapy in breast and lung cancer patients, few are currently used in daily clinical practice. For breast cancer patients, predictive markers used in clinical practice consist of the presence of hormone receptors and amplification of the epidermal growth factor receptor-2 (*ERBB2*, i.e. *HER2*) within a breast tumor [4]. Expression of the estrogen receptor (ER) predicts improved outcome after endocrine therapy [5], while expression of HER2 indicates the presence of *HER2* amplification and predicts improved survival after HER2-targeting drugs, such as Trastuzumab [6,7]. In lung cancer patients, mutations of the epidermal growth factor

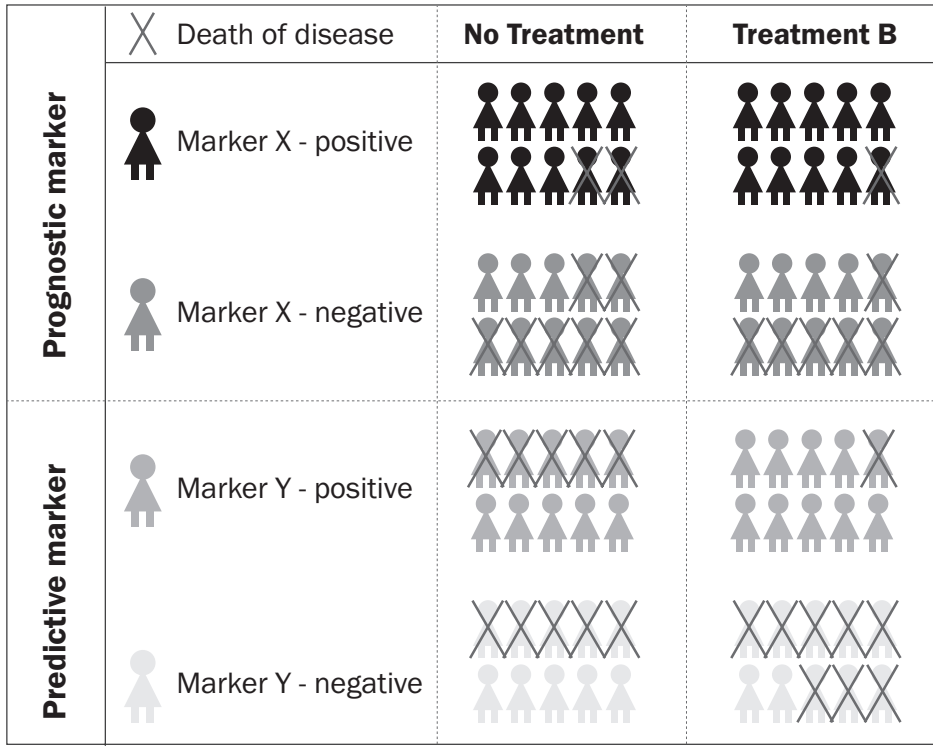

Figure 1. Prognostic versus predictive markers. A prognostic marker describes the natural outcome of a disease, regardless of treatment. In this example it is shown that Marker X is a prognostic marker as it shows that Marker X-positive patients have a better survival with only 20% of the patients dying of disease compared to 70% Marker X-negative patients. This is irrespective of treatment as with treatment, both groups show benefit of treatment but the difference (50%) between Marker X-positive and negative cases stays similar. In this example Marker X tells us *who to treat* because it identifies patients with a worse outcome. A predictive marker ideally predicts response to a specific therapy and consequently will not predict outcome in the absence of this therapy. In this example there is no difference in survival between Marker Y-positive or negative patients, both having a survival of 50%, in the absence of therapy and marker Y therefore does not predict prognosis. However, Marker Y is a predictive factor for therapy B, since Marker Y-positive patients have a better survival in the presence of therapy B, while Marker Y-negative patients fare worse; Marker Y therefore tells us *with what to treat*.

receptor (*EGFR*) in the tumor have been shown to predict for sensitivity to EGFR tyrosine-kinase inhibitors, such as erlotinib and gefitinib [8-10].

Choice of systemic treatment

Current systemic treatment choices are guided by results of large randomized controlled trials in the general breast or lung cancer population. However, these trials do not take into account the molecular heterogeneity of these diseases. Consequently, many patients might not benefit from the systemic therapies used in general treatment guidelines. Furthermore, the benefit of a certain therapy in a

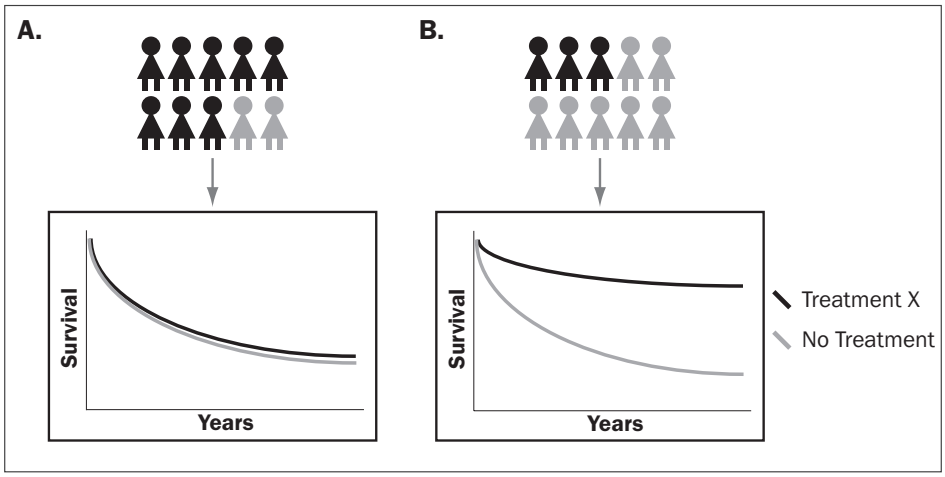

Figure 2. Effect of patient selection on study outcome: dilution of potential benefit. Specific therapies might only be beneficial in a certain subgroup of patients. Testing this therapy in the general population might dilute this potential benefit. Panel A shows the outcome in a population in which only 20% benefits (gray figures) from treatment X: this effect is lost due to the 80% without benefit. In panel B, the study population is pre-selected and includes 70% of patients with potential benefit, suddenly showing a clear benefit of Treatment X.

small percentage of patients may have been diluted by the lack of benefit in the general population (Figure 2). This could have led to discarding therapies that might actually have been very beneficial in a subgroup of patients in the past. Vice versa, by pre-defining a subgroup benefitting from a certain systemic therapy too early in clinical trials, one could run the risk of denying other potentially sensitive patients this specific systemic therapy.

An example of the first, the lack of benefit in the general population while a subgroup might benefit, might have been the desertion of the use of bifunctional alkylating agents and platinum compounds in breast cancer patients. An example of the second, unwarranted restriction of systemic therapy use to a subgroup, might be restricting the use of EGFR-TKIs in lung cancer patients carrying a mutation in *EGFR* in their tumor.

BREAST CANCER AND ALKYLATING CHEMOTHERAPY

Bifunctional alkylating agents and platinum compounds were quickly abandoned after their introduction because of the disappointing results in heavily pretreated metastatic breast cancer patients [11,12]. They made a revival in the late eighties and nineties, being incorporated in high-dose chemotherapy regimens, when the paradigm prevailed that higher dosing would ultimately lead to complete eradication of cancer cells, even in the metastatic setting [13,14]. To allow this higher

dosing, autologous stem cell transplantation was added to the regimen since otherwise the period of bone marrow recovery time and thereby granulopenia would be too long [15]. Subsequently, many phase I and II studies were performed showing positive results in the metastatic setting (reviewed in Peters et al.) [16]; however, when the results of randomized controlled trials became available, high-dose chemotherapy became a controversial subject. Especially, since the first positive results of these trials were shown to be based on fraudulent data [17]. In total, 17 adjuvant randomized controlled trials were performed in which high dose chemotherapy was incorporated in at least one study arm [18]. A recent meta-analysis of these randomized controlled trials in which adjuvant high dose chemotherapy was compared to no additional chemotherapy or conventionally dosed chemotherapy showed no survival benefit in the general population of high risk breast cancer patients [18]. With these results the use of high dose chemotherapy does not seem warranted for breast cancer patients in general. However, understanding the mechanisms of actions of the agents used in high dose chemotherapy, bifunctional alkylating agents and platinum compounds, could help identify a subgroup that *does* benefit from this type of therapy.

Mechanisms of action of bifunctional agents and platinum compounds and DNA repair

Both types of agents are known to cause double strand breaks (DSB) in the DNA by forming interstrand crosslinks. To repair these DSBs without any errors in the DNA a specific repair pathway is needed, namely homologous recombination DNA repair [19]. This repair pathway is further known for its relation to breast and ovarian cancer, as mutations in two genes involved in homologous recombination DNA repair, *BRCA1* and *BRCA2*, account for the majority of hereditary breast and ovarian cancers [20,21]. Deficiency in homologous recombination would render a cell incapable of DSB-repair and therefore could result in hypersensitivity to DSB-inducing agents, such as bifunctional alkylating agents or platinum compounds [22]. Indeed, *in vitro* and *in vivo* experiments have shown that breast cancer cells with a deficient *BRCA1* or *BRCA2* protein, *i.e.* homologous recombination deficient, were hypersensitive to DNA DSB-inducing agents, such as cisplatin.[23,24] Furthermore, breast cancer patients with a *BRCA1* mutation were shown to have a higher pathological complete remission (disappearance of the tumor after chemotherapy) to neoadjuvant cisplatin (*i.e.* cisplatin therapy administered before surgery) than to other neoadjuvant regimens [25]. This (pre)clinical evidence suggests that by applying knowledge acquired in hereditary breast cancer patients of the molecular biology of these well known genetic defects could help identify a subgroup of patients benefitting from alkylating agents and platinum compounds.

LUNG CANCER AND SYSTEMIC THERAPY

The majority of NSCLC patients will not be eligible for curative resection due to either metastases or advanced locoregional invasion [26]. The median overall survival of NSCLC patients with stage III-IV disease (advanced NSCLC) ranges between 6 – 14 months [27]. However, poor performance status (measured by for example the

Karnofsky initial performance status score or the Eastern Cooperative Oncology Group performance status) shortens this survival even further [28,29]. Patients with stage IIIa disease should receive concomitant chemoradiation with cisplatin, since this could lead to downstaging and thereby resectable disease. However, most patients with advanced NSCLC are treated with palliative intent with systemic therapy as it has been shown that chemotherapy prolongs absolute survival with 9% at 12 months [30]. In the Netherlands the choice of first line chemotherapy mainly depends on histology of the tumor. Patients with non-squamous NSCLC should be treated with cisplatin and a third generation chemotherapeutic (such as pemetrexed) but not in combination with gemcitabine, since this combination was shown to have a worse overall survival when compared to cisplatin – pemetrexed in this histologic subgroup. Vice versa, patients with squamous NSCLC should not be treated with cisplatin – pemetrexed combinations, since these patients had a worse overall survival after this combination when compared to cisplatin gemcitabine [26]. However, in case of an EGFR-mutation being present in the tumor, stage-IV NSCLC patients should be treated with an EGFR-TKI as first-line therapy, while in patients with non-mutated tumors this therapy is reserved for second- or thirdline therapy [26]. Besides determining EGFR mutation status no other marker is known to predict benefit of EGFR-TKIs. Understanding the mechanisms of action of EGFR-TKIs could help in the search for new predictive markers of EGFR-TKI response.

LUNG CANCER AND EGFR TKIS

EGFR, also known as *HER1* or *ERBB1*, is part of the receptor tyrosine kinase ErbB family as is *HER2* (*ERBB2*), *HER3* (*ERBB3*), and *HER4* (*ERBB4*). These transmembrane receptors have an extracellular ligand binding domain (except for *HER2*) and an intracellular tyrosine kinase domain (except for HER3) [31]. Binding of ligands to HER-receptors, leads to homodimerisation with the same activated HER family member or, preferentially, to heterodimerisation with another HER family member thereby activating downstream signaling [31].

The ligands of EGFR consist of transforming growth factor alpha (TGFa), epidermal growth factor (EGF), amphiregulin (ARG) or epiregulin [31]. Activation of EGFR leads to phosphorylation of specific sites of the tyrosine kinase domain and thereby activates different pathways such as the mitogen-activated protein kinase (MAPK) pathway or phosphatidylinositol-3 kinase – AKT (PI3K-AKT) pathway resulting in anti-apoptotic signals, proliferation and angiogenesis [32]. Overstimulation of EGFR could therefore lead to constant pro-survival signaling and as such has been linked to cancer in the eighties [32,33]. It was found that EGFR was overexpressed in NSCLC and seemed to predict for a worse prognosis [34,35]. These findings formed the rationale to develop EGFR-targeted therapies consisting of small-molecules inhibiting the adenosine triphosphate binding site of the tyrosine kinase of EGFR (tyrosine-kinase inhibitors, TKIs), of which gefitinib and erlotinib are examples.

EGFR-TKIs in unselected NSCLC patients

In early phase II trials a partial response rate of approximately 10% was observed in NSCLC patients treated with EGFR-TKIs [36-38]. However, in phase III trials no significant benefit was seen with regard to response rate or survival when these agents were added to standard chemotherapy in randomized phase III trials [39,40]. Alternatively, the use of single-agent erlotinib in non-selected NSCLC patients with advanced disease and failure to first- or second-line chemotherapy resulted in a significant improved overall response rate of ~9%, progression-free and overall survival (respectively PFS, OS) when compared to a placebo arm [41]. For gefinitib a similar study was performed in non-selected NSCLC patients [42]. However, against expectations the second study did not show any significant survival advantage, although both EGFR-TKIs resemble each other closely in most laboratory analyses. The reason of this discrepancy has been reviewed (Sharma, et al.) [43]. Briefly, this difference could be explained by these factors: 1) gefitinib was not administered at the maximum tolerated dose, while erlotinib was; 2) the patients recruited in the gefitinib trial more often had progressive disease (45%) than those in the erlotinib trial (28%); and 3), in the gefitinib trial patients had to have progressive disease within 90 days of their last chemotherapy, while the erlotinib trial did not have this time-limit. Subsequently, two studies tested whether the use of gefitinib would result in similar response and survival rates as standard chemotherapy, such as a carboplatin/paclitaxel regimen [44], or in a second-line setting compared to docetaxel [45]. Both studies found that the use of gefitinib was not inferior to the more toxic standard chemotherapy with regard to response rate, PFS or OS.

EGFR-TKIs and predictive markers

Regardless of the different outcomes, these trials and some phase II studies found that especially women, never-smokers, patients of Asian origin and patients with adenocarcinomas benefitted most from these EGFR-TKIs [36,41,42,46,47]. Subsequently, it was found that a somatic activating mutation in EGFR was present within these subgroups of NSCLC patients [8,48,49]. This could explain the high response rate and improved survival within these subgroups, as NSCLC tumors carrying this mutation might depend on continuous activation of EGFR for survival and proliferation. Other manners of EGFR activation, mostly EGFR amplification or overexpression, have also been investigated. It has been determined that response to EGFR-TKIs cannot be predicted based on EGFR overexpression on immunohistochemistry and prediction with amplification levels measured with FISH has given mixed results (reviewed in Cataldo et al.) [50]. On the contrary, results regarding EGFR mutation status used as a predictive marker for sensitivity to EGFR-TKIs have thus far been convincing [8,36,48,51-53]. With these findings a number of trials were performed within the subgroup of EGFR-mutated NSCLC only and showed that a better objective response rate and longer PFS was obtained after EGFR-TKI treatment than after standard chemotherapy (carboplatin/paclitaxel or cisplatin/docetaxel) [54,55]. However, no significant OS benefit was seen in both trials, which could be due to the use of EGFR-TKIs at time of progression for these EGFR-mutated NSCLC patients in the control arm.

Based on these observations one could conclude that EGFR-TKI treatment should be reserved for those NSCLC patients carrying an activating EGFR-mutation. However, there are a number of reasons why selecting patients based on this one feature would not suffice: i) Overall approximately 80-90% of the responders to EGFR-TKI treatment carry an EGFR-mutation, leaving 10-20% of the responders unexplained [8,49,51,56-58]. ii) Stabilization of disease has not been associated with EGFR-mutations while this type of response is considered favorable in the management of NSCLC patients [51,59]. Especially, since treatment options in advanced NSCLC patients with progressive disease on standard chemotherapy are limited in this mostly palliative setting. iii) There is evidence that treatment with EGFR-TKI of NSCLC patients with a wild-type EGFR tumor could be beneficial when compared to standard chemotherapy. The use of EGFR-TKIs in previously treated patients with wild-type tumors resulted in similar survival-rates as compared to standard chemotherapy [52]. This suggests that EGFR-TKI treatment is just as ineffective in this subgroup but because of less side-effects, might be seen as beneficial. Furthermore, in a placebo-controlled trial in which erlotinib was used as maintenance therapy for NSCLC patients acquiring at least stable disease after four cycles of platinum-based chemotherapy, erlotinib was found to also significantly reduce risk of progression and death in patients with wild-type EGFR [53]. Although the benefit was found to be larger in patients with an EGFR-mutated tumor (significant test for interaction), the benefit for NSCLC patients with wild-type EGFR should not be overlooked [53]. iv) Lastly, to select patients based on EGFR mutation status tumor tissue needs to be available. However, acquisition of this material often proves to be difficult and methods not requiring tumor tissue would have added value. These reasons illustrate why it remains important to try to identify new markers predicting response to these targeted-agents.

RATIONALE OF THIS THESIS

Most breast and lung cancer patients receive surgery and radiotherapy to eradicate local tumor growth, yet many patients will still be confronted with locoregional relapsed or metastasized disease. Eradicating vital tumor cells after these local treatments is the main goal of systemic therapy. In the Netherlands current clinical guidelines regarding adjuvant systemic therapies depend on large randomized controlled trials, in which the general breast or lung cancer population should have a five percent survival benefit at 10 years. Although this sounds promising for breast or lung cancer patients in general, for an individual this represents a 95% chance of facing all toxic side-effects of systemic therapy without having any benefits. Identifying markers that would predict therapy benefit for an individual patient is therefore crucial as it will not only prevent unnecessary treatment and as such toxic side-effects, it would also prevent relapse of disease. In case of metastasized disease, the value of predictive markers is further emphasized as in this palliative setting unnecessary toxic-side effects are perhaps even more unwanted and initial on-target therapy would result in prolonged survival.

Systemic therapy in breast cancer consists of endocrine therapy for estrogen-receptor positive tumors, HER2-targeted agents for HER2-positive breast cancer, and chemotherapy. The backbone of chemotherapy consists of classic cytotoxic agents which induce poorly defined cytotoxicity on dividing cells, and as such are used in the general breast cancer population. However, thus far no markers exist that predict response to any of these chemotherapeutics. Insights into the molecular biology of breast cancer coupled to the specific mechanism of action of chemotherapeutic agents might lead to selection of patients for specific chemotherapeutics and thereby a better response and survival.

This thesis describes the discovery of predictive markers using *BRCA1* or *BRCA2*-mutations, well-known molecular defects in breast cancer, as the basis for its search. We evaluated a hypothesis-driven strategy, which was based on knowledge obtained from genome-wide analyses of *BRCA1-/BRCA2*-mutated breast cancers and used these genomic findings as a means to select breast cancer patients benefitting from DSB-inducing agents, such as alkylating chemotherapy or platinum compounds.

Since using a known molecular defect as starting point for the identification of predictive markers proved successful in breast cancer, a similar approach was used in lung cancer patients. Mutations in EGFR are one the most well-studied molecular defects in lung cancer patients. Furthermore, patients having NSCLC with these mutations seem to be hypersensitive to EGFR-targeted therapy. Therefore, we evaluated a different selection strategy to identify NSCLC patients benefitting from EGFR-TKI treatment, incorporating knowledge obtained from EGFR-mutations.

OUTLINE OF THIS THESIS

In the first part of this thesis studies are presented that evaluate the use of predictive markers for DSB-inducing agents in breast cancer patients and their use when applied to lymph node metastases instead of primary tumor tissue. We base this section on the concept that absence of homologous recombination offers a potential drug target for therapies that lead to DSBs, such as alkylating agents or platinum compounds.

In **chapter 2** the background of DNA repair pathways and the impact of defects in these pathways on therapy sensitivity are explained in the context of breast and ovarian cancer. Since germline mutations in *BRCA1* or *BRCA2* predispose to familial breast and ovarian cancer, we studied them together. Furthermore, the efforts already undertaken to identify predictive markers of homologous recombination deficiency and thereby sensitivity to DSB-inducing agents are discussed.

From previous studies it is known that tumors of *BRCA1*-mutated breast cancer patients have a specific genomic pattern of copy number gains and losses, which can be visualized and classified using array comparative genomic hybridization (aCGH), resulting in an aCGH BRCA1-classifier [60-62]. In **chapter 3**, we tested the hypothesis that this aCGH BRCA1-classifier would select patients with sporadic tumors benefitting from DSB-inducing agents, as this classifier

might also identify patients with tumors with functional *BRCA1* loss due to other mechanisms besides mutations, such as methylation of the *BRCA1*-promoter. To test our hypothesis we studied patients with stage-III breast cancer who participated in a trial in which patients were randomized between high-dose (HD-chemotherapy) and conventional chemotherapy. This setting was chosen because of its controlled set-up, thereby enabling predictive marker studies, and because HD-chemotherapy is one of the few regimens which consists of DSB-inducing agents only, namely platinum and alkylating compounds.

As the BRCA1 aCGH classifier, just as *BRCA1*-mutated breast cancer, is highly associated with ER-, PR- and HER2-negative (triple-negative) breast cancers, this classifier was not likely to select patients with ER-positive tumors benefitting from DSB-inducing agents. Since approximately ~70% of *BRCA2*-mutated breast cancers are ER-positive, we evaluated an aCGH BRCA2-classifier (which was trained and validated in a different study to identify *BRCA2*-mutated breast cancers) as predictive marker for DSB-inducing chemotherapy, in **chapter 4**. Furthermore, we explored whether using both BRCA1- and BRCA2 aCGH classifiers as one predictive marker would result in selecting the largest subgroup of patients benefitting from DSB-inducing agents. Patients in this chapter were derived from the same study as mentioned-above.

A genome-wide strategy to visualize DNA copy number aberrations for prediction of chemotherapy benefit is one strategy, another frequently employed one is genome-wide gene expression profiling. Although many efforts have been undertaken with this technique no predictive classifier has been identified thus far. This might be due to heterogeneity of human breast tumors with regard to gene expression. In **chapter 5,** we therefore used genetically engineered mouse models which recapitulate *BRCA1*-mutated hereditary breast cancer. Tumors from these models closely resemble each other as they start out with similar genetic backgrounds and engineered defects facilitating identification of genes determining response. Using a *K14cre;Brca1F/F;p53F/F* model for hereditary breast cancer [63], we searched for genes correlating with cisplatin and docetaxel response with gene expression microarrays and extrapolated one of our findings namely, the association of low *XIST* expression and cisplatin response, to human breast cancers.

All known predictive and prognostic biomarkers are tested on the primary breast tumor; however this might not reflect the biomarker status of their micrometastatic tumor cells. A tumor-positive axillary lymph node status is associated with a higher likelihood of micrometastatic disease, and is generally thought to demonstrate the capacity of tumor cells to migrate to other organs. In **chapter 6**, we investigated the genetic resemblance between primary breast cancers and their paired lymph node metastases using array genomic hybridization on a high resolution 720K platform. We tested the hypothesis that the genetic resemblance might be less for triple-negative breast cancers than for estrogen-receptor positive, HER2-negative breast cancers, since the former subtype is known to be genetically instable. Furthermore, we evaluated the concordance of the aCGH BRCA1-classifier between primary breast cancers and their lymph node metastases.

The second part of this thesis focuses on the identification of predictive markers for EGFR-TKI therapy in NSCLC patients. This part is based on the concept that lung cancer cells with an activated EGFR-pathway, either due to mutations, cross-talk with other receptors or due to ligand binding, would be sensitive to EGFR-TKIs.

In **Chapter 7** we evaluated whether the presence of soluble epidermal growth factor receptor (sEGFR) would correlate with survival in NSCLC patients treated with EGFR-TKIs gefitinib or erlotinib. Furthermore, the prognostic marker CEA was also tested in this respect. However, since this study lacked a controlled set-up we could not differentiate whether these markers were associated with better survival (prognosis) or benefit of EGFR-TKIs (prediction). In **Chapter 8**, we therefore conducted a case – control study in which advanced NSCLC patients treated with erlotinib or gefitinib were studied together with a control group of non-EGFR-TKI treated NSCLC patients which were matched for gender, age and histology. In this study we assessed whether ligands of EGFR or ligands of the insulin-like growth factor receptor (IGFR) measured in serum would predict for response to erlotinib or gefitinib.

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GENOMIC INSTABILITY IN BREAST AND OVARIAN CANCERS: TRANSLATION INTO CLINICAL PREDICTIVE BIOMARKERS

ABSTRACT

Breast and ovarian cancer are among the most common malignancies diagnosed in women worldwide. Together they account for the majority of cancer-related deaths in women. These cancer types share a number of features, including their association with hereditary cancer syndromes caused by heterozygous germline mutations in *BRCA1* or *BRCA2*. BRCA-associated breast and ovarian cancers are hallmarked by genomic instability and high sensitivity to DNA double-strand break (DSB) inducing agents due to loss of error-free DSB repair via homologous recombination (HR). Recently poly(ADP-ribose) polymerase inhibitors, a new class of drugs that selectively target HR-deficient tumor cells, have been shown to be highly active in BRCA-associated breast and ovarian cancers. This finding has renewed interest in hallmarks of HR deficiency and the use of other DSB-inducing agents, such as platinum salts or bifunctional alkylators, in breast and ovarian cancer patients. In this review we discuss the similarities between breast and ovarian cancer, the hallmarks of genomic instability in *BRCA*-mutated and BRCA-like breast and ovarian cancers, and the efforts to search for predictive markers of HR deficiency in order to individualize therapy in breast and ovarian cancer.

Keywords: breast cancer, ovarian cancer, *BRCA1*, *BRCA2*, genomic instability, predictive markers, double-strand break-inducing agents.

INTRODUCTION

Breast and ovarian cancer comprise approximately 10% and 3% of all cancers among women worldwide. Together they account for the majority of cancer-related deaths in women [1,2]. Every year, more than 1.6 million women are diagnosed with breast or ovarian cancer and over 598,000 women die of these malignancies [1,2]. For both cancer types, treatment generally consists of surgery followed by systemic therapy. Most guidelines for current systemic therapies rely on results of large randomized controlled trials in the general breast or ovarian cancer population. However, these trials do not take into account the molecular heterogeneity of these diseases and consequently many patients might not benefit from these general treatment guidelines. Insights into the molecular biology of these cancers may not only yield novel biomarkers to guide treatment choices, but also novel molecular drug targets that permit development of new targeted therapies. Well known targets in the treatment of breast cancer patients are the hormone receptors and the epidermal growth factor receptor-2 (ERBB2, i.e. HER2) [3]. Expression of the estrogen receptor (ER) predicts improved outcome after endocrine therapy [4], while expression of HER2 indicates the presence of HER2 amplification and predicts improved survival after HER2 targeting drugs, such as Trastuzumab [5,6]. Recently, a new targeted agent has been introduced in the form of poly(ADP-ribose)-polymerase inhibitors (PARPi) [7,8], an agent which selectively targets homologous recombination (HR) deficient cells, such as cells with mutations in breast cancer susceptibility genes 1 or 2 (*BRCA1* or *BRCA2*) [9]. Patients carrying germline mutations in *BRCA1* or *BRCA2* have long been recognized for their predisposition to familial breast and ovarian cancer [10-12]. Recent trials have shown that *BRCA1/2*-mutated breast and ovarian cancer patients are indeed sensitive to PARPi [13,14]. However, this sensitivity is not restricted to *BRCA*-mutated tumors but likely applies to all cells with any molecular defect resulting in HR deficiency. These recent findings have led us to evaluate genomic instability as one of the hallmarks of HR deficiency in breast and ovarian cancers.

In this review we will discuss shared features of breast and ovarian cancers, including the DNA repair deficiencies that give rise to genomic instability and chemotherapy sensitivity in specific breast and ovarian cancer subtypes. We also discuss the underlying mechanisms and opportunities to exploit features of HR deficiency as predictive markers to select patients for systemic therapies.

SIMILARITIES BETWEEN BREAST AND OVARIAN CANCER

Besides the fact that breast and ovarian cancers share epidemiologic risk factors and both originate from hormone-responsive tissues, they share many additional features such as tumor heterogeneity, spectrum of mutations and degree of genomic instability (Table 1).

Table 1. Similarities and differences between breast and ovarian cancer.

Features specific for either breast or ovarian cancer		
Features	**Breast cancer**	**Ovarian cancer**
Epidemiologic risk factors	- Period immediately after pregnancy (duration dependent on age of mother) - Recent oral contraceptives use	
Tumor heterogeneity	- Molecular subtype distribution roughly according to hormone receptor and HER2 status	- Molecular subtype distribution roughly according to histology and grade
Familial Cancer	- *BRCA1* mutation risk: 60 – 80% - *BRCA2* mutation risk: 40 – 80%	- *BRCA1* mutation risk: 40 – 50% - *BRCA2* mutation risk: 10 – 20%
Features shared between breast and ovarian cancer		
Epidemiologic risk factors	- Young age at menarche - Older age at menopause - Nulliparity - Inverse correlation with breastfeeding - Hormonal Replacement therapy	
Tumor heterogeneity	- Great histological variation - Different types of histology have different prognosis	
Mutations	- *PI3KCA* mutations (somatic) - *TP53* mutations (somatic) - *BRCA1* or *BRCA2* mutations (germline)	

Abbreviations: HER2, human epidermal growth factor receptor-2.

Tumor subtypes in breast and ovarian cancer

Both breast and ovarian are further characterized by their heterogeneity of disease (Table 1). Firstly, this is illustrated by the histological variety present in both diseases [15,16]. The prognostic relevance of these histological subtypes is indicative of different molecular biological backgrounds within the same disease [16-18]. Secondly, gene expression microarray studies revealed even further heterogeneity by identifying additional subtypes within both cancers. Further insights into the molecular biology of breast cancer were offered by the hallmark paper of Perou and colleagues on the molecular portraits of breast cancer, and follow-up studies in which Sorlie et al. reported the influence of these molecular subtypes on prognosis [19-21]. These papers used gene-expression microarray data to classify breast cancer into five subtypes, which roughly followed the distribution of hormone receptor and HER2 status. The luminal A and B subtypes expressed ER and genes associated with luminal epithelial cells, which was confirmed by positive cytokeratin (CK) 8/18 staining using immunohistochemistry (IHC); the HER2-positive subtype expressed genes associated with the HER2 gene; the normal-like subtype showed many similarities with normal breast tissue on gene expression and the basal-like breast cancer (BLBC) subtype was characterized by high expression of basal CK5/6 and CK17, which was verified by positive IHC staining for CK5/6 [19-21]. Furthermore, BLBCs showed large overlap (70-80%) with tumors lacking expression of ER, progesterone receptor (PgR) and HER2, also

known as triple-negative (TN) breast cancers [22-24]. The BLBC subtype was further characterized by IHC and was found to have the highest concordance (81%) with ER- and HER2-negative and either CK5/6-positive or EGFR-positive staining breast tumors (BLBC-IHC) [25].

Whereas molecular subtypes in breast cancers clustered on hormone receptor and HER2 status, gene-expression profiling of ovarian cancers yielded subtypes that generally followed histology. Unsupervised clustering of epithelial ovarian cancers clearly distinguished clear cell carcinoma and mucinous carcinoma from serous carcinomas; the endometroid subtype showed overlap with all other histological types [26]. Additionally, Tothill et al. performed gene expression profiling of endometroid and serous ovarian carcinomas and found classification by grade [27]. Low grade tumors showed activated signaling of the TP53 pathway which was not observed in high-grade tumors [28].

Mutations reveal similarities between breast and ovarian cancer subtypes

Some mutations are restricted to ovarian cancer only. Examples of these are *BRAF* or *KRAS* mutations, which were found with a high frequency (~60%) in low-grade serous and border-line ovarian carcinomas suggesting a similar etiology. However, these mutations are uncommon in breast cancers and other ovarian subtypes (high-grade serous or clear-cell) [29,30], except for endometroid ovarian carcinomas which show a modest frequency of *BRAF* mutations of 24% [30]. More recently, inactivating mutations in *ARID1A* have been reported in 46% of all clear-cell ovarian carcinomas and 30% of all endometroid ovarian cancers [31]. However, no studies have thus far reported on *ARID1A* mutation frequency in breast cancer and it therefore remains to be determined whether *ARID1A* mutations are specific for these ovarian cancer subtypes.

Interesting parallels can be drawn between breast and ovarian cancer subtypes regarding shared mutations (Table 1 and Table 2). In both cancer types *PI3KCA* mutations appear common but seem to predispose to specific subtypes (Table 2). In ovarian cancer these mutations were mainly present in clear cell ovarian carcinomas [32,33]. In breast cancer, *PI3KCA* mutations were significantly enriched in invasive lobular carcinomas (ILC), a histological subtype of breast cancer that intriguingly metastasizes to the ovaries and gastro-intestinal tract [17,34,35]. Besides mutations, these subtypes seem to share other features, such as chemoresistance [36-38] and poor prognosis [16,18]. Interestingly, clear cell ovarian cancers show relatively few DNA copy number aberrations (CNAs) when visualized by array comparative genomic hybridization (aCGH), especially when compared to high-grade serous carcinomas [39]. Similarly, ILC breast cancers nearly always cluster among the luminal A subtypes [40] in which a low degree of genomic instability is also observed [41,42].

Breast and ovarian cancers also display frequent mutations in *TP53*, predominantly in specific subtypes which again share certain characteristics (Table 2). The frequency of *TP53* mutations is approximately 25% in breast cancer [43,44] and ~50% in ovarian cancer [45-48]. However, this frequency is significantly higher in high-grade serous ovarian carcinomas [46,48] and in BLBCs [20,49]. BLBCs are known to be poorly differentiated, high-grade tumors with a poor prognosis and a high

Table 2. Similarities between *subtypes* of breast and ovarian cancer.

Similarities between basal-like breast cancer and serous ovarian carcinoma
- High frequency of *TP53* mutations (82-92% in BLBC and ~50% in high grade serous carcinoma) - Poorly differentiated (high grade) - Chemotherapy sensitivity - Poor prognosis - Genomic instability - Tumors of *BRCA1* mutation carriers are often found within these subtypes
Similarities between invasive lobular breast carcinoma and clear cell ovarian carcinoma
- High frequency of *PI3KCA* mutations (46-52% in ILC and 33-46% in CCC) - Well or moderately differentiated (low or intermediate grade) - Chemotherapy resistance - Elevated risk of recurrences or metastases - Absence of genomic instability

Abbreviations: BLBCs, basal-like breast cancers; ILC, invasive lobular carcinoma; CCC, clear cell carcinoma.

degree of chemosensitivity [21,23], as are serous ovarian carcinomas [36,50]. Furthermore, both tumor subtypes display a high level of genomic instability shown by the high number of CNAs present in these tumors [41,42,51]. The chemosensitivity and genomic instability phenotypes suggest that a substantial fraction of serous ovarian carcinomas and BLBCs are defective in error-free DNA repair. Fitting with this notion is that *BRCA1* germline mutations predispose to both cancer subtypes. Furthermore, it has been shown that most *BRCA1*-mutated breast and ovarian cancers harbor *TP53* mutations [49,52,53].

Germ-line mutations in BRCA1 and BRCA2 predispose to breast and ovarian cancer

It has been long recognized that within familial breast cancer families the incidence of ovarian cancers was very high [54]. The discovery that this familial predisposition is caused by germline mutations in *BRCA1* or *BRCA2* is probably the foremost reason why breast and ovarian cancers are mentioned together (Table 1) [11,12]. Germline mutations in *BRCA1* or *BRCA2* confer a life-time risk of 40-80% for breast cancer and respectively 25-65% and 15-20% for ovarian cancer [55,56]. *BRCA* germline mutations also increase the risk for other cancer types such as prostate and pancreatic cancer [57,58]. Although *BRCA1* has been implicated in multiple cellular processes, both *BRCA1* and *BRCA2* are mostly known for their role in the HR-pathway, which is responsible for error-free repair of double-strand breaks (DSBs) in the DNA; in case of defects in this repair pathway, i.e. due to *BRCA1/2* mutations, cells call upon alternative error-prone pathways, such as non-homologous end joining (NHEJ), resulting in genomic instability and predisposition to cancer [59]. However, the increased risks associated with *BRCA1/2* mutations are relatively specific for ovarian and breast epithelium in women. This gender- and tissue-specificity cannot be explained by the housekeeping function of *BRCA1* and *BRCA2* in DNA repair. In the next sections we will give a short

overview of the DNA repair pathways and their relation to genomic instability and therapy sensitivity. We will also review potential causes for the tissue- and gender-specificity of *BRCA*-associated cancers.

ROLE OF DNA REPAIR PATHWAYS IN THERAPY RESPONSE AND GENOMIC INSTABILITY

Already more than a century ago, Theodor Boveri suggested that cancer might arise as a consequence of abnormal segregation of chromosomes to daughter cells [60]. The link between genomic instability and cancer was further elucidated when it became clear that many inherited defects in DNA repair genes lead to genomic instability and predispose to malignancies, illustrating the importance of DNA repair pathways for maintaining genomic integrity and preventing cancer [61]. In general DNA repair can be divided into pathways that repair damage of one of the DNA strands (mismatches, subtle base modifications, bulky adducts, single-stranded breaks or gaps) or damage that affects both DNA strands (crosslinks, double-stranded breaks).

Repair of DNA double-stranded breaks

In the presence of a DNA double strand break (DSB), repair systems no longer can depend on the complementary strand for correct repair. DSBs are mostly induced by free radicals, ionizing radiation, chemotherapeutics forming DNA interstrand crosslinks (ICLs) and the conversion of SSBs into DSBs by replication fork collapse during DNA replication [61]. The presence of a DSB is sensed by the MRN complex of MRE11/RAD50/Nijmegen Breakage Syndrome 1 (NBS1) (MRN-complex), which localizes to both DNA ends and subsequently recruits ataxia telangiectasia mutated (ATM), which is responsible for checkpoint activation and cell cycle arrest through TP53. ATM also phosphorylates histone H2AX (γH2AX) resulting in chromatin remodeling around the break and recruitment of DNA damage response (DDR) factors such as BRCA1. Depending on the phase of the cell cycle, DSBs are repaired either by NHEJ, which takes place in G0-G1 phase; or by HR, which takes place in S or G2 phase. For an extensive review on both NHEJ and HR see [62,63].

Non-homologous end joining

Non-homologous end joining (NHEJ) is an error-prone mechanism for ligation of DNA DSBs. In brief, after phosphorylation of γH2AX, a heterodimer of KU70/KU80 binds to both DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The DNA-PKcs proteins on either end of the DSB interact, forming a bridge between both DNA ends [62,63]. The MRN complex has been suggested to play an additional role in NHEJ, probably in stabilizing the two DNA ends [64]. Lastly, the break needs to be sealed by ligating the DNA ends back together; the complex of XRCC4/Ligase 4 is responsible for this step [62,63]. Since NHEJ fuses DNA ends without taking into account the missing DNA or a template, this pathway is error-prone.

Repair by homologous recombination

In contrast to NHEJ, DNA DSB repair by HR is error-free since the homology of the sister chromatid is used for repair. To search for this homology, a long 3'end DNA overhang needs to be created. For this again the MRN complex is needed, which interacts with CtBP-interacting protein (CtIP), EXO1 and the helicase Bloom syndrome protein (BLM) helicase [62,63]. *BRCA1* seems to play a role in the interaction between CtIP and MRN [65]. The created single-stranded DNA ends are subsequently coated with RPA; however to start the search for sequence homology RPA needs to be replaced by RAD51. This process is directly mediated by BRCA2 (also called FANCD1) [62,63]. To facilitate this replacement a complex of BRCA1/BARD1, together having an E3 ubiquitin ligase function, needs to be present. The exact interaction remains unknown, but it is thought that PALB2 (partner and localizer of BRCA2, also known as FANCN) may connect BRCA2 and BRCA1/BARD1. RAD51 subsequently invades the sister chromatid resulting in partial displacement of the non-complementary strand (D-loop). If the second end of the DSB is also captured in the D-loop, a structure called a Holliday junction is formed, enabling DNA synthesis using the sister chromatid as a template. Lastly, the DNA structures formed by the D-loop or Holliday junction are resolved by proteins such as BLM, topoisomerase IIIa, GEN1 and probably also the Werner syndrome protein (WRN) [62,63].

In the presence of DNA ICLs an additional pathway comes into play, consisting of Fanconi Anemia (FA) proteins. Upon DNA damage ATM and ATR activate a complex of eight FA proteins, which function as an E3 ubiquitin ligase that monoubiquitinates FANCD2 and FANCI. These seem to be involved in the recruitment of BRCA2 and RAD51 at the site of the break, although the exact mechanism remains to be resolved [66].

Repair of single-strand lesions

Several DNA repair pathways exist for repair of different types of single-strand lesions such as DNA adducts and mismatched bases. These pathways use the intact complementary DNA strand for error-free repair.

Nucleotide excision repair

The nucleotide excision repair (NER) pathway is responsible for clearing helix-distorting lesions from the DNA, such as those induced by ultraviolet radiation or chemotherapeutics causing bulky intrastrand DNA adducts [61]. Using a broad range of proteins, the NER pathway 1) unwinds ~30 base pairs of DNA around the damage site through helicases, 2) cleaves the DNA using endonucleases, excising the nucleotides of the unwound stretch of DNA including the damaged site, and 3) fills the resulting gap using the complementary DNA strand as a template (see review by Cleaver et al. [67]). Inherited defects in the NER pathway are associated with three autosomal-recessive diseases: xeroderma pigmentosum (XP; mutations in *XPA – XPG*), Cockayne syndrome (CS; mutations in *CSA* and *CSB*) and trichothiodystropy (TTD, mutations in *XPD* (*ERCC2*)). In general, patients with XP have a strongly increased risk of developing skin cancers with a small increased risk of other cancers, while persons heterozygous for the mutation do not show this

phenotype [68,69]. Patients with CS and TTD do not show cancer predisposition but are characterized by neurodegeneration resulting in mental and physical retardation, as well as brittle hair, nails and scaly skin in case of TTD. In contrast, mouse models with engineered mutations specific for CS and TTD do display an increased risk of skin cancer after ultraviolet exposure [70,71]. It has been proposed that this difference is caused by the fact that patients with CS or TTD rarely live long enough to develop cancer, whereas mice display a milder, non-lethal neurodegenerative phenotype that allows enough time for cancer to occur [67]. The genomic instability seen with NER loss has been classified by Lengauer et al. as "subtle sequence instability" [72].

Base excision repair

In contrast to NER, base excision repair (BER) takes care of non-bulky base modifications in the DNA; most frequently this consists of oxidative modifications, methylation or alkylation [73]. Furthermore, BER repairs single-strand breaks (SSB) in the DNA which can be caused by for example ionizing radiation or result from intrastrand crosslinks formed by platinum agents [74-76]. In brief, DNA glycosylases specific for different types of DNA damage cleave the DNA around the damaged base and remove the damaged base from the helix but not from the sugar phosphate backbone. Subsequently apurinic-apyrimidinic endonuclease-1 (APE1) incises the backbone after which polymerase β fills the single nucleotide gap using the complementary DNA strand as a template (short patch BER) and the nick is sealed by the XRCC1-ligase3 complex. In case of a 2-12 nucleotide gap (long patch BER) additionally polymerase δ/ε, proliferating cell nuclear antigen (PCNA), flap endonuclease 1, replication factor C and DNA Ligase1 (LIGI) are being used [61,77,78]. PARP1 is thought to function as a SSB damage sensor binding to the SSB after which repair via mostly long patch BER can take place [79]. Although homozygous deletion of some of the BER genes, such as *Xrcc1*, leads to embryonic lethality in mice [80], only one cancer syndrome has been linked to a defect in the BER pathway. Germline mutations in *MUTYH*, one of the DNA glycosylases, have been shown to increase risk of colorectal tumors in an autosomal recessive manner [81,82].

Mismatch repair

The DNA mismatch repair (MMR) pathway specifically recognizes and repairs erroneous mis-incorporated bases and insertion/deletion loops that can occur during DNA replication; these loops originate from incorrect replication of repetitive sequences, also called microsatellites. In short, four steps have been recognized in MMR 1) hMSH proteins form heterodimers (hMSH2/hMSH6 and hMSH2/hMSH3) which recognize the mismatched bases or loops; 2) these heterodimers recruit a protein complex consisting of MLH1/PMS2, MLH1/PMS1 or MLH1/MLH3 heterodimers, which are thought to facilitate excision of the mismatched DNA by recruitment of an exonuclease; 3) using the complementary strand, which is stabilized by replication protein A (RPA), polymerase δ and PCNA resynthesize the DNA; 4) the remaining nick in the DNA is sealed by LIGI (see [83,84] for extensive review).

The MMR pathway is most commonly associated with familial colorectal cancers. Inherited defects in the MMR pathway (specifically mutations in *MSH2*, *MSH6*, *MLH1* or *PMS2*) result in Lynch syndrome, formerly called hereditary non-

polyposis colorectal cancer (HNPCC). Even though mouse models defective for different MMR genes are all viable (for example [85,86]), HNPCC is an autosomal dominant syndrome. Heterozygous germline mutations in MMR genes (generally resulting in a truncated protein) predispose to mainly colorectal cancer, but also endometrial, gastric and bladder cancer [84]. Inactivation of the remaining wildtype allele by mutation, loss of heterozygosity or promoter methylation results in loss of MMR [84], leading to accumulation of point mutations (mismatches) and insertions and deletions in repetitive sequences (microsatellite instability), finally resulting in cancers displaying the "subtle sequence instability" phenotype [72,87].

DNA repair defects and therapy sensitivity

Several error-free DNA repair pathways are involved in repair of DNA lesions induced by anticancer drugs (Table 3). Defects in DNA repair pathways may therefore offer potential new therapeutic targets, since failure to repair DNA lesions should lead to accumulation of damage and eventually cell death due to apoptosis or mitotic catastrophe, Two studies have used this concept to identify DNA-damaging drugs that selectively kill cells with specific DNA repair deficiencies. Martin et al screened a library of clinically approved drugs for compounds with selective toxicity in MSH2 deficient cells [88]. They found that agents that cause oxidative DNA damage, such as methotrexate, were able to specifically kill MSH2 deficient cells because of their inability to remove the oxidized base 8-hydroxy-2'-deoxyguanosine (8-OHdG) [88]. These oxidative lesions are known to increase G-T transversions, which are thought to persist in the absence of an effective MMR, leading to cell death [88]. Secondly, Evers et al. screened for compounds with selective toxicity in BRCA2-deficient cells [89]. They found that bifunctional alkylators, which form DNA ICLs and subsequently DSBs, were specifically lethal to BRCA2-deficient cells because of their defect in HR mediated DSB repair [89].

Table 3. Involvement of major error-free DNA repair pathways in repair of DNA lesions induced by anticancer drugs

Drug class	Examples	DNA lesions	Error-free repair pathways
Monofunctional alkylators	Monofunctional nitrogen mustards Temozolomide	Base damage, adducts	HR, BER
Bifunctional alkylators	Bifunctional nitrogen mustards Platinum drugs	DSBs, adducts, crosslinks	HR, NER, FA
Topoisomerase I inhibitors	Camptothecins	SSBs, DSBs	HR
Topoisomerase II inhibitors	Anthracyclines, etoposide	SSBs, DSBs	HR
PARP inhibitors	Olaparib	SSBs, DSBs	HR
Replication inhibitors	Aphidicolin, hydroxyurea	DSBs	HR
Antimetabolites	Base analogs, antifolates	Base damage	BER, MMR

Abbreviations: SSBs, single-strand breaks; DSBs, double-strand breaks; HR, homologous recombination repair; BER, base excision repair; NER, nucleotide excision repair; FA, Fanconi anemia; MMR, mismatch repair.

Paradoxically, many proteins involved in DNA repair are tumor suppressors and their loss of function has been proven difficult to target. To solve this, the concept of synthetic lethality has been introduced, which is based on the fact that two events may not affect cell viability when they occur separate from each other but induce lethality when they occur simultaneously. Thus, inhibition of a specific DNA repair pathway may be relatively harmless for normal cells but induce specific killing of tumor cells with defects in another DNA repair pathway. This concept of synthetic lethality potentially provides a large therapeutic window for the treatment of DNA repair defective cancers with small molecule inhibitors of DNA repair pathways. For example, the G-T transversions that accumulate in MMR deficient cells upon oxidative damage can be repaired by BER during replication. Consequently, inhibition of the BER polymerases β and γ induced selective toxicity in MSH2 and MLH1 deficient cells, respectively [90]. Similarly, targeting BER and SSB repair through PARP1 inhibition induces selective toxicity in HR deficient cells due to accumulation of SSBs that may be converted into DSBs following replication fork stalling during S-phase [91]. Whereas wild-type cells can repair these DSBs via homology-directed repair, *BRCA1*- and *BRCA2*-mutated cells will accumulate unrepaired DSBs or DNA rearrangements generated by error-prone repair DSB repair, ultimately leading to mitotic catastrophe [92].

DNA lesions induced by cytotoxic chemotherapy drugs and PARP inhibitors are resolved by several major error-free DNA repair pathways, including HR, BER, NER, MMR, and FA (Table 3). However, since proliferating cells will convert persisting SSBs into DSBs due to replication fork stalling during S-phase, one could argue that targeting the HR pathway would be most effective for selective killing of rapidly dividing tumor cells. This might also explain why *BRCA1*-mutated and TN breast cancers are generally more sensitive to chemotherapy agents, although the level of sensitivity differs for the different classes of compounds, as has for example been shown by the synthetic lethality screen in *BRCA2*-deficient cells [89]. Agents that directly or indirectly induce DSBs might not only be useful for the treatment of BRCA-mutated breast and ovarian cancers, but also for other cancers with defects in HR [93]. For example, genomic analysis of high-grade serous ovarian carcinomas showed that 51% of all cases contained (epi)genetic alterations in one or more HR genes [94]. Similarly, it has been reported that pancreatic cancer patients with a BRCA2 or PALB2 mutation showed a good response to mitomycin C, a DSB-inducing bifunctional alkylator [95,96]. Lastly, also non-BRCA related defects in HR, such as ATM deficiency in leukemia and lymphoma cells, have been shown to cause sensitivity to PARPi [97], and overexpression of FANCF has been shown to lead to resistance to melphalan, a bifunctional alkylator, in multiple myeloma cells [98].

In addition to hypersensitivity to DSB-inducing agents, HR defective tumors have other features that might facilitate their identification. One of these is their genomic instability phenotype.

HR deficiency and genomic instability

Failure of HR will result in error-prone repair of DSBs, resulting in gross chromosomal rearrangements such as deletions and translocations, resulting in genomic instability. The severity of this defect is illustrated by the cancer predisposition

seen in syndromes, such as Ataxia Telengiectasia (AT; caused by mutations in *ATM*), Nijmegen Breakage Syndrome (*NBS1*), Bloom Syndrome (*BLM*), Werner Syndrome (*WRN*), Fanconi Anemia (*FA* genes) and familial breast and ovarian cancers (*BRCA1* or *BRCA2*). These syndromes are caused by mutations in genes mainly involved in HR. All syndromes are autosomal recessive, except for familial breast and ovarian cancer which is caused by a heterozygous germline mutation in *BRCA1* or *BRCA2*. However, a homozygous mutation in *BRCA2* has been described, giving rise to a Fanconi Anemia phenotype rather than breast or ovarian cancer [99]. The clinical presentation of these autosomal recessive syndromes is diverse with congenital abnormalities, immunodeficiency, neurodegeneration as some of the features. However, all syndromes are characterized by a strongly increased risk for cancer, with a preference for hematological malignancies, at a young age and hypersensitivity to ionizing radiation (syndromes are reviewed in [100,101]).

It remains puzzling why little elevated cancer risk is observed in heterozygous relatives of patients with these autosomal recessive syndromes [102,103], especially since heterozygosity for *BRCA1* or *BRCA2* germline mutations results in a strongly increased breast and ovarian cancer risk. This could suggest that there is to some extent redundancy for genes of the above-mentioned syndromes but not for *BRCA1* and only to a very limited extent for *BRCA2*. In this case, tumorigenesis in *BRCA*-mutation carriers would require cell-intrinsic or –extrinsic mechanisms to promote the survival of cells with a second-hit in *BRCA1* or *BRCA2* [104]. It is tempting to speculate that cell-extrinsic survival mechanisms are also responsible for the gender- and tissue-specificity of *BRCA1*- and *BRCA2*-associated cancers, as hormonal signaling might foster proliferation and/or survival of BRCA-deficient breast and ovarian epithelial cells [104].

GENDER- AND TISSUE-SPECIFICITY OF BRCA-ASSOCIATED CANCERS

Mechanisms that have been implicated in gender- and tissue-specificity of *BRCA*-associated cancers are the ER, PgR, androgen receptor (AR) and the X-chromosome.

Tissue specificity and the estrogen receptor

In mice, puberty and pregnancy but also supplementation with estrogen (E2) and progesterone after ovariectomy induced *Brca1* expression in the mammary gland, linking BRCA1 with hormonal signaling [105,106]. In addition, *BRCA1* expression was shown to cause reduced expression of estrogen-responsive element (ERE)-containing luciferase reporter genes and endogenous E2 responsive genes after E2 stimulation. This might be mediated by direct interaction of ERα with the amino-terminal region of BRCA1 [107,108], which contains the RING domain required for BRCA1-BARD1 interaction and E3 ubiquitin ligase activity. Interestingly, ERα is an *in vivo* substrate of the BRCA1-BARD1 ubiquitin ligase [109] and monoubiquitinated ERα is targeted for degradation [110]. Together these findings suggest a model in which ERα activation by E2 stimulates proliferation through transcriptional activation of target genes including *BRCA1*, which subsequently counteracts

this signal by monoubiquitinating ERα and targeting it for degradation. Hence, functional loss of BRCA1 would result in both defective HR and sustained ERα signaling due to loss of a negative feedback loop. However, this model does not explain the fact that most *BRCA1*-mutated breast cancers are ERα-negative [111,112] and the recent finding that *BRCA1*-associated breast cancer originates from luminal ERα-negative progenitor cells [113,114].

Functional links between BRCA2 and ER have been less well studied. It has been reported that ERα may activate transcription of *BRCA2* through histone deacetylation [115], but the significance of this finding for the tissue specificity of *BRCA2*-associated tumorigenesis remains unclear.

Tissue specificity and the progesterone receptor

BRCA1 has also been linked to PgR signaling. Exogenous *BRCA1* expression was found to reduce transcriptional activity of PgR-responsive genes, possibly through direct interaction between BRCA1 and both isoforms of PgR [116]. In conditional *Brca1*-deficient mice BRCA1 was found to regulate PgR stability through polyubiquitination [117]. Moreover, treatment of these mice with PgR antagonist RU486 abrogated mammary tumorigenesis, suggesting a causal relation between the PgR signaling and *BRCA1*-associated tumorigenesis [117]. In patients contradicting evidence has been found. Although one study reported that PgR expression in adjacent tissue was significantly higher in *BRCA1*-mutated breast tumors compared to sporadic tumors [118], another study showed a reduced expression of PgR in normal breast tissue of *BRCA1* or *BRCA2* mutation carriers [119]. Moreover, since PgR is an ERE [120] it remains difficult to determine the individual effects of ERα and PgR on *BRCA*-associated breast cancer risk.

Although the precise interactions remain to be elucidated, a picture emerges in which extensive crosstalk exists between *BRCA1/2* and ER or PgR. Some of these interactions may explain the specific susceptibility of E2/progesterone-responsive proliferating tissues such as breast and ovaries to *BRCA*-associated tumorigenesis. The importance of this hormonal stimulation is further illustrated by the finding that BRCA1-deficient mice showed substantially decreased tumorigenesis after ovariectomy [121]. In patients, meta-analysis showed that bilateral salpingo-oophorectomy in *BRCA1/2*-mutation carriers reduced the risk of ovarian/fallopian tube cancer with 80% and breast cancer with 50% [122].

Tissue specificity and the Androgen Receptor (AR)

Besides the female sex hormones, androgens have also been suggested to play a role in the tissue specificity of *BRCA*-associated breast and ovarian cancers. Both *BRCA1* and *BRCA2* can act as co-activators of Androgen Receptor (AR) mediated transcription [123-125]. Furthermore, *BRCA1*-mutated breast cancers were shown to have reduced AR expression compared to sporadic or *BRCA2*-mutated breast cancers [126].

Tissue specificity and X chromosome dosage

Evidence that the X-chromosome dosage is linked to breast cancer was provided by studies on cancer incidence in patients with numerical sex chromosome

abnormalities. Men with Klinefelter syndrome, caused by an extra X-chromosome (XXY), were strongly predisposed to developing male breast cancer [127,128]. In contrast, women with Turner syndrome, caused by lack of one X-chromosome (X0), were found to be at a decreased risk for breast cancer [129]. Interestingly, in a small study it was noted that none of the 62 Turner women who received 20 to 40 years of continuous HRT developed breast cancer, even though this would have been expected based on breast cancer incidence and increased risk after HRT [130].

Another link between X chromosome dosage and tissue specificity was forged by the observed loss of the Barr body in breast and ovarian cancers (reviewed by Pageau et al. [131]). The Barr body is the heterochromatic inactive state of the X-chromosome (Xi), which is triggered by *X-inactivation specific transcript* (*XIST*), a non-coding RNA, in order to control gene dosage of X-linked genes. Loss of Xi and reduced *XIST* levels have both been linked to *BRCA1*-mutated hereditary breast cancer and sporadic BLBC, the molecular subtype resembling *BRCA1*-mutated breast cancer [132,133]. Although, BRCA1 seemed to co-localize with XIST [132], 3D analysis revealed that BRCA1 was located adjacent to XIST [134,135]. Nevertheless, it remains possible that BRCA1 has an indirect effect on the localization of *XIST* to the Xi [131].

Effects of BRCA1/2 founder mutations on tissue specificity

Given the large number of reported associations between *BRCA1/2* and ER, PgR, AR or the X-chromosome, it is possible that multiple factors underlie the tissue specificity of *BRCA*-associated tumorigenesis. Moreover, the tissue specificity might vary with specific sites of a *BRCA1* or *BRCA2* mutation. For example it was found that different *BRCA1* founder mutation sites (N-terminal, central or C-terminal) confer different risks to breast and ovarian cancers [136]. Furthermore, there might well be an interplay between general breast cancer risk factors (present for example on the X-chromosome as suggested by the Klinefelter and Turner syndrome studies), which may be enhanced in the presence of *BRCA1/2* mutations, and more direct mechanisms involving ER, PgR and/or AR signaling. All these issues complicate the elucidation of the exact mechanisms underlying gender and tissue specificity of *BRCA*-associated cancer.

IDENTIFICATION OF PREDICTIVE MARKERS FOR HR DEFICIENCY AND THEIR CLINICAL UTILITY IN BREAST AND OVARIAN CANCER

The development of PARP inhibitors (PARPi) offers new opportunities for treatment of *BRCA1*- and *BRCA2*-related breast and ovarian cancers. Recent clinical trials have indeed shown sensitivity to PARPi for this specific subgroup [13,14,137]. However, sensitivity is likely not restricted to familial *BRCA*-mutated cancers. All tumors incapable of error-free DSB repair, i.e. tumors with a defect in the HR repair pathway, should be sensitive to PARPi. A recent placebo-controlled study with the PARP inhibitor olaparib (AZD2281) in ovarian cancer patients with relapsed platinum-sensitive, high-grade serous ovarian cancer showed a significant

improvement in progression-free survival after olaparib treatment compared to the placebo-arm [138]. This is indicative that there is a subgroup of sporadic ovarian cancers in which sensitivity to PARPi might be caused by other defects in the HR pathway. Indeed, integrated genomic analyses of 489 high-grade serous ovarian carcinomas showed that 51% of all cases contained defects in HR genes [94]. In breast cancer the HR-deficient phenotype (BRCAness) is thought to be present in ~30% of all cases [93]. Conversely, HR-deficient cancer cells are likely not only sensitive to PARPi but to all agents that directly or indirectly cause DNA DSBs. Examples of these DSB-inducing agents are bifunctional alkylators or platinum agents, which are known to form DNA crosslinks that cause DSBs. Adequate selection of patients with HR-deficient cancers might place these "old" classes of chemotherapeutics in a new perspective.

An important question is how to adequately select HR-deficient breast or ovarian cancer patients who may benefit from targeted therapy involving PARPi or other DSB-inducing agents. For this review we evaluated three strategies for selecting predictive markers for DSB-inducing agents in breast or ovarian cancer patients: 1) use of knowledge-driven, mostly gene-based markers; 2) use of genomic instability as a general hallmark of HR deficiency; and 3) use of functional readouts of HR activity (Figure 1). Below we will describe these different approaches to identify predictive markers for DSB-inducing agents, described in the literature. Although some agents (e.g. bifunctional alkylating agents and platinum agents) are considered to be stronger DSB-inducing agents than others, we will consider a wide range of chemotherapeutics in our overview.

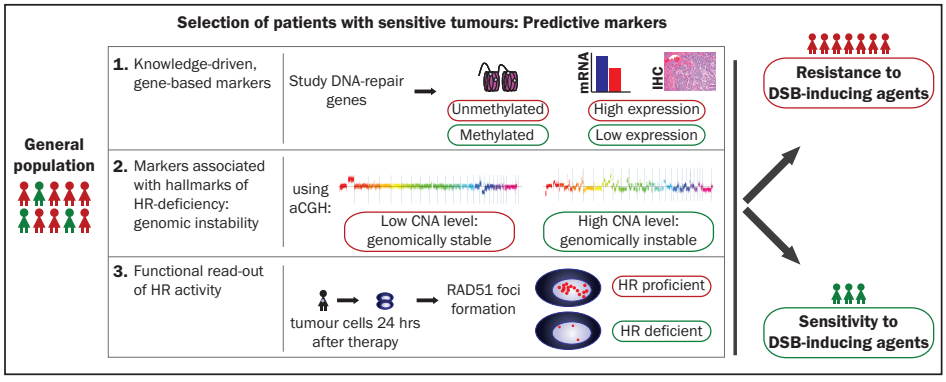

Figure 1. Predictive markers are needed to adequately select those patients benefitting from DSB-inducing agents (such as bifunctional alkyaltors, platinum salts or PARPi) out of the general breast or ovarian cancer populations. In general three ways of predictive marker studies can be distinguished. Examples are given for all three options (In example 2 aCGH plots are depicted, with on the x-axis the chromosomes and on the y-axis the log2-ratio of tumour DNA over normal DNA).
Abbreviations: IHC, immunohistochemistry; CNA, copy number aberration; HR, homologous recombination; hrs, hours; DSB, double-strand break.

Predictive versus prognostic markers

For the description of these approaches it is important to distinguish between predictive markers and prognostic markers. Prognostic markers are informative of the natural outcome of disease irrespective of treatment. In other words, prognostic markers will tell us who will have a recurrence, metastasis or die of disease, and they will therefore tell us *who should be treated* (Figure 2A). In contrast, predictive markers are treatment specific: these markers will tell us which tumors will respond to a specific therapy and which won't and therefore tell us *how to treat* (Figure 2B) [139]. Ideally, predictive markers do not show any differential effect on outcome in the absence of treatment but only in the presence of the specific treatment.

In general, there are two settings to study predictive markers: the neoadjuvant setting and the adjuvant setting. In neoadjuvant studies, systemic treatment is given *before* surgery. Response to neoadjuvant therapy can directly be measured by comparing tumor size on radiologic imaging before therapy to the pathologic tumor size after therapy. Response in the neoadjuvant setting is a good measure of the sensitivity or resistance of the bulk of the tumor, and complete disappearance of disease (pathologic complete remission, pCR) is predictive of good outcome of disease [140]. However, response to neoadjuvant therapy does not inform us of total disease eradication and therefore does not eliminate poor outcome. In the adjuvant

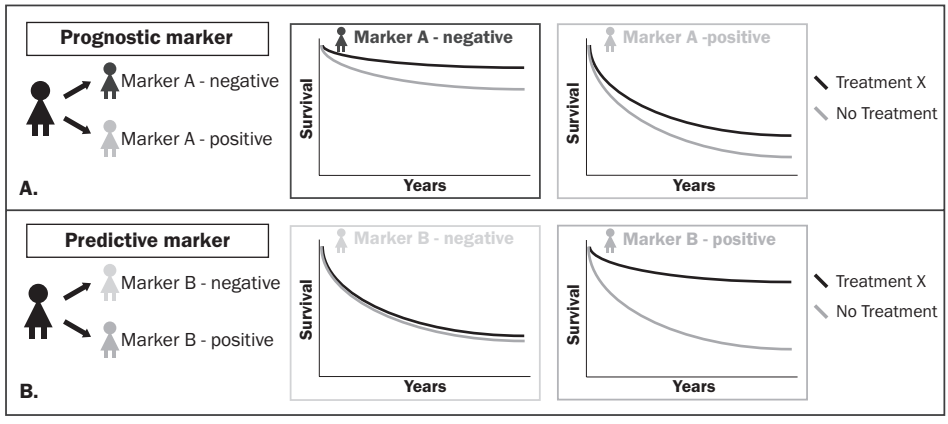

Figure 2. A) A prognostic marker predicts outcome of the natural history of a disease, regardless of treatment and therefore tells you whom to treat. (In this example: Marker A is a prognostic marker, as it predicts for a worse survival for Marker A-positive cases compared to Marker A-negative cases, irrespective of treatment. Consequently, Marker A-positive cases should be treated as these are more likely to die of disease. However the marker does not tell how to treat). B) A predictive marker predicts outcome in the presence of a specific therapy only but not in the absence of that specific treatment. It therefore tells you *how* (with what specific therapy) the patient should be treated (In this example: Marker B is a predictive marker, as it predicts an improved outcome to Treatment X over no treatment in Marker B-positive cases, while no such benefit is seen in Marker B-negative cases; This difference in treatment effect between Marker B-positive and -negative cases should be significant on a test of interaction).

setting, chemotherapy is given after surgery and the endpoint of study is usually disease-free, progression-free, recurrence-free or overall survival (DFS, PFS, RFS or OS, respectively), the ultimate proof of disease eradication. To study predictive markers in both settings, patients should be randomized between two treatments (randomized controlled trials, RCT). For markers associated with improved outcome (adjuvant) or pCR (neoadjuvant), the RCT design permits discrimination between markers that are prognostic or predicting general chemosensitivity and markers that are predicting response to specific treatments. Prognostic markers are related to natural outcome of disease and will therefore also correlate with improved outcome regardless of treatment. Markers for general chemosensitivity will show similar correlation with increased survival or pCR in both treatment arms. On the other hand, predictive markers that are related to a specific treatment will correlate with increased survival or pCR for one specific treatment arm. Sargent et al. has described four clinical adjuvant RCT designs for predictive marker studies [141]. Up till now most predictive marker studies were not part of the adjuvant RCTs design and therefore not prospectively performed. However, predictive markers can be assessed retrospectively in adjuvant RCTs, by testing whether the treatment effect on survival observed in the presence of the marker is significantly different from the treatment effect observed in the absence of the marker, using a statistical test for interaction [141]. We used four criteria to evaluate predictive marker studies: 1) Preclinical/clinical evidence existed that the marker of interest plays a role in the pathway targeted by the treatment of interest; 2) The marker was studied in at least two different treatment populations; 3) The marker studied was reproducible; 4) The marker–treatment relation was studied with regard to survival (see Table 4). Although predictive markers can only truly be evaluated in a RCT, we will also consider single-arm treatment studies in this overview, since they can be considered as hypothesis-generating. A comprehensive overview of all predictive marker studies cited is shown in Supplemental Table 1.

Knowledge-driven studies to identify predictive markers: BRCA1 and BRCA2

BRCA mutation status

Early on it was observed in ovarian cancer patients that BRCA-mutation carriers had a better survival rate compared to non carriers after cisplatin treatment **[142,143]**. Interestingly, survival rates also seemed to differ by mutation site although the numbers were small **[142]**. The initial good response of ovarian cancers to platinum-based chemotherapy might be explained by the relative high frequency of BRCA germline mutations of ~13% [144]. Moreover, the presence of somatic BRCA mutations was recently found to raise the frequency of *BRCA* mutations to 23% in high-grade serous ovarian cancers [53]. However, since the standard of care for ovarian cancer consists of treatment with a platinum agent, it was difficult to distinguish whether the observed association of *BRCA* mutation status with increased survival was due to sensitivity to the specific treatment (predictive) or favorable tumor features (prognostic) **[143]**. Since regimens without a platinum agent or a bifunctional alkylator are not often used, it is difficult to compare regimens with and without DSB-inducing agents. Further evidence that BRCA-

Table 4. Criteria which can be used to judge or set up predictive marker studies.

Criteria essential for predictive marker studies
1. Preclinical and/or clinical data show involvement of the marker in the pathway targeted by the treatment of interest Has a relevant treatment regimen been studied with regard to the marker of interest? Is the dosing of the drug of interest relevant with regard to the marker of interest?
2. The marker was studied in at least two different treatment populations, preferentially in a RCT Was the marker tested in patients treated **with** the therapy of interest and in a similar patient population **without** the therapy of interest (or preferably but uncommon: without any treatment)? Was the treatment-effect tested in a marker-negative population? In case the study design did not consist of a RCT: was the marker tested in a well matched case-control study (i.e. matching of patient characteristics treated with the therapy of interest to those without the therapy of interest)?
3. The marker studied was reproducible Were the results concordant when tested twice on the same patient population with respect to the marker? Were the results from independent studies concordant with respect to the marker?
4. The marker – treatment relation was studied with regard to either pCR rate but preferably survival. Was the outcome (RFS, DFS, PFS, OS) or pCR rate improved in marker-positive patients treated with the therapy of interest, compared to those without the therapy of interest? And was this treatment-related survival benefit or increased pCR rate absent in the marker-negative population (i.e. was the statistical test for interaction significant)? The ultimate goal of treatment remains long-term survival as this is the most objective read-out. Although pCR after neoadjuvant therapy has been related to long-term survival, the survival of partial responders cannot be predicted.

Abbreviations: RCT, randomized controlled trial; pCR, pathological complete remission; RFS, recurrence-free survival; DFS, disease-free survival; PFS, progression-free survival; OS, overall-survival.

mutated ovarian cancers are sensitive to DSB-inducing agents is provided by the recent PARPi studies. In the phase I study of the PARPi olaparib, 9 out of 15 *BRCA*-mutated ovarian cancer patients showed an objective response [137], an astonishing result for a phase I study, which was confirmed in a phase II study [13].

Evidence that *BRCA*-mutation status forms a predictive marker for DSB-inducing agents for breast cancer is complicated by the fact that bifunctional alkylators or platinum agents are not commonly used as systemic treatment, except for cyclophosphamide which is mostly given in a relatively low dose. Until now two neoadjuvant studies reported on the use of platinum in *BRCA*-mutated breast cancer. Byrski et al. showed in a cohort study that 10 out of 12 *BRCA1*-mutated breast cancer patients achieved a pCR after neoadjuvant cisplatin, while this was much lower (7–22%) after other treatments (cyclophosphamide-methotrexate-5-flourouracil (CMF), doxorubicin-docetaxel (AT), doxorubicin-cyclophosphamide (AC) and 5-fluoruracil-AC (FAC)) [145]. However, direct comparison could not be made since this study was not randomized and cisplatin-treated patients had more favorable patient characteristics compared to patients with other treatments

(for example previous chemotherapy was given in ~18% of other treatments compared to 0% in the cisplatin arm) [145]. Furthermore, the fact that no comparison was made to non-mutated cases precluded evaluation of *BRCA1* mutation status as a predictive marker. Silver et al showed that 2/2 BRCA1-mutated breast cancer patients achieved a pCR in a neoadjuvant study in which TN breast cancer patients received cisplatin and markers for response were investigated [146]. However, numbers remain small and perhaps the most convincing hint that BRCA-mutated breast cancers are sensitive to DSB-inducing agents is the phase II PARPi study. In this study of metastatic breast cancer patients, an objective response rate of 41% was seen in the olaparib 400 mg arm [14].

Mutations are not the only cause of functional loss of BRCA1 or BRCA2 and many studies have investigated different read-outs of BRCA1 and BRCA2 and their influence on chemotherapy response/outcome. Since immunohistochemical analyses of BRCA1 and BRCA2 proteins have given conflicting results due to lack of specificity of antibodies [147], we did not include them in this overview.

BRCA1 promoter methylation

Studies investigating ovarian cancers for *BRCA1* promoter hypermethylation have given contradictory results; patients with BRCA1-methylated ovarian cancers were shown to have a worse survival [148] but also a better clinical response [149,150] after platinum-based chemotherapy. However, these studies were all hampered by small numbers of tumors (max methylated tumors n=15), a different mix of control patients compared to methylated cases and different outcome measurements (clinical response versus adjusted survival analyses). In one study, in which control patients were matched to patients with *BRCA1*-mutated (n=40) and –methylated (n=19) tumors, little difference in survival after platinum agents was found between subgroups [151]. However, control patients were also matched for residual disease, which might have biased this study [151], since residual disease itself relates to survival. In breast cancer, one study investigated methylation of *BRCA1* and 13 other genes in patients treated with neoadjuvant doxorubicin and found no association with breast cancer specific survival (BCSS) [152]; this might be due to the type of treatment (doxorubicin) used. Silver et al. tested multiple markers for response in TN breast cancer patients who received neoadjuvant cisplatin [146]. *BRCA1*-promoter methylation, but also low *BRCA1* mRNA expression was significantly associated with better tumor response in the neoadjuvant setting. However, since this was a small study (n=28) investigating multiple markers without any multivariate adjustments, it should be considered as hypothesis-generating [146].

Next to *BRCA1*-related markers specific types (nonsense or frameshift) of *TP53* mutations were shown to be predictive of a good response [146]. Interestingly, specifically these types of *TP53* mutations have been found in high frequencies in *BRCA1*-mutation carriers [52] and might function as an alternative marker for *BRCA1* deficiency. In line with this reasoning, it was found that a yeast-based screen for functional *TP53* mutations could predict for pCR in a cohort of three different series of breast cancer patients treated with neoadjuvant intensified cyclophosphamide (a bifunctional alkylator), while it predicted resistance to anthracyclines alone [153].

BRCA1 mRNA expression

In breast cancer two studies examined *BRCA1* mRNA expression as a predictive marker with mixed outcomes (i.e. high levels better clinical response [154] and low levels prolonged survival [155]). However, both studies used different read-outs (clinical response vs survival), used an arbitrary cut-off for low versus high expression [154] and consisted of small subgroups (highest n=17). In ovarian cancer, Quinn et al. observed first in cell lines that loss of *BRCA1* mRNA expression increased cisplatin sensitivity and taxane resistance **[156]**; subsequently, they found in ovarian cancer patients (n=70) that low *BRCA1* mRNA expression gave a survival benefit after platinum-based chemotherapy (significant after adjustment for potential confounders) **[156]**. A similar association of low *BRCA1* expression and improved survival was seen in ovarian cancer patients with little residual disease after surgery [157]; however this study was small (n=51) with the number per subgroup unknown and a heterogeneous population regarding treatment and stage of disease [157].

The above-mentioned studies regarding *BRCA1* promoter methylation or gene expression as markers of response to DSB-inducing agents were all limited by small numbers of patients, and in some cases also by the treatment schedules and statistical shortcomings (for details see Supplementary Table 1), making firm conclusions impossible. Larger studies, preferably within RCTs, with a statistical sound set-up should be performed to investigate the performance of these markers.

Knowledge-driven studies: genome-wide effects of BRCA1 or BRCA2 deficiency

Gene expression classifiers

Instead of studying *BRCA1* mRNA expression levels, several studies used genome-wide gene expression profiling as starting point to identify predictive markers in sporadic tumors. In breast cancer patients, a previously published gene expression signature of BRCA1-mutated breast cancer [158] was used to develop a DNA repair gene expression profile [159]. This profile was associated with pCR after anthracyclines in TN breast cancer patients from two neoadjuvant studies and with resistance after a neoadjuvant taxane-based regimen [159]. However, the experimental design of this study was flawed for various reasons, for example resistance was not defined and patient characteristics (and subsequently adjustment for potential confounders) were not shown but, more importantly, ability of the signature to identify *BRCA1*-mutated cases was not investigated [159]. A somewhat similar strategy in ovarian cancer was employed, in which differences in gene expression between *BRCA1/2*-mutated and sporadic ovarian cancers were used to develop a BRCAness profile **[160]**. The presence of this BRCAness profile in sporadic ovarian cancers (n=70) was associated with a significant longer DFS and OS after platinum-based chemotherapy **[160]**.

aCGH classifiers

We employed a similar genome-wide strategy by using DNA copy number aberrations (CNAs) as predictors for BRCAness [161,162]. In a retrospective analysis of an RCT in which breast cancer patients were randomized between

intensified carboplatin-thiotepa-cyclophosphamide (CTC) and conventional FEC chemotherapy, we tested the performance of an aCGH classifier characteristic for *BRCA1*-mutated breast cancer (BRCA1-like^{CGH}) as a predictive marker for recurrence-free survival after CTC [162]. We found that HER2-negative breast cancer patients with a BRCA1-like^{CGH} tumor were eight times less likely to have a recurrence after CTC compared to FEC, while no difference in treatment was observed in non-BRCA1-like^{CGH} breast cancer patients (test for interaction p<0.01) **[162]**. While this BRCA1-like^{CGH} profile was highly associated with TN status, it was still able to predict outcome *within* TN breast cancer patients only, making this marker more than just a readout for TN breast cancer. Although the use of intensified chemotherapy in breast cancer is controversial [163], our data strongly suggest that certain subgroups may specifically benefit from this treatment regimen [153,164]. However, whether this survival benefit is due to the type of DSB-inducing agents used in these intensified regimens or the dosing itself, should be further investigated. In accordance with the FEC results of the above mentioned RCT, this BRCA1-like^{CGH} profile was not correlated with pCR in TN patients after anthracycline-based chemotherapy as part of a neoadjuvant trial [161]. Interestingly, in this study an aCGH profile for *BRCA2*-mutated breast cancer [165] identified both ER-positive and TN breast cancer patients. Furthermore, this BRCA2-like profile showed a trend for prediction of pCR after anthracycline-based chemotherapy in ER-positive breast cancer patients [161]. In line with the fact that *BRCA1*-methylated breast cancers were shown to display similar aCGH patterns as *BRCA1*-mutated breast cancers [166], the BRCA1-like^{CGH} profile identified several sporadic breast cancer patients with a *BRCA1*-methylated tumor [161,162].

DNA and histone methylation

Since *BRCA1* has additional functions besides DNA repair, these features could also be explored in the search for predictive markers. As such *BRCA1* has been shown to play a role in chromatin remodeling through interaction with HDAC1 and HDAC2 [167] and the SWI/SNF-complex [168]. Moreover, multiple studies have shown that *BRCA1*-mutated breast cancers show less DNA methylation when compared to sporadic breast cancers [169] or other familial breast cancers [170]. In a study including a larger number of sporadic breast cancers it was observed that this lower methylation state was not restricted to *BRCA1*-mutated cancers but also present in sporadic BLBCs, suggesting that a BRCA1-like defect might also be present in these tumors [171]. In this study no difference between methylation frequency in sporadic and *BRCA1*-mutated breast cancer could be found. Mixed results have been reported for patterns of gene promoter methylation in ovarian cancers and their association with response or survival after platinum-based chemotherapy [149,150,172]. Increased numbers of methylated genes were associated with a shorter PFS [172], platinum sensitivity (PFS interval of >12 months) [150] but not with clinical response [149]. However, these studies did not measure genome-wide methylation or investigate the same set of genes. It therefore remains to be determined whether general methylation status can be used as a predictive marker.

Identification of chromatin remodeling factors as predictive markers might become even more interesting since it was recently shown that cells could become reversibly drug-tolerant, i.e. survive treatment, through demethylation of H3K4 [173]. Moreover, histone deacetylase inhibitors could eradicate these drug-tolerable populations by inducing γH2AX [173]. These findings offer a new perspective and candidate therapeutic targets for disease eradication.

Knowledge-driven markers: other DNA repair genes

Besides the *BRCA* genes, other genes involved in DNA repair have been studied for their predictive potential for sensitivity to DSB-inducing agents. These include genes involved in MMR, NER, BER and HR.

HR genes

Although most predictive marker studies have focused on the *BRCA* genes, a few studies have investigated other HR genes in relation to DSB-inducing agents. Two separate studies on the same dataset, consisting of a RCT in which breast cancer patients were randomized between CMF or local radiotherapy used IHC to measure expression of firstly, BRCA1, BRCA2 and RAD51 and secondly, MRE11, NBS1, RAD50 and ATM **[174,175]**. It was found that patients with tumors with low RAD51 expression or with high expression of nuclear ATM or RAD50 benefited significantly from radiotherapy compared to CMF, while no differential benefit was seen in patients with tumors expressing high RAD51 or low ATM/RAD50 (no significant test for interaction) **[174,175]**. While the RAD51 data was consistent with the hypothesis that lack of RAD51 corresponds to impaired DNA repair and sensitivity to radiotherapy **[174]**, the ATM/RAD50 data are counterintuitive **[175]**. As stated by the authors, the ATM/RAD50 data could be explained by the fact that the MRN/ATM complex is responsible for cell cycle arrest and subsequently apoptosis. Lack of this complex could therefore result in radiotherapy resistance due to failure to induce apoptosis. In ovarian cancer, sensitivity to platinum agents was further linked to *FANCF* methylation [176]. Although *FANCF* methylation was shown to be variable and thought to disappear on progression of disease [176], it was found in ~20% of ovarian cancers [176,177]. Unfortunately, only one study investigated association of *FANCF* methylation with survival, and in this study only seven methylated cases were found [178], resulting in too small numbers to draw any conclusion.

A recent study in high-grade serous ovarian carcinomas has illustrated that many defects in HR related genes have thus far been undiscovered. In this study, using integrated genomic analyses of 489 high-grade serous ovarian carcinomas, it was found that (epi)genetic somatic alterations/mutations in BRCA1, BRCA2, EMSY, RAD51C, ATR, ATM, PALB2 and several FA genes were present in 51% of all the cases [94]. Identifying these patients will be essential, since they will have selective benefit of DSB-inducing agents.

NER genes

Since NER is responsible for the repair of bulky DNA adducts or intrastrand crosslinks, many studies investigated whether loss of NER through reduced ERCC1 activity in ovarian cancers would result in sensitivity to crosslinking agents such as platinum drugs. Most studies indicate that good platinum response is

associated with low levels of ERCC1 (measured by mRNA expression, IHC or analysis of single nucleotide polymorphisms (SNPs) associated with low mRNA expression) [179-183]. However, these studies again have some limitations, making it impossible to draw firm conclusions from the reported data. While some studies investigated relatively small subgroups [179,180,183], others showed association of ERCC1 with CA-125 levels response but showed no difference in survival [182].

Besides ERCC1, ERCC5 has also been studied as a predictive marker in ovarian cancer patients **[184]**. Using genome-wide LOH analysis, LOH at a region on chromosome 13q was found to correlate with a prolonged PFS after platinum-based chemotherapy. Based on the biological functions of genes present in this 13q region, *ERCC5* was selected for further investigation. Reduced ERCC5 expression was associated with a prolonged PFS after platinum-based chemotherapy **[184]**.

In conclusion, although published studies suggest involvement of *ERCC* genes in platinum response in ovarian cancer patients, it remains difficult to draw firm conclusions on the predictive value of these genes. However, the potential involvement of NER in platinum response is supported by studies in other cancer types such as lung cancer [185]. Additional studies, preferably RCTs, in larger cohorts of ovarian cancer patients would therefore remain interesting.

BER genes

In the hope of identifying new potential drug targets next to PARP1, other BER genes have been tested for their predictive capacity of chemotherapy response. A good example is an exploratory study which investigated APE1 in ovarian cancer patients treated with platinum compounds [186]. Although nuclear expression of APE1 was found to be associated with worse OS and platinum resistance (i.e. progression on therapy or relapse within 6 months after start of therapy), this study should be considered as hypothesis-generating as it consisted of a heterogeneous patient population and no adjustment for potential confounding factors was performed [186]. Two breast cancer studies investigating SNPs in BER genes yielded opposing associations of XRCC1 variants with survival: the _AA variant of XRCC1_1196G>A was associated with favorable survival **[187]** but also with a worse survival [188]. However, both studies suffered from relatively heterogeneous treatment regimens and small subgroups (for details see Supplementary Table 1), leaving the true association between these XRCC1 SNPs and survival unsolved.

MMR genes

Loss of MMR has been associated with resistance to platinum agents by loss of MMR-dependent apoptosis *in vitro* [189]. Consequently, multiple studies investigated MMR deficiency in breast and ovarian cancer (via IHC, methylation or microsatellite instability detection) in relation to response or outcome after chemotherapy [172,190-197]. These studies reached opposing conclusions based on associations of low MMR gene expression with both clinical progressive disease (e.g. [191]) and longer OS (e.g. [197]). The reason for these contradictory results is that the quality of all studies was compromised by analysis of small subgroups within heterogeneous patient populations (for example varying chemotherapy regimens within studies [190,195]), risk of unnoticed associations with other potential prognostic patient characteristics, or studying only clinical response (e.g. [196]; for details

regarding above mentioned studies see Supplementary Table 1). Interestingly, prospective analysis of *hMLH1* methylation in ovarian cancer patients as part of a RCT showed an increased methylation frequency in DNA isolated from blood plasma recovered at time of progression compared to plasma DNA recovered prior to carboplatin treatment [193]. This increase was associated with an increase of microsatellite instability, a more direct read-out of MMR activity [193]. Moreover, the acquisition of *hMLH1* methylation was associated with a shorter progression-free interval (PFI) and a worse OS [193]. Unfortunately, it could not be studied whether *hMLH1* methylation status in blood plasma reflected methylation status in the actual tumor. Nevertheless, these results suggest an acquired resistance mechanism via loss of MMR, which is supported by the *in vitro* data and warrants further investigation.

Markers based on general hallmarks of HR deficiency

Alternative strategies for developing predictive markers of DSB-inducing chemotherapy sensitivity in breast and ovarian cancer focus on general aspects of homology-directed DSB repair deficiency rather than on activity of single genes. Several studies have investigated genomic instability as a central hallmark of HR deficiency.

DNA copy number aberrations

One way to assess genomic instability is by measuring DNA copy number aberrations (CNAs) in tumors. It has been shown in multiple studies that *BRCA1*- and *BRCA2*-mutated breast cancers display characteristic DNA copy number gains and losses [165,198-200]. Subsequently, it has been found that the total number of CNAs, also referred to as the chromosomal instability (CIN) score or the genomic instability index, differs between *BRCA*-associated familial cancers and sporadic tumors. Mainly *BRCA1*-mutated breast tumors were observed to have the highest number of CNAs compared to other tumors [198-200]. Furthermore, the type of CNAs differed between familial and sporadic tumors, with a higher frequency of large deletions being present in the *BRCA*-mutated breast cancers [199]. Interestingly, high numbers of CNAs were also found in sporadic ER-negative/TN/basal-like breast cancers [42,199-201] and these tumors clustered with the *BRCA1*-mutated group [42,199]. Similarly, the number of CNAs in ER-positive breast cancers was found to be highest in Luminal B tumors, which co-clustered with *BRCA2*-mutated breast cancers [42,201]. These findings suggest that a subset of sporadic breast cancers share features of *BRCA*-mutated cancers, which could include HR deficiency. Evidence of this is provided by the aCGH patterns of sporadic *BRCA1*-methylated breast cancers, which were shown to resemble *BRCA1*-mutated breast cancers [162,166,199] and to display a high genomic instability index [199]. Whether genomic instability measured by number of CNAs can be employed for response prediction has thus far only been investigated in one small study, in which the total number of chromosomal breakpoints was associated with response to neoadjuvant cisplatin in breast cancer patients [202]. Also in ovarian cancer, specific CNAs as well as type of CNA (large deletions) are associated with *BRCA1*-mutation status [203]; however these features have never been related to therapy response.

DNA rearrangements

With the advent of next-generation massively parallel sequencing (MPS) techniques, new ways of investigating genomic instability can be explored. Especially, since this technology also permits evaluation of copy number neutral alterations such as point mutations, balanced translocations and inversions [204]. In-depth analysis of cancer genomes using MPS might reveal association of specific types of DNA rearrangements or mutations associated with HR deficiency, which would enable development of sequencing-based tests for identifying genomic instability and predicting response to DSB-inducing agents. Two studies have used MPS to catalogue genomic rearrangements in breast cancer. Using paired-end MPS of 9 breast cancer cell lines and 15 human breast cancer samples, Stephens et al showed that intrachromosomal rearrangements and tandem duplications were most frequent in TN breast tumors (n=4) compared to ER- or HER2-positive breast tumors but not in *BRCA*-mutated cancers (n=4) [205]. This high frequency of tandem duplications was not observed in a subsequent paired-end MPS analysis of genetically engineered mouse mammary tumors recapitulating *BRCA*-mutated hereditary breast cancer and *BRCA*-proficient sporadic breast cancer [206]. Furthermore, the type nor the frequency of genomic rearrangements was different between *BRCA*-deficient and –proficient tumors, suggesting that HR deficiency can actually not be identified using this method [206]. However, in the mouse study it seemed that microhomology in non-amplicon related rearrangements was higher in BRCA1-deficient tumors compared to BRCA1-proficient tumors [206]. It is difficult to draw firm conclusions from these two studies, because the numbers of tumors analyzed were very low. Furthermore, the heterogeneity was very high, even between tumors from the same breast cancer subtype or genetically engineered mouse model. More extensive studies involving larger collections of tumors will be required to evaluate the potential of MPS for the identification of HR deficiency and development of predictive markers.

Functional assays for homologous recombination status

Until now we have described markers which are indirectly related to HR deficiency or DSB-inducing agent sensitivity. Identification of these markers might be hampered by the fact that control groups are likely to be contaminated with sporadic HR-deficient tumors, thereby diminishing the chances of finding predictive markers. This could be the case in for example sporadic TN breast cancers, of which ~60% showed a BRCA1-like aCGH profile [162].

Thus far only few studies have pursued the identification of markers that directly test the functionality of HR. One study used seven pre-treatment breast cancer biopsies for *ex vivo* analysis of radiation-induced RAD51, BRCA1 and FANCD2 nuclear foci [207]. Although defective foci formation was found in 4 cases, of which 3 were TN tumors, the quantification of foci formation was shown to be heterogeneous [207]. Similarly, Asakawa et al. analyzed γH2AX, BRCA1 and RAD51 nuclear foci in breast cancer biopsies obtained prior to treatment and 18-24 hours after the first cycle of epirubicin-cyclophosphamide [208]. They found that clinical response was negatively correlated with the presence of RAD51 foci post chemotherapy or the presence of BRCA1, γH2AX or RAD51 foci prior to

chemotherapy [208]. Unfortunately, correlations between more reliable endpoints, such as pathological response or survival, were not studied [208]. In ovarian cancer, PARPi-induced formation of γH2AX and RAD51 foci was studied on *in vitro* cultured cells from ascitic fluid from patients [209]. An increase in RAD51 foci formation after PARPi treatment was found to be associated with increased cell survival and reduced growth inhibition; however, it is unclear how this *in vitro* response to PARPi translates to PARPi responses in the actual patients [209]. Recently, a neoadjuvant study investigating breast cancer biopsies 24 hours after anthracycline-based chemotherapy found that low RAD51 foci formation in proliferative cells correlated with TN status and a higher pCR rate [210]. However, numbers were again small and therefore this marker could not be tested next to markers known to be associated with pCR (high grade and TN status).

Although these first results indicate that functional assays hold promise as potential markers to identify all sensitive patients based on HR deficiency, implementation in the clinic might be difficult because most of these assays require extra biopsies. Furthermore, inter- and intra-tumor heterogeneity might complicate unambiguous tumor classification based on numbers of nuclear foci.

Challenges for the future

Above we have tried to summarize most efforts undertaken thus far to identify predictive markers for HR deficiency and sensitivity to DSB-inducing agents. For this overview, we have restricted ourselves to studies performed in patient settings. Although the level of evidence differs substantially per marker, it is clear that some candidate markers are very promising and should be studied with high priority in translational prospective studies. Based on the evidence obtained in breast and ovarian cancer, interesting candidate markers are: expression of BRCA1/2 and other HR genes, *BRCA1* promoter hypermethylation, aCGH classifiers and functional approaches using RAD51 foci induction.

However, even with highly specific and sensitive predictive markers for sensitivity to DSB-inducing agents many challenges lay ahead (Figure 3). Firstly, previous studies have shown that even the presence of *BRCA* mutations (supposedly the gold standard for HR deficiency) does not guarantee lasting therapy sensitivity, since secondary mutations in *BRCA1/2* can restore expression of functional protein and thereby confer resistance to DSB-inducing agents [211,212]. Secondly, it could well be that even within *BRCA*-mutated cancers, the level of HR deficiency might affect therapy sensitivity. The fact that risk of breast and ovarian cancer differ by *BRCA* mutation position [136], suggests that different *BRCA* founder mutations might also be associated with differences in level of HR deficiency. In support of this, studies in ovarian cancer patients showed that different *BRCA* founder mutations correlated with different survival rates after platinum-based chemotherapy [142]. This phenomenon could also contribute to the variation in response to PARPi observed in the phase II trials [13,14]. Thirdly, a recent study showed that restoration of HR and concomitant therapy resistance in *BRCA*-mutated tumors might also be caused by other DDR pathway aberrations, such as loss of 53BP1 [213]. Bouwman et al. showed that loss of 53BP1 in *BRCA1*-deficient cells restored partial functionality of HR [213]. Furthermore, it was shown in breast cancer patients that 15% and

11% of *BRCA1*- and *BRCA2*-mutated tumors, respectively, had reduced 53BP1 expression as measured by IHC; and this frequency was even higher in TN breast cancer [213]. Fourthly, the current focus has been on genes and pathways directly related to DNA repair; however, it has been proposed that loss of PTEN might also lead to HR deficiency and sensitivity to DSB-inducing agents [214]. Lastly, acquired resistance mechanisms might greatly influence the response and outcome of metastatic disease in patients, even when predictive markers are capable of adequate selection of (neo)adjuvant therapy. It has for example been shown that upregulation of P-glycoprotein confers acquired resistance to the clinical PARPi olaparib in a mouse model of *BRCA1*-associated breast cancer [215].

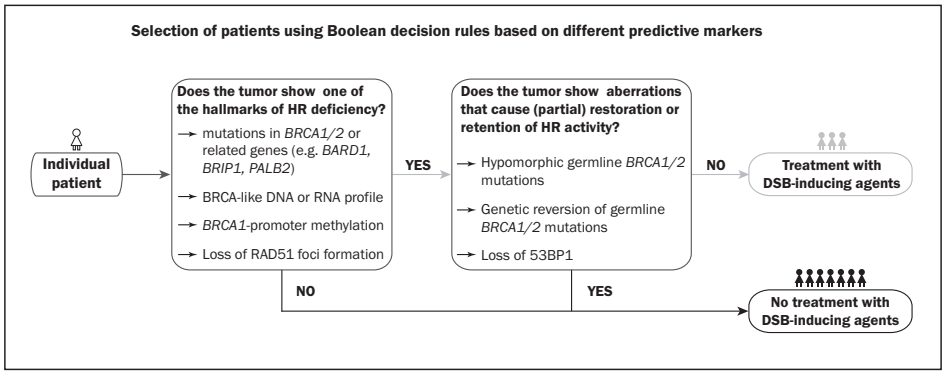

Figure 3. In the future, therapy choice might be guided by combinations of predictive biomarkers. In the presented example, the presence of one feature and the absence of a second feature might guide treatment with DSB-inducing agents.
Abbreviations: HR, homologous recombination; DSB, double-strand break.

CONCLUDING REMARKS

In conclusion, specific subgroups of breast and ovarian cancer show remarkable resemblance in terms of hormonal regulation, driver mutations, genomic instability and chemotherapy sensitivity. Hence, knowledge obtained in one cancer type might very well be applicable to the other cancer type, thereby accelerating the process of developing tailored therapies and companion diagnostic biomarkers for prediction of therapy response. Many studies have investigated predictive markers in breast and ovarian cancer for sensitivity to DSB-inducing agents, such as platinum salts, bifunctional alkylators and recently PARPi. However, the identification of truly predictive markers has proven difficult since most studies were not based on randomized controlled trials (RCTs) or lacked a control group. With the growing interest in personalized cancer therapy, correct trial designs will become increasingly important. However, given all challenges presented above one could envision that one biomarker will not suffice. Instead, decision rules based on combination of multiple markers using Boolean operators might be applied to treatment decisions in clinical practice in the future (Figure 3). While

these biomarker combinations are expected to improve the outcome of breast and ovarian cancer patients, they will also present a challenge with regard to cost-effectiveness and implementation in clinical practice.

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Supplementary Table: Overview all patient studies correlating markers to outcome.

Marker type	Regimen(s) [mg/m2]	RCT (y/n)	N (tested)	Cancer	Marker(s) used
NEOADJUVANT					
1. BRCA	Cis [75] (3wk/4 cycles), CMF, AC, FAC, AT	No	102	Breast	BRCA1-mutation
1. BRCA 2. aCGH	Cis [75] (3wkly)	No	28	Breast (TN)	BRCA1: Mutation, Methylation and Gene Expression; ΔNp63/TAp73 ratio Type P53 mutation Molecular Inversion Probe assay (Affymetrix, (ref 228))
1. BRCA	Doxorubicin [14] wkly, 16wks (test) Unknown (validation)	No	94 test (75) 163 validation of association clinicopathological features (162)	Breast	Methylation: ABCB1, ATM, BRCA1, CDH3, CKN2A, CXCR4, ESR1, FBXW7, FOXC1, GSTP1, IGF2, HMLH1, PPP2R2B, PTEN
1. BRCA	Intensified EC [75-1200]; FAC [500-50-500]; Epirubicin [100]	No	244	Breast	P53 mutation status (yeast-based assay)
1. BRCA	FEC	No	86 (41)	Breast	BRCA1 gene expression (FFPE)
1. gw-effect BRCA	4x AC [60-600] (n=16), OR RCT: 6x FEC [500-100-500], vs (3x [100] doce, 3x ET [90-70])	Partly	3 Test sets: 174 TN 2 validation sets: 16 TN – 89 TN (total 3 sets publically available)	Breast	Genome-wide effect BRCA-mutated tumors: RNA gene expression microarray
1. gw-effect BRCA	dd AC	No	60 TN 103 ER-pos/ HER2-neg	Breast	Genome-wide effect BRCA1- or BRCA2-mutated tumors: aCGH; BRCA1 gene expression and methylation; EMSY amplification
1. MMR	FAC, FA, FEcis	No	36 (29 paired samples)	Breast	Pre and post therapy IHC hMLH1

	Outcome for marker pos vs neg group	Of note	ref
	pCR /total number of patients of each treatment regimen: 1/14 CMF; 2/25 AT; 11/51 FAC or AC; 10/12 Cis	Cohort study, no randomization → direct comparison not possible (tumor size lower in cis-treated group compared to others). No comparison to non-mutated group	145
	Miller Payne score for response: 2/2 BRCA1-mutation carriers pCR; tumours with low BRCA1 gene expression (lowest quartile n=5), BRCA1 methylation (n=8) and nonsense of frameshift P53-mutations → better response; Genomic CNA separated patients on degree of response (p<0.001)		146 202
	ATM-methylation 0%; BRCA1-methylation: no association BCSS. Methylation FoxC1 or GSTp1: ↑ BCSS (adjusted) in doxorubicin-treated cohort. P53-mutations: association ↓ BCSS (adjusted) in doxorubicin-treated cohort.		152
	ER-neg and intensified EC → ↑ pCR; Significant interaction: P53-mutation status ←→ cyclo intensity; In patients with ER-neg &P53-mutated tumors: pCR 71% intensified EC (15/21), 6% FAC (1/17), 12% Epirubicin (7/57)	Case study; Type of P53-mutations have been associated with BRCA1-mutated breast tumours	153
	Total 1 pCR; Lower levels BRCA1 → ↑ TTP (adjusted hazard ratio intermediate group (n=14): 7.68, p=0.001; high group (n=13) hazard ratio: 4.52, p=0.007) and OS (adjusted hazard ratio: 4.52, p=0.007, respectively 2.94, p=0.05)	No association observed between patient characteristics and BRCA1 levels (other studies: association ER-status)	155
	Genes of BRCA1-like classifier correlated expression RT-PCR and Low-density arrays (LDA). BRCA-like → Validation 1: 7/8 near/pCR vs 2/8 non-BRCA-like after AC; Validation 2, BRCA-like: → pCR (area under ROC: 0.61) after FEC (n=50) → resistance (ROC 0.65) to randomized taxane-arm (n=39).	Resistance not defined; number pCR of validation set 2 unknown; no interaction test; not adjusted for potential confounders; performance of BRCA1-mutations identification not validated.	159
	Significant association: BRCA2-like^{CGH} status and pCR / near pCR; on multivariate analyses trend (odds ratio: 2.4, p=0.11)	BRCA1-like^{CGH} status, low BRCA1 expression and BRCA1-promoter methylation significantly associated in TN cases	161
	Significant reduction hMLH1 post therapy; Low post hMLH1 levels and difference in pre versus post → ↓ DFS (adjusted)	19/36 Grade III;	190

Supplementary Table: Overview all patient studies correlating markers to outcome.

Marker type	Regimen(s) [mg/m2]	RCT (y/n)	N (tested)	Cancer	Marker(s) used
1. MMR	FAC [500-50-500]	No	31	Breast	Pre and post therapy IHC: hMLH1, hMSH2, hMSH6, PMS2
3. RAD51	4x EC [80-600] followed by 4x doce [75]	No	60	Breast	IHC pre and 18-24 hours post therapy: γH2AX, RAD51, BRCA1, conjugated ubiquitin,
3. RAD51	Anthracycline-based CT	No	68 (57)	Breast	Immunofluorescence: pre and 24 hours post therapy: geminin, RAD51 foci
ADJUVANT					
1. BRCA	Platinum-based combination CT	No	896 (778)	Ovarian	BRCA-mutation
1. BRCA	Not mentioned (most certainly platinum-based)	No	779	Ovarian	BRCA-mutation
1. BRCA	Platinum-based CT	No	63	Ovarian	BRCA1: Mutation Methylation hMLH1 methylation
1. BRCA	Platinum-based CT	No	106 (70 with response data)	Ovarian	Methylation: 24 genes DNA-repair, apoptosis, cell cycle; of interest: BRCA1, FANCF
1. BRCA	Platinum-based CT	No	35	Ovarian	Methylation: BRCA1, MGMT, hMLH1, RASSF1A, p16
1. BRCA	Platinum-based CT	No	118	Ovarian	BRCA1: Mutation and Methylation (mutated and methylated group had low BRCA1 gene expression)

	Outcome for marker pos vs neg group	Of note	ref
	No significant difference pre and post levels; Clinical response (pCR only n=2): high post treatment levels PMS2 → poor clinical response	Low hMLH1 and hMSH2 → less differentiation; (5/31 poorly differentiated)	196
	EC treatment induced foci of H2AX, conjugated ubiquitin, RAD51; Presence of H2AX, BRCA1, RAD51 before treatment and RAD51 foci after treatment → ↓ clinical response	Clinical response; no association with other tumor features	208
	RAD51 score: % geminin positive cells also positive for RAD51; Low RAD51 score (<10%) → ↑grade and TN; Patients low RAD51 score: 4/12 pCR, high RAD51 score 1/36;		210

	carriers (n=229) vs non-carriers (n=549) : 3-yr survival rate : 66% vs 52% In stage III/IV only, median OS: 51 vs 33 months (multivariate significant)	Survival difference between patients with different BRCA1-mutations observed (smallest subgroup n=19)	142
	Carriers (n=213) vs non-carriers (n=392) Ashkenazi population: 5-yrs survival rate in stage III/IV: 38% vs 25% Adjusted hazard ratio : 0.72, p-value : 0.005		143
	Patients with BRCA1-methylated tumours (n=11) → ↓ DFS and ↓ (adjusted) OS compared to BRCA1-mutated cases (n=22), p=0.02; similar trend compared with wild-type cases (n=30), p=0.08. hMLH1 not separately investigated;		148
	13/106 BRCA1-methylated; no FANCF-methylation detected. BRCA1-methylation→ borderline significantly associated with clinical response (in n=70, partial/complete, p=0.05).	Unsupervised: subset frequently co-occuring methylated genes (in- dependent of BRCA1-methylation) no association response	149
	15/35 BRCA1-methylated; 2/35 hMLH1 methylated; BRCA1-methylation correlated with chemoresponsiveness (PFS interval >12 months), no correlation with disease status (evidence of disease) at last FU. ↑ methylated genes → chemosensitivity and no evidence of disease.	prognostic factors, such as residual disease were not significantly associated with sensitivity or evidence of disease at last FU	150
	Control patients (n=59) were matched (on age, stage, histology, grade, site of disease, site of P53 mutation and residual disease) to patients with germline BRCA1-mutated (n=24), somatic BRCA1-mutated (n=16) and BRCA1-methylated (n=19) tumors:→ no difference in survival between groups.	Matching on residual disease might have biased study. Residual disease itself is related to response and survival. It could well be that matched on all criteria except residual disease, the BRCA1-mutated group had significantly more patients with small residual disease and thereby a better survival.	151

Supplementary Table: Overview all patient studies correlating markers to outcome.

Marker type	Regimen(s) [mg/m2]	RCT (y/n)	N (tested)	Cancer	Marker(s) used
1. BRCA	Platinum-based CT Platinum-based CT / Pacli	No	46 24	Ovarian	BRCA1 gene expression (FF)
1. BRCA 1. NER	Platinum-based CT	No	51	Ovarian	BRCA1 and ERCC1 gene expression (dichotomized on MCF7 levels, FF)
1. gw-effect BRCA	Platinum-based CT	No	61 (test) 6 (validation) 70 (validation)	Ovarian	Genome-wide effect BRCA-mutated tumors: RNA gene expression microarray
1. gw-effect BRCA	5xFEC [500-90-500] vs 4xFEC [500-90-500] +1xCTC [6000-480-1600]	Yes	230 HER2-neg (60 TN, 170 ER-pos)	Breast	aCGH pattern associated with BRCA1-mutated tumors
1. gw-effect BRCA	Platinum-based CT	No	234 (215 malignant, 19 low malignant potential)	Ovarian	Methylation: P16, BRCA1, IGFBP3, GSTP1, ERα, hMLH1 (FF)
1. NC-DNA-repair	CMF [100-40-600] vs RT	Yes	224	Breast	IHC: MRe11, NBS1, RAD50 (MRN complex), ATM, BRCA1, BRCA2, RAD51 (FFPE)

	Outcome for marker pos vs neg group	Of note	ref
	Expression divided into low-intermediate-high; Univariate: OS ↓ with ↑ in expression level. Low and intermediate grouped (n=47) → high level (n=23) associated with ↓ OS (adjusted hazard ratio: 2.63, p=0.006). Taxane treatment trend to ↑ OS in BRCA1 high group.	Numbers too small to distinguish between both treatment regimens	156
	Low BRCA1 expression median OS 46 months versus high 33 months (p=0.03), especially in patients with <2cm residual disease. No difference in TTP. ERCC1 similar direction of results, all non-significant		157
	BRCA-like classifier tested in 6 samples; 4 paired: accurate distinction of platinum sensitivity in 8/10; 9/12 accurate RAD51 foci formation in cell-lines; Non-BRCA-like patients → ↓ DFS and OS compared to BRCA-like patients (adjusted hazard ratio: 2.65, p=0.016, respectively 3.39, p=0.009)	BRCA-like classifier developed upon n=34 BRCA-mutated and 27 non-mutated cases.	160
	BRCA1-like^{CGH} patients → significantly ↑ RFS after intensified CTC versus FEC (hazard ratio: 0.12, p=0.001) compared to Non-BRCA1-like^{CGH} patients (hazard ratio: 0.78, 95%CI: 0.5-1.2); → difference in treatment effect between subgroups significant (test for interaction: p=0.006)	Test for interaction significant; BRCA1-like^{CGH} status associated with BRCA1-promoter methylation	162
	p16 methylation (n=89) → disease progression (adjusted hazard ratio PFS: 1.56, 95%CI: 1.0-2.4) hMLH1 methylation (n=19) → disease progression (adjusted hazard ratio PFS: 2.04, 95CI: 1.0-4.0), both not significant with OS. Increase in number of genes methylated (excluding ERα, high in both malignant and low malignant) → worse OS (≥ 3 methylated genes approximately 2x to 4x ↑ risk of death, P=0.04)		172
	↓ RAD51 levels ↓ local recurrences after RT vs CMF (relative risk: 0.38, p=0.02), while in ↑ RAD51 no treatment differences (relative risk: 0.83, p=0.71), test for interaction: 0.22; ↑ levels MRN complex or nuclear ATM: ↓ local recurrences after RT vs CMF (relative risk: 0.27, p<0.01, respectively 0.24, p=0.03), while in ↓ MRN complex or nuclear ATM no treatment differences (relative risk: 0.87, p=0.73, respectively 0.55, p=0.11), interaction-test MRN-complex: 0.07.	Low RAD51 levels and low MRN-complex → ↑ grade and ER-neg,	174 175

Supplementary Table: Overview all patient studies correlating markers to outcome.

Marker type	Regimen(s) [mg/m2]	RCT (y/n)	N (tested)	Cancer	Marker(s) used
1. NC-DNA-repair	Cis-cyclo [80-800] vs cis-pacli [80-175]	Yes	640 stage III and IV (53)	Ovarian	FANCF methylation
1. NER	Platinum-based CT	No	28 27	Ovarian	Gene expression: ERCC1, XPA, XPB, CSB (FF)
1. NER	Carbo-pacli (AUC 5-[175])	No	60	Ovarian	SNP: ERCC1_354G>A (genomic DNA)
1. NER	Carbo-Cyclo ([500]-AUC4) vs ([500]-AUC8)	Yes	159	Ovarian	IHC: ERCC1; SNP: ERCC1_354G>A (FFPE)
1. NER	Carbo-Pacli (AUC5-[175])	No	101	Ovarian	IHC: ERCC1 (FFPE)
1. NER	Platinum-based CT	No	20 (genome-wide) 52 (candidate) 90 (gene expression)	Ovarian	Genome-wide LOH analysis, genotyping ERCC5, gene expression ERCC1-2-3-4-5-6
1. BER	Platinum-based CT	No	157 (135)	Ovarian	IHC: APE1 (FFPE)
1. NER / 1. BER	CMF FAC FEC	No	180	Breast	SNP: ERCC1-2-4-5, XRCC1-3, BRCA1, CCNA2, CCND1, CCND3, TP53 (FFPE normal breast)
1. MMR	Platinum-based CT	No	54	Ovarian	IHC pre and post therapy: hMLH1, hMSH2 (FFPE)
1. MMR	CEC [60-60-750]	No	38 (34)	Ovarian	IHC: hMLH1 (FFPE)
1. MMR	Pacli [175] vs Doce [75] + Carbo (AUC 5)	Yes	480 (149)	Ovarian	Methylation hMLH1 and MSI in plasma DNA pre therapy and at time of relapse

	Outcome for marker pos vs neg group	Of note	ref
	7/53 cases FANCF methylation; In cis-cyclo arm methylation → PD (3/4); FANCF methylation → ↓ PFS (adjusted HR 3.7, p=0.001); not significant OS; test for interaction not significant	Adjustment for 5 factors; numbers of methylated cases only 7	178
	Clinical unresponsive patients (SD+PD, n=13/12): ↑ levels of ERCC1 (p=0.06), ↑ levels of XPAC (p=0.01), ↑ levels XPB (p=0.001), ↑ levels CSB (p=0.03).		179 180
	_CT + _TT (n=25) reduced risk of resistance (progression on therapy or recurrence within 6 months; adjusted odds ratio: 0.17, p=0.02); no difference in OS or TTP between genotypes		181
	High ERCC1 levels → ↑ CA-125 non-responders (n=12, p=0.03); trend for better OS seen in 1st years after therapy; _TT → ↑ CA-125 response (p=0.05); no survival difference;	High grade: low ERCC1 levels; No correlation SNP with IHC; treatment effect not studied	182
	High ERCC1 levels (n=14) → resistance (PD during treatment or recurrence within 6 months (p=0.001) and ↓ PFS (adjusted hazard ratio: 3.6, p=0.006); similar trend in OS (not significant)		183
	LOH blocks associated with PFS; after adjustment: 13q region: ERCC5 in this region chosen as candidate, LOH ERCC5 → ↑ pCR; Low mRNA levels ERCC5 (non of the other ERCC's) ↑ PFS (adjusted hazard ratio: 0.49, p=0.03);	Expression levels non-significantly correlated with LOH (p=0.08)	184
	Nuclear expression correlated with ↓ optimal debulking; High expression: ↓ OS (median OS 52 months vs 71 months in low cases)	Expression: significantly different between histologic subgroups; No adjusted test performed	186
	Univariate SNPs in XRCC1, ERCC4 and CCND3 associated with response (Non-response: recurrence, metastasis or cancer death); Adjusted: XRCC1_1196_AA (n=27) significantly ↑ response (adjusted odds ratio: 0.19, 95%CI: 0.1-0.6); ↑ EFS (hazard ratio: 0.62, 95%CI: 0.4-0.9)		187
	Significantly lower post levels compared to pre levels → hMLH1, hMSH2; High post hMLH1 levels → clinical PD, low levels → CR (r=-0.36, p=0.008); No association OS	Pre levels of both proteins associated with histological subtype (serous type: 39%) FIGO 4: 18%	191
	Low hMLH1 levels median OS 55 months ←→ High hMLH1 levels median OS 12 months (adjusted, p=0.006); no association response or PFS	Stage III-IV patients	192
	Increase in hMLH1 methylation at time of relapse → 11% to 33%; MSI increased at time of relapse; Acquisition hMLH1 methylation (n=31) → shorter OS (hazard ratio 1.83, p=0.02; significant adjusted for histology as well)	Methylation levels measured in plasma DNA; Treatment effect not studied	193

Supplementary Table: Overview all patient studies correlating markers to outcome.

Marker type	Regimen(s) [mg/m2]	RCT (y/n)	N (tested)	Cancer	Marker(s) used
1. MMR	Platinum-based CT Not platinum-containing	No	75 (46 platinum-based)	Ovarian	MSI, hMLH1 methylation, gene expression: hMLH1, hMSH2, hMSH3, hMSH6, PMS2
1. MMR	Platinum-based CT	No	43 (30 post therapy)	Ovarian	IHC pre and post therapy: hMLH1, hMSH2, caspase-3, BCL-xl, P53, p21, p63 (FFPE)
METASTATIC					
1. BRCA	Olaparib 400mg bd Olaparib 100mg bd	No	33 24	Ovarian	BRCA-mutation
1. BRCA	Olaparib 400mg bd Olaparib 100mg bd	No	27 27	Breast	BRCA-mutation
1. BRCA	Olaparib	No	21	Ovarian	BRCA-mutation
1. BRCA	EC [60–600]	No	51	Breast	BRCA1, BRCA2 mRNA; Biopsy pre therapy: FF
1. BER	Combinations of cyclo, anthracyclines, 5-FU, mitox + Intensified: mitox, vinblastine, cyclo, carbo, pacli or CTC	No	134 (95)	Breast	SNPs: XRCC1_1196G>A, XRCC3_722C>T ; CCND1_870A>G (genomic DNA)
1. MMR	EICE [500-4000-50-50] +2x intensified VIC [1500-12000-1500]	No	39	Breast	MSI; IHC: hMLH1, hMSH2; P53 mutations (FFPE)

Marker types: 1. Knowledge-driven, mostly gene-based pursuit; 2. Markers based on hallmarks of HR-deficiency; 3. Markers testing the functionality of HR.
Abbreviations: **5FU,** 5-flourouracil; **AC**, doxorubicin-cyclophosphamide; **aCGH**, array, comparative genomic hybridization; **AT**, doxorubicin-docetaxel; **bd,** bi-daily; **BER**, base excision repair; **Carbo**, carboplatin; **CEC**, cisplatin-epibrubin-cyclophosphamide; **Cis**, cisplatin; **CMF**, cyclophosphamide-methotrexate-5-fluorouracil; **CNA**, copy number aberrations; **CR**, complete remission; **CT**, chemotherapy; **CTC**, cyclophosphamide-thiotepa-carboplatin; **Cyclo**, cyclophosphamide; **dd**, dose-dense; **DFS,** disease-free survival; **Doce**, docetaxel; **EC**, epirubicin – cyclophosphamide; **EFS**, event-free survival; **EICE**, etopside-ifosfamide-cisplatin-epirubicin; **ER**, oestrogen; **ET**, epirubicin, docetaxel; **FA**, 5-flourouracil-doxorubicin;

	Outcome for marker pos vs neg group	Of note	ref
	No MSI seen, i.e. no correlation with hMLH1 methylation (n=7); No association of low expression with clinical response (n=7 non responders vs 28 responders)		195
	No difference observed in pre and post IHC levels hMLH1 or hMSH2; High pre hMSH2 levels (n=12) → ↓ OS and PFS (univariate). High post hMLH1 levels (n=8) → ↑ OS (adjusted hazard ratio: 0.02, p=0.01); High post hMSH2 levels (n=13) → ↓ OS (adjusted hazard ratio: 12.1, p=0.04); High pre p21 levels, low caspase-3 levels and High post caspase-3 levels → longer OS (adjusted respectively p=0.004, p=0.02 and p=0.03)	Low numbers per subgroup, many markers tested. Serous ovarian cancer 86%; No FIGO 4; various in grade (1: 16%, 2: 42%, 3: 42%) but not correlated to features investigated.	197
	(Not randomized on dosing) All BRCA-mutated: Objective response rate 11/33 in 400mg cohort And 3/24 in 100mg cohort	Poor prognostic features in 100mg group; no comparison to non-mutated group	13
	All BRCA-mutated: Objective response rate 11/27 in 400mg cohort 6/27 in 100mg cohort	Not randomized on dosing; no non-mutated group	14
	16 BRCA-mutated, 15 evaluated for response: 9/15 Radiologic or CA-125response or SD	Phase I study	137
	Locally advanced breast cancer; clinical CT response; BRCA1 mRNA levels higher in responders (n=25, CR+PR)		154
	XRCC1_1196_AA (n=16) ↓ BCSS (adjusted hazard ratio: 2.3, 95%CI 1.3-4.2) and ↓ PFS (univariate); XRCC2_722_TT (n=14) ↓ BCSS (adjusted hazard ratio: 2.4, 95%CI 1.3-4.5) and ↓ PFS (adjusted hazard ratio 1.9, 95%CI 1.1-3.6); CCND1_807_GG ↓ BCSS (univariate) and ↓ PFS (univariate)	Relatively small numbers per subgroup and many different treatment schedules	188
	MSI not correlated to hMLH1 or hMSH2 levels; presence of MSI → shorter OS (p<0.001, remained significant after adjusting) and PFS (p=0.02)	hMLH1 and hMSH2 association not studied in relation to survival	194

FAC, 5-flourouracil-doxorubicin-cyclophosphamide; **FEC**, 5-fluorouracil-epirubicin-cyclophosphamide; **FEcis**, 5-flourouracil-epirubicin-cisplatin; **FF**, fresh-frozen; **FFPE**, fresh-frozen paraffin-embedded; **FU**, follow-up; **gw,** genome-wide; **IHC**, immunohistochemistry; **mitox,** mitoxantrone; **MMR**, mismatch repair; **MSI**, microsatellite instability; **NC-DNA-repair**, non complementary strand DNA-repair; **neg,** negative; **NER**, nucleotide excision repair; **OS**, overall survival; **Pacli**, paclitaxel; **pCR,** pathological complete remission; **PD**, progressive disease; **PFS**, progression-free survival; **pos,** positive; **PR**, partial remission; **RFS,** recurrence-free survival; **ROC**, receiver operating characteristic curve; **RT,** radiotherapy; **SD**, stable-disease; **SNP,** single nucleotide polymorphism; **TN**, triple-negative; **TTP**, time to progression; **VIC**, etoposide-ifosfamide-carboplatin; **wk**, week. References in bold are described more elaborately in the text, due to either their large patient numbers, the set-up of the study or the chemotherapy regimen used.

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3

AN aCGH CLASSIFIER DERIVED FROM *BRCA1*-MUTATED BREAST CANCER AND BENEFIT OF HIGH-DOSE, PLATINUM-BASED, CHEMOTHERAPY IN HER2-NEGATIVE BREAST CANCER PATIENTS

ABSTRACT

Background: Breast cancer cells deficient for *BRCA1* are hypersensitive to agents inducing double-strand DNA breaks (DSB), such as bifunctional alkylators and platinum agents. Earlier we had developed a Comparative Genomic Hybridization (CGH) classifier based on *BRCA1*-mutated breast cancers. We hypothesized that this BRCA1-like^{CGH} classifier could also detect loss-of-function of *BRCA1* due to other causes besides mutations and, consequently, might predict sensitivity to DSB-inducing agents.

Patients and Methods: We evaluated this classifier in stage-III breast cancer patients, who had been randomized between adjuvant high-dose platinum-based (HD-PB) chemotherapy, a DSB-inducing regimen, and conventional anthracycline-based chemotherapy. Additionally, we assessed *BRCA1*-loss through mutation or promoter methylation and immunohistochemical basal-like status in the triple-negative subgroup (TN-subgroup).

Results: We observed greater benefit from HD-PB-chemotherapy versus conventional chemotherapy among patients with BRCA1-like^{CGH} tumors (41/230=18%, multivariate HR=0.12, 95%CI 0.04-0.43), compared to patients with Non-BRCA1-like^{CGH} tumors (189/230=82%, HR=0.78, 95%CI 0.50-1.20), with a significant difference (test for interaction p=0.006). Similar results were obtained for overall survival (p-interaction=0.04) and when analyses were restricted to the TN-subgroup. 63% (20/32) of assessable, BRCA1-like^{CGH} tumors harboured either a *BRCA1*-mutation (n=8) or *BRCA1*-methylation (n=12).

Conclusions: *BRCA1*-loss as assessed by CGH analysis can identify patients with substantially improved outcome after adjuvant DSB-inducing chemotherapy when compared to standard anthracycline-based chemotherapy in our series.

INTRODUCTION

Most evidence for benefit of adjuvant systemic treatment comes from large clinical trials performed in the general breast cancer population [1]. However, these trials do not generally consider the molecular heterogeneity of breast cancers which may be related to treatment benefit of individual patients. The disadvantage of these traditional trials can be best illustrated with the example of trastuzumab. Its efficacy among HER2-positive breast cancer patients would likely have been overlooked in analyses of the general population, since a large percentage of breast cancers is HER2-negative and therefore does not benefit from trastuzumab. Several systemic treatments might therefore have been discarded in the past, although they may have been proven beneficial if tested in a predefined targeted population.

Among these discarded agents are bifunctional alkylators and platinum salts, which are not commonly used, with the exception of cyclophosphamide, due to their relatively high toxicity and low level of efficacy in unselected breast cancer patients [2-5]. These agents act via formation of DNA cross-links resulting in DNA double strand breaks (DSBs). Preclinical and clinical evidence has emerged that a possible target of DSB-inducing agents are tumors with a non-functional BRCA1-protein, such as tumors with *BRCA1*-mutations [6-9]. *BRCA1*-mutated tumors showed hypersensitivity to these agents which may be related to the role of *BRCA1* in homologous recombination, a conservative mechanism for error-free repair of DSBs. Absence of homologous recombination, such as in *BRCA1*-mutated tumors, prohibits error-free repair of DSBs which is reported to lead to cell death [10].

Furthermore, defects in homologous recombination activates alternative, more error-prone mechanisms such as non-homologous-end-joining, presumably leading to genomic instability [11-13]. *BRCA1*-loss related instability can be visualized by array Comparative Genomic Hybridization (aCGH) showing characteristic copy number aberrations (CNAs) in defined genomic loci in a tumor [14-17].

We have previously developed an aCGH BRCA1-like classifier aimed to differentiate between *BRCA1*-mutated and sporadic breast cancers with reasonable accuracy based on their characteristic CNAs [17]. This test has been shown to have a relatively high sensitivity but a somewhat lower specificity for *BRCA1*-mutated tumors. We hypothesized that tumors testing "false-positive" with the classifier could represent tumors with functional *BRCA1*-loss due to other causes than mutations, such as *BRCA1*-promoter methylation. If true, the BRCA1-like^{CGH} classifier would identify a larger fraction of breast cancer patients, who might benefit from DSB-inducing agents.

The aim of this study was to determine whether the BRCA1-like^{CGH} classifier was capable of identifying patients benefiting from DSB-inducing agents. For this purpose we studied a representative sample of stage-III, HER2-negative breast cancer patients who had been randomized between two treatment arms; high-dose, platinum-based, alkylating chemotherapy (HD-PB-chemotherapy), which is a DSB-inducing regimen, and a standard anthracycline-based regimen (conventional chemotherapy) in a trial with long term follow-up [18]. We restricted our analyses to HER2-negative patients, as in the pivotal study HER2-positive

patients did not benefit from HD-PB-chemotherapy [18]. Since patients in our study had been randomized, we could differentiate between selective HD-PB-chemotherapy benefit and general chemotherapy benefit. Accordingly, we evaluated whether the effect of HD-PB-chemotherapy on survival differed by BRCA1-like^{CGH} classification based on multivariate proportional hazards regression with an interaction term. To explore the biology of BRCA1-like^{CGH} classified tumors, we studied their association with other markers for *BRCA1*-loss. We studied basal-like status defined by immunohistochemistry since this had been associated with *BRCA1*-mutated breast cancers [19,20]. Secondly we assessed *BRCA1*-promoter methylation which has been reported as an alternative mechanism for reduced *BRCA1*-expression in basal-like breast cancer [21,22]. Lastly, *BRCA1*-mutation status was determined. Since we found a strong association between the BRCA1-like^{CGH} classified tumors and triple-negative status and these markers have all been associated with triple-negativity, we investigated them in the triple-negative subgroup (TN-subgroup).

PATIENTS AND METHODS

BRCA1-like^{CGH} classification

A BRCA1-like^{CGH} classifier, which calculates the probability of belonging to the *BRCA1*-mutated class, had previously been constructed (see Appendix B). We determined the optimal cut-off of the BRCA1-like^{CGH} probability-score to identify breast cancer patients likely to benefit from DSB-inducing agents (for details see Appendix B). For this purpose we studied metastatic breast cancer (MBC) patients who had participated in phase-II studies of HD-PB-chemotherapy (n=39, MBC-series described in Appendix B) [23-25]. We performed BRCA1-like^{CGH} class detection on each individual aCGH tumor profile, resulting in either a BRCA1-like^{CGH} or a Non-BRCA1-like^{CGH} score.

Patient Selection

We studied stage-III HER2-negative breast cancer patients from a large randomized controlled trial (RCT) performed in the Netherlands between 1993 and 1999 in the adjuvant setting (stage-III series). Eligibility criteria have been published previously [18] (see Appendix A). Patients were randomized between conventional chemotherapy (5*FEC: 5-fluorouracil 500mg/m^2, epirubicin 90mg/m^2, cyclophosphamide 500mg/m^2) and HD-PB-chemotherapy (4*FEC, followed by 1*CTC: cyclophosphamide 6000mg/m^2, thiotepa 480mg/m^2 and Carboplatin 1600mg/m^2) [18].

Due to practical (financial) constraints we did not evaluate all 621 HER2-negative breast cancer patients, but randomly selected 320 HER2-negative patients (320 / 621 HER2-negative cases, 51%). Patient samples were included in analyses if formalin-fixed paraffin-embedded (FFPE) primary tumor tissue consisting of more than 60% of tumor cells was available and if they had been treated per-protocol. Figure 1 summarizes the flow of patients through the study. All trials described in this manuscript were approved by the Institutional Review Board of the Netherlands Cancer Institute. This study was designed according to

the REMARK guidelines [27] following the predictive marker trial design of "Indirect assessment: Marker by treatment interaction design, test of interaction" as described by Sargent et al.[28].

Comparative Genomic Hybridization

Genomic DNA was extracted from FFPE primary tumors as previously described [29]. For 11 patients, only lymph-node tissue containing primary tumor tissue, removed at first diagnosis, was available. Of 11 samples DNA concentration were too low for direct aCGH-analysis and these samples were amplified with the *BioScore*™ Screening and Amplification Kit (42440, Enzo Life Sciences). Tumor DNA and reference DNA were labelled and hybridized as published previously (see Appendix A) [30]. To determine the quality of each CGH profile and to be able to compare experiments, we used a profile-quality and hybridization quality score (see Appendix A).

Mutation and Methylation Analyses

We screened for 38 known *BRCA1*-mutations using allelic discrimination and multiplex-PCR accounting for 853 of 1166 *BRCA1*-families (~73%) in the Netherlands (Supplementary Table 1) [manuscript in preparation; Schmidt MK, et al.]. Each putative mutation identified was validated using capillary sequencing.

Hypermethylation of the *BRCA1*-promoter was assessed using a custom methylation specific MLPA-set according to the manufacturer's protocol (ME005-custom; MRC-Holland, the Netherlands). Probe sequences of the MLPA-set are available on request (info@mlpa.com). DNA fragments were analyzed on a 3730 DNA Analyzer (AB, USA). For normalization and analysis the Coffalyzer program was used (MRC-Holland); peak heights below 250 were excluded from further analyses. When both *BRCA1*-probes showed methylation (threshold of 0.2 (MRC-Holland)), we classified the result as *BRCA1*-promoter methylation.

Histopathology

Haematoxylin&Eosin-slides were scored for tumor percentages. Oestrogen-receptor (ER), progesterone-receptor (PR), P53, and HER2 status were determined by immunohistochemistry (IHC) as described previously [18,32]. We used Pronase pretreatment for the epidermal growth factor receptor (EGFR Ab-10 clone 111.6; 1:200; Neomarkers; EGFR clone 31G7, 1:400; Zymed) and the standard procedure for cytokeratin 5/6 staining (CK5/6, clone D5/16 B4, M7237, 1:200, Dako). CK5/6 and EGFR were considered positive if any staining of tumor cells was observed. Tumors were classified as basal-like according to the Nielsen basal-like breast cancer IHC definition [33].

Statistical Analysis

Differences between groups of interest were tested using Fisher's exact tests. Survival curves were generated using the Kaplan-Meier method and compared using log-rank tests. Hazard ratios (HR) were calculated using Cox-proportional hazards regression.

Table 1. Patient characteristics distributed by treatment arm and BRCA1-classification of the stage-III series.

	Patients with Non-BRCA1-like^{CGH} tumors						
	Conventional Chemotherapy		High-Dose Chemotherapy		Total		*P* value *
Variable	**N**	**%**	**N**	**%**	**N**	**%**	
Total	**95**	**50.3**	**94**	**49.7**	**189**	**100.0**	
Age in categories							
≤ 40 years	21	22.1	22	23.4	43	22.8	n.s
> 40 years	74	77.9	72	76.6	146	77.2	
Type of surgery							
Breast conserving therapy	16	16.8	18	19.1	34	18.0	n.s
Mastectomy	79	83.2	76	80.9	155	82.0	
Pathological tumor classification							
pT1 or pT2	80	84.2	78	83.0	158	83.6	n.s
pT3	15	15.8	14	14.9	29	15.3	
Unknown	0	0.0	2	2.1	2	1.1	
No. of positive lymph nodes							
4-9	66	69.5	59	62.8	125	66.1	n.s
≥ 10	29	30.5	35	37.2	64	33.9	
Histologic grade							
I + II	63	66.3	63	67.0	126	66.7	n.s
III	30	31.6	27	28.7	57	30.2	
Not determined	2	2.1	4	4.3	6	3.2	
Triple negative status							
ER or PR positive (≥10%)	82	86.3	81	86.2	163	86.2	n.s
Triple negative	13	13.7	13	13.8	26	13.8	
Unknown	0	0.0	0	0.0	0	0.0	
Nielsen basal-like breast cancer definition							
Negative	89	93.7	85	90.4	174	92.1	n.s
Basal-like	6	6.3	9	9.6	15	7.9	
Unknown	0	0.0	0	0.0	0	0.0	
P53 status							
Negative (<10%)	51	53.7	65	69.1	116	61.4	0.05
Positive (≥10%)	40	42.1	26	27.7	66	34.9	
Unknown	4	4.2	3	3.2	7	3.7	

P values: patients with unknown values were omitted and *P* values were calculated using the Fisher's exact test.; * Association within subgroup; † Association between subgroups.
Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

Patients with BRCA1-like^{CGH} tumors							
Conventional Chemotherapy		High-Dose Chemotherapy		Total		*P* value *	
N	%	N	%	N	%		*P* value †
23	**56.1**	**18**	**43.9**	**41**	**100.0**		**n.s**
11	47.8	9	50.0	20	48.8	n.s	0.002
12	52.2	9	50	21	51.2		
8	34.8	6	33.3	14	34.1	n.s	0.03
15	65.2	12	66.7	27	65.9		
19	82.6	17	94.4	36	87.8	n.s	n.s
4	17.4	1	5.6	5	12.2		
0	0.0	0	0.0	0	0.0		
15	65.2	11	61.1	26	63.4	n.s	n.s
8	34.8	7	38.9	15	36.6		
4	17.4	1	5.6	5	12.2	n.s	<0.001
19	82.6	14	77.8	33	80.5		
0	0.0	3	16.7	3	7.3		
4	17.4	1	5.6	5	12.2	n.s	<0.001
18	78.3	16	88.9	34	82.9		
1	4.3	1	5.6	2	4.9		
7	30.4	2	11.1	9	22.0	n.s	<0.001
15	65.2	15	83.3	30	73.2		
1	4.3	1	5.6	2	4.9		
8	34.8	7	38.9	15	36.6	n.s	0.02
12	52.2	8	44.4	20	48.8		
3	13.0	3	16.7	6	14.6		

Recurrence-free survival (RFS) was calculated from randomization to appearance of local or regional recurrence, metastases or to death from any cause [18]. All other events were censored. Overall survival (OS) was time from randomization to death from any cause, or end of follow-up. Median RFS and OS were 7.6 and 8.2 years, respectively, for all 230 patients. Patients alive at last follow-up were censored at that time. All treatment comparisons were based on patients who completed their assigned treatment (per-protocol analysis) to secure the correct correlation between molecular subtype and treatment received. We assessed whether the effect of HD-PB-chemotherapy versus conventional chemotherapy on survival, expressed as the HR, differed by BRCA1-like^{CGH} status based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders. All calculations were performed using the statistical package SPSS 15.0 (for Windows).

RESULTS

Of the 320 randomly selected patients, 90 could not be analyzed with aCGH due to unavailability or low quality of tumor tissue (i.e. tumor percentage, DNA yield, quality of DNA reflected by the aCGH quality score); In Figure 1 reasons for dropout are listed. Our selection held more ER- and PR-negative patients than the HER2-negative patients not selected for these analyses. Otherwise, characteristics and treatments of these 230 cases did not differ from those HER2-negative cases of the RCT not in current analyses (Supplementary Table 2).

Forty-one of 230 tumors (18%) were scored as BRCA1-like^{CGH}. Patient characteristics did not differ by treatment arm within the BRCA1- or Non-BRCA1-like^{CGH} subgroups (Table 1). When compared to patients with Non-BRCA1-like^{CGH} tumors, patients with BRCA1-like^{CGH} tumors were generally younger and more often treated with breast conserving surgery; their tumors were more often poorly differentiated, triple-negative, basal-like and P53-positive (Table 1).

Outcome According to Treatment in Stage-III Series by BRCA1-like^{CGH} classification

The beneficial effect of HD-PB-chemotherapy compared to conventional chemotherapy differed between patients with BRCA1-like^{CGH} tumors and those with Non-BRCA1-like^{CGH} tumors (adjusted test for interaction p=0.006). Among patients with BRCA1-like^{CGH} tumors the risk of recurrence was 8-fold decreased after HD-PB-chemotherapy compared to conventional chemotherapy (adjusted HR 0.12, 95%CI 0.04-0.43, Table 2, Figure 2B), while in patients with Non-BRCA1-like^{CGH} tumors no significant treatment difference was observed (adjusted HR 0.78, 95% CI 0.50-1.20, Table 2, Figure 2A). Similar results were observed for overall survival (Figure 2C and 2D, adjusted test for interaction p=0.04, data not shown). All analyses were adjusted for pathological tumor size, number of positive lymph-nodes, Bloom-Richardson grade, triple-negative status and treatment as these were significantly associated with RFS (supplementary Table 3).

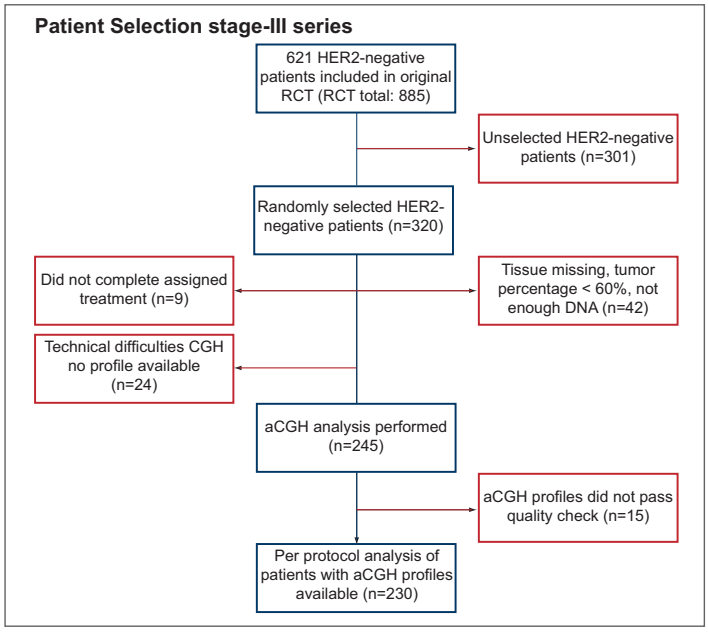

Figure 1. Flow diagram of patients in the study. Flow of patients through the study including number of patients in each stage. Reasons for dropout are listed.
Abbreviations: FEC, 5-fluorouracil, epirubicin, cyclophosphamide; aCGH, array comparative genomic hybridization.

Association of the BRCA1-like^{CGH} classifier within the triple-negative subgroup with BRCA1-mutation status, BRCA1-promoter methylation status and basal-like Nielsen phenotype

In the TN-subgroup (n=60), eight of thirteen *BRCA1*-mutated tumors had a BRCA1-like^{CGH} profile (Table 3). All twelve tumors with methylation of the *BRCA1*-promoter displayed a BRCA1-like^{CGH} profile (Table 3). All *BRCA1*-mutated tumors had an unmethylated *BRCA1*-promoter. TN BRCA1-like^{CGH} tumors displayed in 88% (30/34) a basal-like phenotype. Conversely, 33% (15/45) of the basal-like tumors scored as Non-BRCA1-like^{CGH} (Table 3). To explore the predictive potential of above markers and to put the BRCA1-like^{CGH} classifier in perspective, we assessed whether the effect of HD-PB-chemotherapy on recurrence-free survival (RFS) differed by each separate marker with an interaction term.

Outcome according to treatment in the triple-negative subgroup by different markers

Influence of the BRCA1-like^{CGH} classifier on differential treatment effect in the TN-subgroup was similar to that observed in the total group of 230 patients (p-interaction=0.05). Subsequently, no substantial modification was seen of the HRs for RFS in BRCA1-like^{CGH} (adjusted HR: 0.17, 95%CI 0.05-0.60, Figure 2F, Table 4) and Non-BRCA1-like^{CGH} patients (adjusted HR: 0.88, 95%CI 0.30-2.57,

Table 2. Multivariate Cox proportional-hazard analysis of the risk of recurrence (RFS) in the stage-III series.

	All patients Stage-III series			
Variable	**No. Events / No. Patients**	**Hazard Ratio**	**95% CI**	***P* value**
Lymph Nodes				
4 - 9	61 / 151	1.00		
≥ 10	43 / 79	1.71	1.13 – 2.59	0.01
p T-stage				
1 or 2	82 / 194	1.00		
3	22 / 34	1.95	1.19 – 3.22	0.009
Histologic grade				
I + II	55 / 131	1.00		
III	47 / 90	1.54	0.98 – 2.40	n.s.
Hormone receptor status				
ER and PR negative (<10%)	32 / 60	1.00		
ER or PR positive (≥10%)	71 / 168	0.74	0.43 – 1.25	n.s.
aCGH classifier				
Non-BRCA1-like^{CGH} tumor	83 / 189	1.00		
BRCA1-like^{CGH} tumor	21 / 41	2.07	1.02 – 4.17	0.04
BRCA1-like^{CGH} tumor				
Conventional chemotherapy	17 / 23	1.00		
High-dose chemotherapy	4 / 18	0.12*	0.04 – 0.43	0.001
Non-BRCA1-like^{CGH} tumor				
Conventional chemotherapy	47 / 95	1.00		
High-dose chemotherapy	36 / 94	0.78*	0.50 – 1.20	n.s.

Homogeneity of both hazard ratios was rejected based on an interaction term with * p-valued =0.006; Number of events is not equal for all variables, since some patients have missing data; maximum missing variables (i.e. events) of All patients Stage-III series is 2/104 events. *Abbreviations:* No, number; CI, confidence interval; p, pathological; ER, estrogen receptor; PR, progesterone receptor; n.s., non significant.

Figure 2E, Table 4). *BRCA1*-methylation interacted significantly with the effect of HD-PB-chemotherapy on RFS in the TN-subgroup (interaction p=0.02; Table 4). HD-PB-chemotherapy effects differed less strongly by basal-like status or *BRCA1*-mutation status and homogeneity was not rejected (p-interaction: p=0.83, p=0.76, respectively, Table 4).

Toxicity of HD-PB-chemotherapy and marker status

There was no correlation between *BRCA1* status as assessed by mutation, methylation or aCGH analysis and early or late (non-)haematological toxicity of HD-PB-chemotherapy.

Table 3. Distribution of patients with a *BRCA1*-mutation, a *BRCA1*-methylation and basal-like status between BRCA1-like^{CGH} and Non-BRCA1-like^{CGH} patients

	Patients with Non-BRCA1-like^{CGH} tumors		Patients with BRCA1-like^{CGH} tumors		
Variable	**N**	**(%)**	**N**	**(%)**	**P value**
BRCA1*-mutation status					
No mutation detected	19	73.1	26	76.5	n.s.
Mutation present	5‡	19.2	8	23.5	
Undetermined	2	7.7	0	0.0	
BRCA1*-promoter methylation status					
Unmethylated	25	96.2	20	58.8	0.001
Methylated	0	0.0	12	35.3	
Undetermined	1	3.8	2	5.9	
Nielsen basal-like breast cancer definition *					
Negative	11	42.3	4	11.8	0.01
Basal-like	15	57.7	30	88.2	

* Analyses performed in the triple-negative subset of the stage-III series (n=60). In 7 BRCA1-like^{CGH} tumors only ~ 62% of the types of *BRCA1*-mutations prevalent in The Netherlands were determined due to technical difficulties instead of the intended ~73%. Similarly, of 1 Non-BRCA1-like^{CGH} tumors ~40% of the type of *BRCA1*-mutations could be tested. § Two of the *BRCA1*-mutated patients identified in our analysis had been tested and identified by the familial cancer clinic. ‡ One patient scored just below the pre-determined threshold of 0.63 the BRCA1-like^{CGH} classifier (score: 0.61). BRCA1-mutations were not necessarily germ-line mutations since we tested DNA derived from the tumors. In all undetermined cases all DNA had been used for aCGH analysis and no additional analyses could be performed. *P* values: patients with unknown values were omitted and *P* values were calculated using the Fisher exact test.
Abbreviations: n.s., non significant.

DISCUSSION

In this study we observed that a BRCA1-like^{CGH} classifier, derived from *BRCA1*-mutated tumors, was capable of selecting HER2-negative patients who had a significantly better outcome after HD-PB-chemotherapy compared to conventional chemotherapy while there was no such evidence for unselected, Non-BRCA1-like^{CGH} patients (significant *P*-interactions, RFS and OS). We found a similar high proportion of triple-negative cases within BRCA1-like^{CGH} tumors (34/39, 87%) as in *BRCA1*-mutated tumors [34,35] and therefore examined the classifier's association with *BRCA1*-mutation, *BRCA1*-methylation and basal-like status in the TN-subgroup. We found that 63% (20/32) BRCA1-like^{CGH} tumors harboured either a *BRCA1*-mutation (n=8) or *BRCA1*-methylation (n=12), and these features were mutually exclusive. Furthermore, *BRCA1*-methylation status showed potential for the identification of patients with selective benefit of HD-PB-chemotherapy; however due to the small numbers these data should be interpreted with caution and no conclusions can be drawn at this stage.

The BRCA1-like^{CGH} classifier displayed two characteristics required for efficacy in clinical practice. It selected a substantial number of patients (41/230).

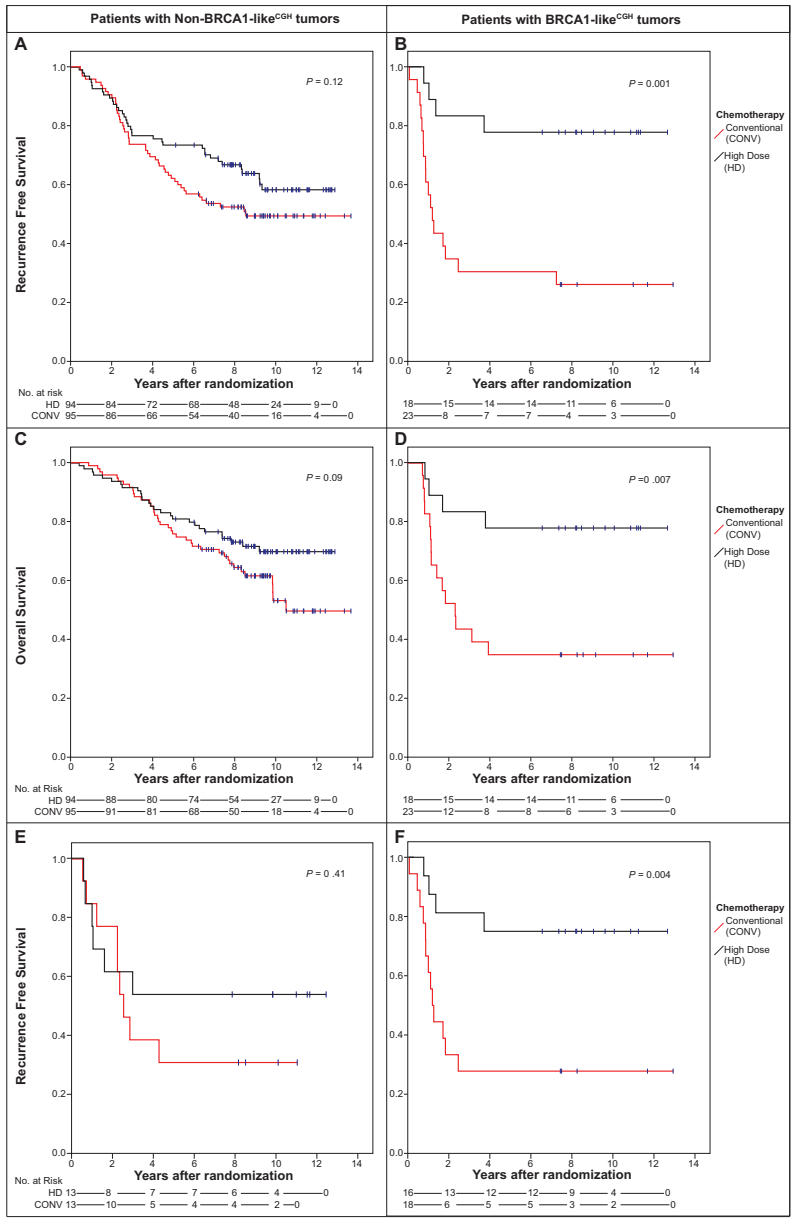

Figure 2. Association of BRCA1-like^{CGH} classification with outcome after HD-PB-chemotherapy and conventional chemotherapy in all patients of the stage-III series and the triple-negative subgroup. Kaplan Meier survival curves according to BRCA1-classification of patients who had been randomized between HD-chemotherapy and conventional chemotherapy. A) Recurrence-free survival of Non-BRCA1-like^{CGH} HER2-negative patients. B) Recurrence-free survival of BRCA1-like^{CGH} HER2-negative patients. C) Overall survival of Non-BRCA1-like^{CGH} HER2-negative patients. D) Overall survival of BRCA1-like^{CGH} HER2-negative patients. E) Recurrence-free survival of Non-BRCA1-like^{CGH} *triple-negative* patients. F) Recurrence-free survival of BRCA1-like^{CGH} *triple-negative* patients.

Table 4. Multivariate Cox proportional-hazard analysis of the risk of recurrence (RFS) for multiple markers in the triple-negative subgroup.

Variable *	No. Events / No. Patients	Hazard Ratio	95% CI	*P* value
Nielsen basal-like tumor				
Conventional chemotherapy	15 / 21	1.00		
High-dose chemotherapy	8 /24	0.36*	0.14 – 0.94	0.04
Non-basal-like tumor				
Conventional chemotherapy	7 / 10	1.00		
High-dose chemotherapy	2 / 5	0.45*	0.09 – 2.30	n.s.
BRCA1-like^{CGH} tumor				
Conventional chemotherapy	13 / 18	1.00		
High-dose chemotherapy	4 / 16	0.17†	0.05 – 0.60	0.006
Non-BRCA1-like^{CGH} tumor				
Conventional chemotherapy	9 / 13	1.00		
High-dose chemotherapy	6 / 13	0.88†	0.30 – 2.57	n.s.
***BRCA1*-mutated tumor**				
Conventional chemotherapy	3 / 6	1.00		
High-dose chemotherapy	3 / 7	0.48§	0.08 – 2.98	n.s.
No mutation found in tumor				
Conventional chemotherapy	19 / 25	1.00		
High-dose chemotherapy	6 / 20	0.35§	0.13 – 0.91	0.03
***BRCA1*-methylated tumor**				
Conventional chemotherapy	6 / 7	1.00		
High-dose chemotherapy	0 / 5	0.00‡	0 - 0.17$^{\|}$	<0.001$^{\|}$
Unmethylated tumor				
Conventional chemotherapy	15 / 23	1.00		
High-dose chemotherapy	9 / 22	0.55‡	0.23 – 1.31	n.s.

* All analyses shown were adjusted for marker of interest, lymph-node status, pathological T-stage and histologic grade as in Table 2. Homogeneity of both hazard ratios was tested with an interaction term resulting in: *p=0.833, † p=0.05, §p=0.760, ‡ p=0.020. ‖ The upper confidence bound is based on a model restricted to patients with methylated tumors because it could not be calculated in the model including methylated and unmethylated tumor patients. Number of events is not equal for all variables, since some patients have missing data; maximum missing variables (i.e. events) is 2/32. *Abbreviations:* No, number; CI, confidence interval.

Secondly, in this series it predicted a large differential treatment effect; selected patients showed an improved outcome after HD-PB-chemotherapy when compared to standard anthracycline-based adjuvant chemotherapy and, just as importantly, unselected patients did not seem to have any advantage over standard chemotherapy as demonstrated by their hazard ratios being close to one. Furthermore, it showed a large overlap with the other markers, 8/13 *BRCA1*-mutated tumors scored as BRCA1-like^{CGH}. Why all *BRCA1*-mutated tumors did not score as BRCA1-like^{CGH} is a matter of speculation. In two cases, the tumor cell

content was estimated to be below 60% at blinded repeat examination, which may have caused excess 'dilution' of the tumor DNA by normal DNA. In a third case, a BRCA1-like^{CGH} score of 0.61 was found, while 0.63 was the pre-determined threshold for a BRCA1-like^{CGH} status. In tumors with a low tumor percentage or tumors scoring near the threshold confirmation of the test results by *BRCA1* sequencing may be advisable. In addition, many BRCA1-like^{CGH} tumors had a basal-like phenotype based on the Nielsen definition [33] in our series (~ 75%). However, basal-like phenotype and BRCA1-like^{CGH} do not seem to be identical markers, since a substantial amount, one-third (15/45), of the basal-like tumors scored as Non-BRCA1-like^{CGH}. Of the *BRCA1*-methylated tumors 12/12 scored as BRCA1-like^{CGH}; given the small numbers it could well be that the accuracy of the BRCA1-like^{CGH} classifier for identifying *BRCA1*-methylated cases is overestimated. However, it should be noted that in our study one-third of the BRCA1-like^{CGH} tumors showed *BRCA1*-promoter methylation, supporting our hypothesis that the classifier also identifies patients with *BRCA1*-loss conferred by causes other than mutations. This hypothesis was further strengthened by a recent publication with a similar approach, in which *BRCA1/2*-mutated ovarian cancers were used to develop a gene expression profile of BRCAness [36]. In this study 20/70 sporadic ovarian cancer patients scored as having BRCAness and had a significantly longer disease-free survival after platinum agents [36].

Our study is in line with previous findings in which *BRCA1*-methylation was associated with good response to a platinum agent in 28 triple-negative breast cancer (TNBC) patients in the neoadjuvant setting [37]. In that study using tumor response according to Miller-Payne criteria as a surrogate endpoint for outcome, 2/2 TNBC patients with a *BRCA1*-mutation achieved a pathological complete remission (pCR) on conventionally-dosed cisplatin [37]. Similarly, Byrski et al. studied a cohort of 102 *BRCA1*-mutation carriers from 16 hospitals who had received various chemotherapy regimens in the neoadjuvant setting [9]. Ten out of twelve patients (83%) achieved pCR on cisplatin monotherapy, while only 11/51 (22%) patients who had received an anthracycline-based regimen achieved pCR [9]. Byrski et al. cautioned however, that their study was an observational study and patients in the cisplatin group had smaller tumors, were more often node-negative and none of them had received prior chemotherapy, making direct comparison among treatment groups difficult [9]. We did not observe a greater beneficial effect of platinum-based HD-PB-chemotherapy over conventional chemotherapy in *BRCA1*-mutated compared to non-*BRCA1*-mutated breast cancers in the context of a RCT, which might at least partly be caused by the small numbers. Furthermore, we studied survival data with a median follow-up time of 7 years as the endpoint, instead of response to neoadjuvant chemotherapy. Additionally, it has been suggested that mutation site in the *BRCA1*-gene could influence sensitivity to these agents [38]. Similarly, secondary mutations restoring the *BRCA1* reading frame in *BRCA1*-mutated cancers could lead to resistance, as has been described for ovarian cancers [39]. The low incidence of *BRCA1*-mutated breast cancer will make it challenging to resolve these remaining questions.

We did not find a significantly different benefit of HD-PB-chemotherapy over conventional chemotherapy between basal-IHC and non-basal-IHC patients

within the TN-subgroup. In contrast, Diallo-Danebrock et al. found an improved outcome after high-dose chemotherapy compared to dose-dense chemotherapy in high risk breast cancer patients with a basal-IHC phenotype [40]. However, this was not studied in the TN-subgroup of patients and high-dose chemotherapy used in this study did not include a platinum salt. It is important to dissect TNBC in at least two subgroups, as TNBC has been shown to derive substantial benefit from addition of taxanes [41,42], while in preclinical studies relative resistance against taxanes has been demonstrated for breast cancer cells lacking functional BRCA1 [6,7]. We hypothesize therefore that BRCA1-like^{CGH} TNBC patients should receive DSB-inducing regimens, while Non-BRCA1-like^{CGH} TNBC patients should receive taxane-based regimens. A neoadjuvant study has been initiated to test this hypothesis [NCT01057069].

The resolution of the CGH platform used in our study was lower compared to newer commercially available platforms. Nevertheless, it is unlikely that findings based on low resolution disappear on high resolution. Moreover, as we tested an existing classifier developed several years ago, we were confined to using the same platform. A limitation of our study was that it consisted of an unplanned subgroup analysis in a RCT. However, the use of a RCT allowed us to determine whether the association between markers and improved survival was related to either selective sensitivity to high-dose platinum-based chemotherapy, or to general chemotherapy sensitivity/resistance.

Despite increased toxicity of HD-PB-chemotherapy in the whole group, we did not observe a difference between patients with or without a BRCA1-like^{CGH}, *BRCA1*-methyled or *BRCA1*-mutated tumor. This corroborates with the synthetic lethality concept in which cells with a functional *BRCA1*-protein maintain their homologous recombination function and are capable of repairing the DSBs induced by the HD-PB-chemotherapy, like normal tissues of *BRCA1*-mutation carriers that did not lose the wild-type allele. In an era where we have largely abandoned HD-PB-chemotherapy as a toxic regimen with no survival benefit, it is tempting to disregard the potential of the predictive markers investigated in our study, especially given the controversy surrounding this subject [43-45]. However, recurrence-free survival differences observed between HD-PB-chemotherapy and conventional, anthracycline-based chemotherapy in the BRCA1-like^{CGH} are remarkable. Presumably this difference observed is an overestimation of the actual effect and should be confirmed in other studies. Because of constraints of the trial, unfortunately, we could not determine whether the platinum-based DSB-inducing regimen would have resulted in a similar improved outcome had it been conventionally dosed. We can only speculate that given the molecular background of the aCGH-classifier (derived from *BRCA1*-mutated tumors) the type of agents is mandatory, all causing DSBs in the DNA, and explains the beneficial effect of HD-PB-chemotherapy. This is particularly interesting given the fact that, recently a far less toxic, new DSB-inducing agent has been introduced in the form of poly(ADP)(PARP)-ribose inhibitors, which has been shown to target *BRCA1*-mutated breast cancer[8,46]. Therefore, it would be interesting to consider the subgroup identified by the aCGH classifier for studies with PARP-inhibitors only or in combination with alkylating or platinum agents. To assess the usefulness of these markers

for prediction of PARP-inhibitor benefit we have initiated a small pilot study in patients treated with an olaparib-containing regimen in the metastatic setting.

In conclusion, our data suggest that the BRCA1-like^{CGH} classifier might be predictive for selective HD-PB-chemotherapy benefit, a DSB-inducing regimen. However, what the role of *BRCA1*-methylation, basal-like and *BRCA1*-mutation status is remains unclear due to small numbers. This is the first study in breast cancer patients in which all these markers were evaluated in the context of a RCT with long term outcome. However, these findings do not justify the introduction of HD-PB-chemotherapy as a standard treatment option for breast cancer patients with a BRCA1-like^{CGH} tumor. The use of the aCGH classifier as a predictive marker for HD-PB-chemotherapy, but especially for other DSB-inducing regimens (such as other alkylators, preferably in combination with PARP-inhibitors) and the additive value of additional biomarkers, such as *BRCA1*-methylation, separately and in combination warrants further investigation and validation, preferably in prospective RCTs.

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CONFLICT OF INTEREST

S.C. Linn, M.A. Vollebergh, and P.M. Nederlof are named inventors on a provisional patent application for the aCGH BRCA1-like^{CGH} classifier used in this study; above authors have declared no further conflicts. All other authors of this paper have declared no conflicts of interest.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Mutation Analysis: mutations tested.

BRCA1 mutations analyzed*	
IVS21-36del510 †	2316del5
2804delAA	3600del11
5382insC	3889delAG
2312del5	1438delT
185delAG	2329delC
1411insT	3939insG
185insA	2355insG
2138delA	2809insA
3867 G>T (E1250X)	2845delA
2457 C>T (Q780X)	1438insT
3109insAA	2057del8
3604delA	2080insA
4446 C>T (R1443X)	2331del4
5622 C>T (R1835X)	2845insA
2841 G>T (E908X)	2846del4
1406insA	2883del4
3938insG	3940insG
2080delA	5389del7
3875del4	IVS20+1G>A
Total: 73.2% ‡	

* In short, frame shift and splice site mutations were detected by the Detection of Small Deletions and Insertions (DSDI) method while substitutions were detected by the Allelic Discrimination (AD) assay. We used tumour DNA isolated initially for aCGH analysis for mutation analysis. For 7 samples extra DNA was isolated to complete the analysis, 4 of which did not contain any tumour cells and was used in the AD assay only. In total 10 genomic *BRCA1* fragments were amplified with fluorescent-labelled primers in 7 multiplex PCR reactions for the DSDI-assay. One *BRCA1* fragment only amplified when the large genomic deletion around exon 22 was present [31]. PCR products were pooled, run in one lane on an ABI PRISM 3730 DNA analyzer and analyzed with Genemapper 4.0 (Applied Biosystems, Foster City, CA, U.S.A.). For AD, 6 *BRCA1* fragments were amplified with primers and 'Minor Groove Binding' probes (Applied Biosystems, Warrington Cheshire, UK) and run on the ABI PRISM 7500 Fast Real-Time PCR system. Direct sequencing of suspect mutations identified by DSDI or AD was performed on the ABI PRISM 3730 DNA analyzer. † IVS21-36del510, Hogervorst F. on behalf of the Netherlands DNA diagnostic laboratories, personal communication. ‡ Percentage accounting for number of families with these specific germ-line mutations in relation to pathogenic germ-line *BRCA1* mutations of all known families.

Supplementary Table 2. Distribution of clinicopathological variables between HER2-negative patients analyzed with aCGH and not in the current analysis from the stage-III series.

Variable	Total		In analysis with aCGH BRCA1-classifier		Not in current analysis		
	N	(%)	N	(%)	N	(%)	*P* value*
Total	621	100	230	37.0	391	63.0	
Treatment							
Conventional chemotherapy	298	48.0	118	51.3	180	46.0	n.s.
High Dose chemotherapy	294	47.3	112	48.7	182	46.5	
Not treated according to protocol	29	4.7	0	0.0	29	7.4	
Age in categories							
≤ 40 years	160	25.8	63	27.4	97	24.8	n.s.
> 40 years	461	74.2	167	72.6	294	75.2	
Type of surgery							
Breast conserving therapy	138	22.2	48	20.9	90	23.0	n.s.
Mastectomy	483	77.8	182	79.1	301	77.0	
Tumour classification							
T1 or T2	519	83.6	194	84.3	325	83.1	n.s.
T3	92	14.8	34	14.8	58	14.8	
Unknown	10	1.6	2	0.9	8	2.0	
No. of positive lymph nodes							
4-9	404	65.1	151	65.7	253	64.7	n.s.
≥ 10	217	34.9	79	34.3	138	35.3	
Histologic grade							
I + II	373	60.1	131	57.0	242	61.9	n.s.
III	229	36.9	90	39.1	139	35.5	
Not determined	19	3.1	9	3.9	10	2.6	
Oestrogen receptor status							
Negative (<10%)	148	23.8	65	28.3	83	21.2	0.05
Positive (≥10%)	472	76.0	165	71.7	307	78.5	
Unknown	1	0.2	0	0.0	1	0.3	
Progesterone receptor status							
Negative (<10%)	225	36.2	98	42.6	127	32.5	0.02
Positive (≥10%)	383	61.7	130	56.5	253	64.7	
Unknown	13	2.1	2	0.9	11	2.8	
P53 status							
Negative (<10%)	342	55.1	131	57.0	211	54.0	n.s.
Positive (≥10%)	239	38.5	86	37.4	153	39.1	
Unknown	40	6.4	13	5.7	27	6.9	

* *P* values: patients with unknown values were omitted and *P* values were calculated using the Fisher exact test.

Supplementary Table 3. Univariate Cox proportional-hazard regression analysis of the risk of Recurrence (RFS) after randomization in the stage-III series.

Variable	No. of events / No. of patients	Hazard Ratio	95% CI	*P* value
Age				
≤ 40 years	32 / 63	1.00		
> 40 years	72 / 167	0.74	0.49 – 1.12	n.s.
Type of surgery				
Breast conserving therapy	21 / 48	1.00		
Mastectomy	83 / 182	1.03	0.64 – 1.67	n.s.
Pathologic tumour classification				
pT1 & pT2	82 / 194	1.00		
pT3	22 / 34	2.12	1.32 – 3.39	0.002
No. of positive lymph nodes				
4-9	61 / 151	1.00		
≥ 10	43 / 79	1.61	1.09 – 2.38	0.02
Histologic grade				
I & II	55 / 131	1.00		
III	47 / 90	1.55	1.05 – 2.29	0.03
Hormone receptor status				
ER and PR negative (<10%) (TN)	32 / 60	1.00		
ER or PR positive (≥10%)	71 / 168	0.57	0.38 – 0.87	0.009
P53 status				
Negative (<10%)	61 / 131	1.00		
Positive (≥10%)	37 / 86	0.92	0.61 – 1.38	n.s.
Treatment				
Conventional Chemotherapy	64 / 118	1.00		
High Dose Chemotherapy	40 / 112	0.55	0.37 – 0.82	0.003
aCGH classifier				
Non-BRCA1-like^{CGH} tumour	83 / 189	1.00		
BRCA1-like^{CGH} tumour	21 / 41	1.56	0.97 – 2.52	n.s.

Abbreviations: CI, confidence interval; ER, oestrogen receptor; PR, progesterone receptor, TN, triple-negative.

APPENDIX A. SUPPLEMENTARY METHODS

Patient Selection

Patient series

We studied patients who had been treated with high-dose, platinum-based, alkylating chemotherapy (HD-PB-chemotherapy), a DNA DSB-inducing regimen. We determined the optimal cut-off for the probability score of belonging to the *BRCA1*-mutated class of the BRCA1-like^{CGH} classifier to identify *metastatic* patients with a long-term survival after HD-PB-chemotherapy (MBC-series, for details see online Appendix B). To determine whether the resulting BRCA1-like^{CGH} classifier was capable of identifying patients benefiting from DSB-inducing agents, we applied the classifier to a representative sample of stage-III, HER2-negative breast cancer patients who had been randomized between HD-PB-chemotherapy and conventional chemotherapy (stage-III series). Since patients in our study had been randomized between treatment arms, we could differentiate between selective HD-PB-chemotherapy benefit and general chemotherapy benefit.

Characteristics Stage-III series

Patients were included from a large multicentre randomized controlled trial performed in the Netherlands between 1993 and 1999, in which patients were randomized to conventional chemotherapy of that time or to high-dose alkylating chemotherapy (CTC) with autologous stem cell support [18]. For this trial patients had to have at least four axillary lymph nodes with metastases but no distant metastases (stage-III disease); Eastern Cooperative Oncology Group–Zubrod performance status:0-1; Chemotherapy had to begin within six weeks after the last surgery; No other cancers were allowed except adequately treated in situ carcinoma of the cervix or basal-cell carcinoma of the skin.

Inclusion criteria Stage-III series

We randomly selected a group of 320 stage-III breast cancer patients (Fig. 1). Since BRCA1-like^{CGH} tumors were highly associated with triple-negativity, we enriched slightly for triple-negative cases. Patients were included if formalin-fixed paraffin-embedded (FFPE) primary tumor tissue consisting of more than 60% of tumor cells.

Exclusion criteria Stage-III series

Patients were excluded from analysis if they did not complete the assigned treatment to secure the correct correlation between molecular subtype and treatment received (Fig. 1).

Treatments Stage-III series

Patients had been treated as previously described [18]. In short, conventional chemotherapy consisted of 5-fluorouracil 500mg/m^2, epirubicin 90mg/m^2, cyclophosphamide 500mg/m^2 ($FE_{90}C$) every three weeks for five courses. In the HD-PB-chemotherapy group stem cells were mobilized and harvested after the third course of $FE_{90}C$. The high-dose alkylating regimen consisted of 6000 mg/m^2 cyclophosphamide, 480 mg/m^2 thiotepa and 1600 mg/m^2 Carboplatin (CTC) and

was administered after 4 courses of $FE_{90}C$. Both groups received radiotherapy and, in case of hormone receptor positive breast cancer, tamoxifen after chemotherapy.

METHODS: Comparative Genomic Hybridization

Specimen characteristics

DNA was used from FFPE primary tumor tissue of the patients. This DNA was extracted as published previously [29]. Of 11 samples the DNA concentration was too low and these samples were amplified with the *BioScore*™ Screening and Amplification Kit (42440, Enzo Life Sciences). For the reference DNA, DNA was isolated from peripheral blood leucocytes from six apparently healthy female individuals. It was pooled and sonicated until its median fragment length was similar to that of tumor DNA. After isolation all samples were stored at 4°C.

Assay methods

DNA was labeled according to the manufacturers' instructions (Kreatech Biotechnology, Amsterdam, http://www.kreatech.com/) and used for aCGH as published previously [30]. Slides were scanned with an Agilent DNA Microarray Scanner BA on the same day. Data processing included signal intensity measurement in ImaGene Software; Computation of the profiles included local background subtraction, Cy5/Cy3 ratio, 2log-transformation and subarray normalisation to its median. Bad morphology or uniformity spots were flagged in ImaGenes. When flagged spots accounted for >5% of all spots, hybridizations were excluded.

Quality control procedures

To determine the quality of each CGH profile and to be able to compare experiments, we used a profile-quality score. This score is based on the median multiplied by the standard deviation of the distance between the log2 ratios and the ratios of the underlying ploidy level estimated by CGH-segmentation [29]. The logarithm of the minus square root of the median score had to be higher than 0.85 to pass the quality check. For the quality of hybridization we assessed a statistic based on the variance observed between the triplo-spotted BAC-clones, an empirically defined cut-off (on former CGH analyses experiences) of 1.0 is used in our institute.

Reproducibility assessments of the platform

During the hybridizations reference samples were run against themselves (self - self hybridizations) to ensure that all BAC-clones reported on target and that labeling and hybridization protocol were correct. All 10 self-self hybridizations showed a straight band with log2ratios of the CGH-segmentations of zero.

Reproducibility assessments of the classifier

To test the reproducibility of the BRCA1-like^{CGH} classifier 19 tumor DNA samples were hybridized two times. In total two tumors switched classes from the Non-BRCA1-like^{CGH} group to the BRCA1-like^{CGH} group upon second hybridization.

Scoring protocols

The BRCA1-like^{CGH} classifier (Appendix B) was run on all individual aCGH profiles and used a shrunken centroid algorithm (value of centroid is based on the previously built classifier, see Appendix B). A BRCA1-like^{CGH} probability score between 0 and 1 was calculated for every aCGH profile, i.e. patient. We trained the cut-off of the BRCA1-like^{CGH} probability score on the MBC-series to obtain the highest positive predictive value for response (determined as a PFS longer than 24 months, the median overall survival of metastatic breast cancer patients). The cut-off was set at higher than or equal to 0.63 (Appendix B, figs. B2.1 and B2.2).

Blinding

Since the classifier was built on an independent dataset, the algorithm was blinded to the study-endpoint. To determine the cut-off the outcome (<24 months or $\geq$24 months) was not blinded to the researcher. For the stage-III series the algorithm was already available and the cut-off was set with no prior knowledge of the outcome in RFS.

Handling of marker value in analysis

We trained the cut-off on the MBC-series to obtain the highest positive predictive value for response (determined as a PFS longer than 24 months, the median overall survival of metastatic breast cancer patients). The cut-off of the BRCA1-like^{CGH} score determined in the MBC-series was tested in the stage-III series.

APPENDIX B

Online files of BRCA1-like^{CGH} classifier
BRCA1-like classifier_shrunken centroids.txt
BRCA1-like classifier_values Si.txt

Development of original BRCA1-like^{CGH} classifier

Previously, regions of differential gain or loss between *BRCA1*-mutated breast tumors and control cases were identified using metaphase CGH [14,16]. To increase sensitivity and specificity of detection of *BRCA1*-mutated tumors and to pinpoint the specific regions of gains and losses associated with separating BRCA1-mutated and control tumors, we changed to a 3.5k Human BAC array (NCBI's Gene Expression Omnibus platform number: GPL4560) and constructed a BAC classifier using the shrunken centroids algorithm [26]. A training set of 18 *BRCA1*-mutated tumors and 32 sporadic breast tumors (not further described in this manuscript) was employed for this purpose. Samples are classified based on the shrunken centroids algorithm [26], employing the shrunken centroids as provided in the file 'BRCA1-like classifier_shrunken centroids.txt', the pooled within-class standard deviation for each BAC, denoted by si and provided in the file 'BRCA1-like classifier_values Si.txt' and a positive constant (s0 = 0.16461). This algorithm calculates, for each sample, the probability of belonging to the *BRCA1*-mutated class, which is, per definition, a real value, between 0 and 1. 95% reference intervals were calculated based on the class-probability distribution in the training set; a sample was assigned to the BRCA1-like^{CGH} class when the probability of belonging to the *BRCA1*-mutated class exceeded 0.8. This BRCA1-like^{CGH} classifier was validated on an independent set of 10 *BRCA1*-mutated tumors and 17 sporadic tumors (not further described in this manuscript). The classifier assigned 10/10 *BRCA1*-mutated tumors to the correct BRCA1-like^{CGH} class and 1/17 sporadic tumors was incorrectly classified as BRCA1-like^{CGH}. The original version of this classifier was optimized in this study to identify breast cancer patients likely to benefit from DSB-inducing agents (see below). Simultaneously, the original classifier was refined to use as a pre-selection tool for the identification of *BRCA1*-mutation carriers, which resulted in a slight modification [17].

Optimization of BRCA1-like^{CGH} classifier by determining the optimal cut-off of the BRCA1-like^{CGH} probability score to predict benefit of DSB-inducing agents

For the optimization, we studied metastatic breast cancer (MBC) patients participating in phase-II studies of high-dose platinum-based chemotherapy (HD-PB-chemotherapy) [23-25]. Eligibility criteria and patient characteristics have been published previously (see for details Table B1) [23-25]. Patient samples were included in the analysis if formalin-fixed paraffin-embedded (FFPE) primary tumor tissue consisting of more than 60% of tumor cells was available and if they had received at least one HD-PB-chemotherapy course. Figure B1 summarizes the flow of the MBC patients.

We optimized the classifier by determining the optimal cut-off of the BRCA1-like^{CGH} probability score of the classifier to obtain the highest positive predictive value for identification of patients with a progression-free survival (PFS) of at least two years after HD-PB-chemotherapy (the median overall survival of MBC patients). PFS was defined as time from the first CTC-course to appearance of first progression of disease. Patients who did not experience progression were censored at the end of follow-up (no patient was lost or died before progression).

RESULTS

Obtaining optimized cut-off in MBC-series

Figure B2.1 and B2.2 shows the performance of different cut-offs of the BRCA1-like^{CGH} probability score to identify those patients (out of 39 patients in total) with

Table B1. Patient selection of MBC-series.

Patient Selection
Characteristics MBC-series
Patients were included from three studies carried out in the Netherlands Cancer Institute between 1993 and 2004 [23-25]. From all patients written informed consent was obtained. Treatment protocols of these three studies resembled each other closely. Inclusion criteria for these studies were: biopsy proven stage IV breast cancer, age <55 years, performance status: Zubrod Scale 0-1 and either hormone receptor negative tumors or, if positive, failure of at least one adequate hormonal therapy. In 2003, the inclusion criteria were narrowed by 3 new criteria: 1) patients with HER2-negative tumors; 2) presence of 'oligometastatic disease' (defined as patients in whom all detectable tumor localizations could either be resected or irradiated with curative intent); 3) no involvement of the central nervous system. (one patient in our current study was included after 2003).
Inclusion criteria MBC-series
Availability of formalin-fixed paraffin-embedded (FFPE) primary tumor tissue containing more than 60% of tumor cells. Patients had to have received at least one course of platinum-containing therapy.
Exclusion criteria MBC-series
Failure to receive at least one course of platinum-based chemotherapy (i.e. progressive disease on the first chemotherapy regimen consisting of fluorouracil, epirubicin and cyclophosphamide ($FE_{120}C$)); treatment-related death; contralateral or stage-IIIc breast cancer and unavailability or ineligibility of primary tumor tissue.
Treatments MBC-series
Patients were treated as previously described [23-25]. In short, treatment was started with 5-fluorouracil 500mg/m2 ($FE_{120}C$), epirubicin 120mg/m2, cyclophosphamide 500mg/m2. The second course of $FE_{120}C$ was used for stem cell mobilization. One patient in our study received 6 courses of adriamycine-vincristine, methotrexate-5-fluorouracil instead of $FE_{120}C$. When patients responded to $FE_{120}C$, including stable disease, the alkylating regimen consisting of a total dose of 1060 mg/m2 carboplatin, 320 mg/m2 thiotepa and 4000mg/m2 cyclophosphamide (CTC) was administered. The majority of patients received three courses of this so-called 'tiny' CTC regimen. Resection or irradiation of residual disease was performed whenever necessary and possible after the last course. In our current study five patients were included from the second study and additionally received reinfusion of autologous lymphocytes with GM-CSF after CTC and one patient received oral paclitaxel as part of the third study. Patients attended the hospital every four to eight weeks.

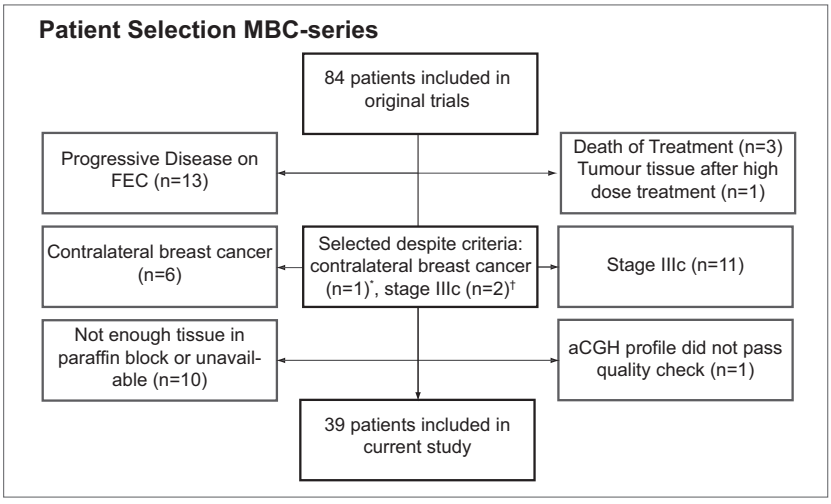

Figure B1. Flow diagram of metastatic breast cancer patients. Flow of patients through the study including number of patients at each stage. Reasons for dropout are listed. Three patients who did not satisfy our predetermined selection criteria were not removed from the analysis: * Two would have had stage IIIc disease according to current criteria, but had stage IV disease according to the pertinent version of the American Joint Committee on Cancer (AJCC) Staging Manual at the time of inclusion in the phase II study. † One had a second breast cancer.

a PFS >24 months. We considered tumors with a BRCA1-like^{CGH} probability score $\geq$0.63 as BRCA1-like^{CGH} (n=16, 41%) and others as Non-BRCA1-like^{CGH}.

Characteristics of MBC patients according to BRCA1-like^{CGH} status using optimized cut-off

Compared with Non-BRCA1-like^{CGH} tumors, BRCA1-like^{CGH} tumors were more often HER2-negative (p=0.056), ER-negative (p=0.017), basal-like (p=0.0004) and had a significantly better response to CTC-treatment, defined by achievement of complete remission (p=0.020) (Table B2). To give an impression of PFS, the Kaplan Meier curve of the BRCA1-like^{CGH} classifier is shown in Figure B3; the analysis itself does not hold much merit since we trained the cut-off of the classifier on PFS. To ascertain that the BRCA1-like^{CGH} classification was not equivalent to mutation status, we screened for *BRCA1*-mutations. We identified 2 *BRCA1*-mutated tumors, both of which had a BRCA1-like^{CGH} profile (Table B1).

Quality of the aCGH platform and Reproducibility of the optimized BRCA1-like^{CGH} classifier For this specific series, a quality control measure was provided by the known HER2-receptor status; nine patients were found to be HER2-receptor positive on immunohistochemistry of which 7 were reconfirmed using CISH. The HER2-receptor gene is located on the aCGH platform. Of all known HER2-positive patients the lowest log2-ratio for this location on the platform was 1.22 (CISH: 6) and the highest 2.56 (CISH>10). This corresponds to respectively 5 to 12 copies of the *HER2*-gene, verifying the precision of the aCGH platform.

Table B2. Patient characteristics by profile of the MBC-series.

	Patients with Non-BRCA1-like^{CGH} tumors		Patients with BRCA1-like^{CGH} tumors		
Variable	**N**	**%**	**N**	**%**	***P*‡**
Total	**23**	**100**	**16**	**100**	
Age at CTC*					
≤ 40 years	7	30.4	8	50.0	0.32
> 40years	16	69.6	8	50.0	
Metastatic disease*					
≤ 2 sites of metastases	12	52.2	10	62.5	0.74
> 2 sites of metastases	11	47.8	6	37.5	
Histological grade†					
Grade 1 and 2	9	39.1	4	25.0	0.49
Grade 3	14	60.9	12	75.0	
HER2 status†					
Negative	15	65.2	15	93.8	0.06
Positive	8	34.8	1	6.3	
Estrogen receptor status†					
Negative	11	47.8	14	87.5	0.02
Positive	12	52.2	2	12.5	
Progesterone receptor status†					
Negative	11	47.8	12	75.0	0.24
Positive	6	26.1	2	12.5	
Unknown	6	26.1	2	12.5	
Nielsen basal-like breast cancer definition †					
Negative	22	95.7	7	43.8	<0.001
Positive	1	4.3	9	56.2	
Prior Chemotherapy§					
No	13	56.5	14	87.5	0.08
Yes	10	43.5	2	12.5	
Number of CTC courses					
< 3 courses	9	39.1	3	18.8	0.29
3 courses	14	60.9	13	81.3	
CTC Response‖					
All other responses	14	60.9	3	18.8	0.02
Complete Remission	9	39.1	13	81.3	
BRCA1*-mutation status*					
No mutation present	22	95.6	14	87.5	0.16
BRCA1-mutation present	0	0.0	2	12.5	
Unknown	1	4.4	0	0.0	

***** at start first CTC treatment. † Of primary tumor, except for two patients of whom only the lymph node metastasis tissue of the primary tumor was available. § Prior chemotherapy, in all cases consisted of cyclophosphamide, methotrexate and fluoruracil (CMF) in the adjuvant setting, except one case who received five courses of adjuvant $FE_{90}C$. ‡ *P* values: patients with unknown values were omitted and *P* values were calculated using the Fisher exact test. ‖ Complete remission was defined as disappearance of all evaluable tumor mass assessed by physical examination and imaging studies ** In 3 BRCA1-like^{CGH} tumors only ~ 63% of the types of *BRCA1*-mutations prevalent in The Netherlands were determined due to technical difficulties. Similarly, of 3 Non-BRCA1-like^{CGH} tumors ~64% of the type of *BRCA1*-mutations could be tested instead of the intended ~73%. *Abbreviations*: CI, confidence interval; HER2, human epidermal growth factor receptor 2; CTC, carboplatin-thiotepa-cyclophosphamide.

Figure B2.1

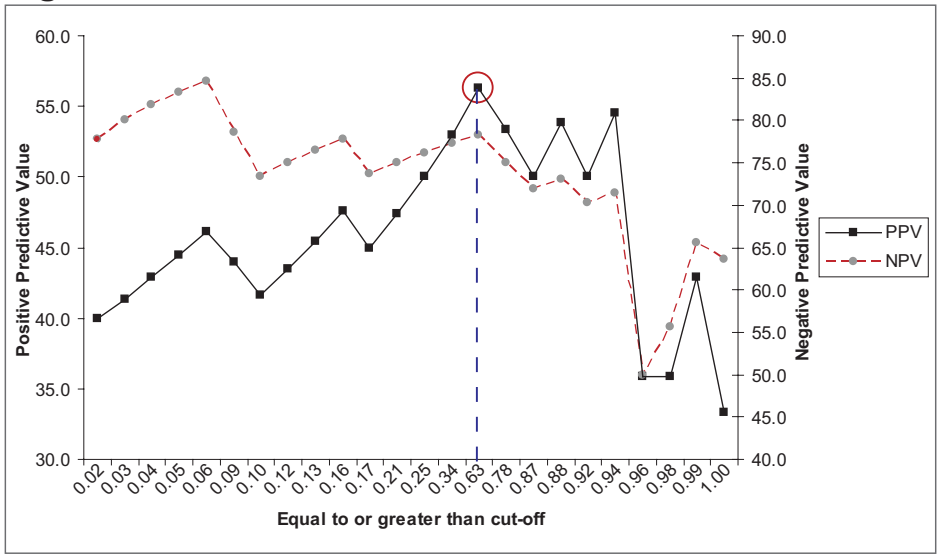

Figure B2.2

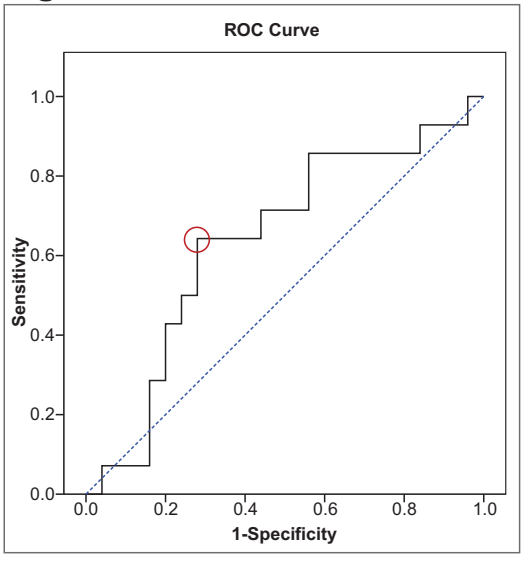

Figure B2. Performance of different cut-offs of the BRCA1-like^{CGH} probability score to identify patients with a progression free survival of more than 24 months. B2.1. Positive predictive values and negative predictive values at different cut-offs of the BRCA1-like^{CGH} probability score. **B2.2.** Receiver operating curve. Circle corresponds to cut-off chosen for further analysis

The reproducibility of the optimized BRCA1-like^{CGH} classifier was tested by hybridizing 19 tumor DNA samples two times. In total two tumors switched classes from the Non-BRCA1-like^{CGH} group to the BRCA1-like^{CGH} group upon second hybridization.

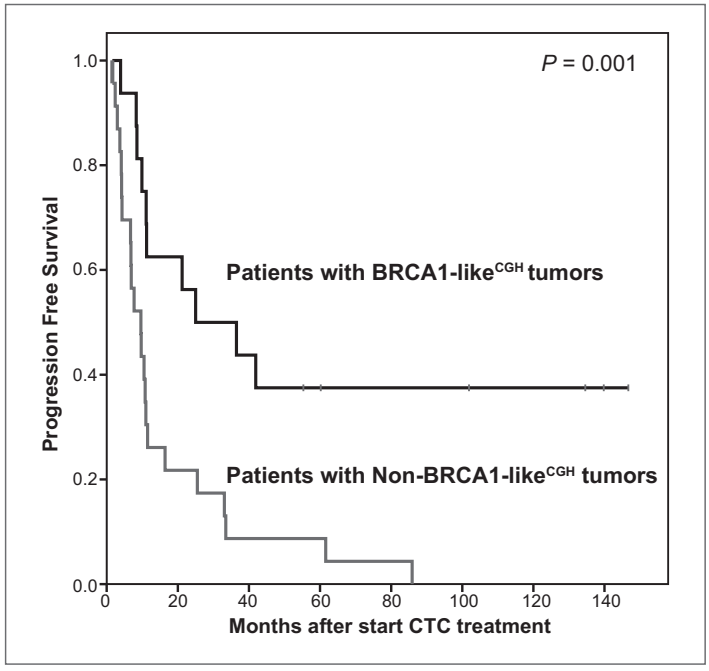

Figure B3. Kaplan-Meier curves for progression free survival by BRCA1-like^{CGH} and Non-BRCA1-like^{CGH} classification in the MBC-series using optimized cut-off. *P* value represents log-rank test of equal survival.

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Submitted

4

GENOMIC PATTERNS RESEMBLING BRCA1- AND BRCA2-MUTATED BREAST CANCERS PREDICT BENEFIT OF INTENSIFIED CARBOPLATIN-BASED CHEMOTHERAPY IN HER2-NEGATIVE BREAST CANCER PATIENTS

ABSTRACT

Objectives: To investigate whether genomic patterns specific for *BRCA1* or *BRCA2*-mutated breast cancers would identify a subgroup of sporadic breast cancer patients with triple-negative (TN) as well as estrogen-receptor (ER)-positive, HER2-negative tumors that would be sensitive to intensified, double-strand-break (DSB)-inducing chemotherapy. To identify this subgroup, studies have focused on sporadic TN breast cancers as these resemble *BRCA1*-mutated breast cancer closely and therefore might also share this hypersensitivity. However, ways to identify estrogen-receptor (ER)-positive breast cancers have remained elusive.

Design: retrospective case-control study of a double-blind randomized controlled trial

Participants: 249 stage-III patients, who had participated in a randomized controlled trial of adjuvant high-dose (HD) cyclophosphamide-thiotepa-carboplatin (CTC) versus 5-fluorouracil-epirubicin-cyclophosphamide ($FE_{90}C$) chemotherapy.

Main outcome measures: Previously published array comparative genomic hybridization (aCGH) patterns resembling *BRCA1/2*-mutated breast cancers were used to divide patients into two groups. Patients with tumors with similar aCGH patterns as *BRCA1*- and/or *BRCA2*-mutated breast cancers were defined as having a BRCA-like^{CGH} status, others as Non-BCRA-like^{CGH}. We evaluated whether the effect of HD-CTC versus $FE_{90}C$ on overall survival (OS) differed by BRCA-like^{CGH} status.

Results: Among the 81 patients (81/249, 32%) with BRCA-like^{CGH} tumors, we observed a significant benefit of HD-CTC compared to $FE_{90}C$ regarding OS (adjusted HR 0.19, 95%CI: 0.08-0.48). HD-CTC was not superior among patients with a Non-BRCAlike^{CGH} tumor (adjusted HR 0.90, 95%CI: 0.53-1.54). The difference was statistically significant (p-interaction: 0.004). Sensitivity analyses showed that the aCGH test was not dependent on small changes in tumor percentage or thresholds. Half of all BRCA-like^{CGH} tumors were ER-positive.

Conclusion: Distinct aCGH patterns differentiated between HER2-negative patients with a markedly improved outcome after adjuvant treatment with an intensified DNA-crosslinking regimen (BRCA-like^{CGH} patients) and those without benefit (non-BRCA-like^{CGH} patients).

INTRODUCTION

Adjuvant systemic treatment decisions for early breast cancer are generally based on results of large randomized clinical trials conducted in the general breast cancer population. Such trials do not take into account the molecular heterogeneity present in breast cancer [1]. Consequently, some treatment strategies that are highly beneficial to a small percentage of the general breast cancer population may have been discarded in the past. An example of such a treatment strategy might be intensified alkylating chemotherapy [2,3]. Here we investigated whether a subgroup of breast cancer patients with tumors that resemble *BRCA*-mutated breast cancer, might derive substantial benefit from intensified platinum-based chemotherapy (IPBC).

Maintenance of genomic integrity depends on homologous recombination, a conservative mechanism for error-free repair of DNA double-strand breaks (DSBs). In the absence of homologous recombination, error-prone DSB-repair mechanisms such as non-homologous end joining are invoked, leading to genomic instability [4-6]. This instability is thought to predispose to familial breast cancer in patients carrying germline mutations in *BRCA1* or *BRCA2,* genes involved in homologous recombination. Absence of homologous recombination offers a potential drug target for therapies that lead to DSBs during the DNA replication phase, when homologous recombination is the dominant DSB repair mechanism. Examples of these therapies are bifunctional alkylating agents and platinum compounds which cause DNA crosslinks leading to DSBs during DNA replication, and poly(ADP-ribose)polymerase (PARP)-inhibitors [7,8], which inhibit repair of single-strand DNA breaks also resulting in DSBs during replication. Recent evidence indeed shows that *BRCA1/-2*-mutated breast cancers are particularly sensitive to such agents [8-11]. This sensitivity is likely not restricted to *BRCA1/-2*-mutated breast cancers, as it is thought that up to 30% of sporadic (germline *BRCA*-wild type) breast cancers have defects in homologous recombination repair, a phenotype which is often referred to as 'BRCAness' [12]. In order to identify sporadic breast cancers sensitive to DSB-inducing agents, many studies have focused on *BRCA1*-mutated breast cancers, since this group of tumors is relatively homogeneous, clustering within the basal-like, hormone-receptor negative and HER2-negative (*e.g.* triple-negative, or TN) molecular subtype [13,14]. Consequently, multiple trials with DSB-inducing agents have been performed in patients with TN breast cancer and indeed have shown good responses not only in *BRCA1*-mutation carriers [9,10]. *BRCA2*-mutated breast cancers show a similar distribution across the breast cancer subtypes as sporadic tumors (~70% estrogen-receptor (ER)- or progesterone-receptor (PR)-positive) [15], and ways to select patients with sporadic ER-positive tumors sensitive to DSB-inducing agents have been lacking thus far.

We have previously employed array Comparative Genomic Hybridization (aCGH) to assess the genomic profiles of *BRCA1*-mutated breast cancers [16]. We found that BRCA1-like CGH patterns were also present in sporadic breast cancers [17,18]. Interestingly, we showed that patients with breast tumors exhibiting a BRCA1-like CGH pattern had a much larger benefit from a high-dose (HD) cyclophosphamide-thiotepa-carboplatin (CTC) regimen (compared to conventional 5-fluorouracil-

epirubicin-cyclophosphamide ($FE_{90}C$) chemotherapy) than patients whose tumor did not display this pattern. Furthermore, the BRCA1-like CGH pattern was highly associated with a TN phenotype [18]. Recently, a BRCA2-like CGH pattern was defined and found to be present in sporadic breast cancers[17]. In contrast to the BRCA1-like CGH pattern, the BRCA2-like CGH pattern was frequently observed in ER-positive tumors.

Given the association of the BRCA1-like CGH pattern with benefit from HD-CTC chemotherapy, we hypothesized that a positive BRCA-like^{CGH} status (the presence of a BRCA1-like and/or BRCA2-like CGH pattern) might identify, besides ER-negative, also ER-positive breast cancer patients who could benefit from DNA crosslinking agents. To explore this, we studied tumor specimens of breast cancer patients from a large randomized controlled trial who had either received adjuvant $FE_{90}C$ followed by HD-CTC, a DNA crosslinking regimen, or conventional $FE_{90}C$ chemotherapy only [19].

To enrich for cases likely to derive benefit from HD-CTC, we selected patients with HER2-negative tumors, for whom a significant benefit of HD-CTC had already been reported [20]. By employing the BRCA-like^{CGH} status, we identified a subgroup of breast cancer patients with a remarkably good outcome after adjuvant HD-CTC compared to conventional $FE_{90}C$ chemotherapy, irrespective of hormone-receptor status. Vice versa, we identified the subgroup that did not seem to derive any benefit from adjuvant HD-CTC.

METHODS

Patients

Patients were part of a multicenter randomized controlled trial (RCT) performed in the Netherlands (1993 – 1999) [19]. In this trial, 885 breast cancer patients with at least four tumor-positive axillary lymph nodes but no distant metastases (stage-III disease) had been randomized to conventional $FE_{90}C$ chemotherapy or the same therapy of which the last course was replaced by HD-CTC chemotherapy with autologous stem cell support.

For this study we randomly selected a group of 320 (out of 621) HER2-negative breast cancer patients. This group was used previously to study the association of the BRCA1-like CGH pattern with benefit from HD-CTC [18]. Patients were included in the current study if formalin-fixed paraffin-embedded (FFPE) primary tumor tissue contained more than 60% tumor cells. Of these 320 patients we obtained aCGH profiles of 249 patients; the flow of the study and reasons for dropout are depicted in Figure 1. Patients selected for analyses did not differ in patient characteristics or treatment from those not selected for analyses (Supplementary Table 1).

Treatment

Conventional chemotherapy consisted of five courses of 5-fluorouracil 500mg/m^2, epirubicin 90mg/m^2, cyclophosphamide 500mg/m^2 ($FE_{90}C$) given every three weeks [19]. The HD-CTC arm consisted of four $FE_{90}C$ courses, after which HD-CTC was administered (6,000 mg/m^2 cyclophosphamide, 480 mg/m^2 thiotepa and

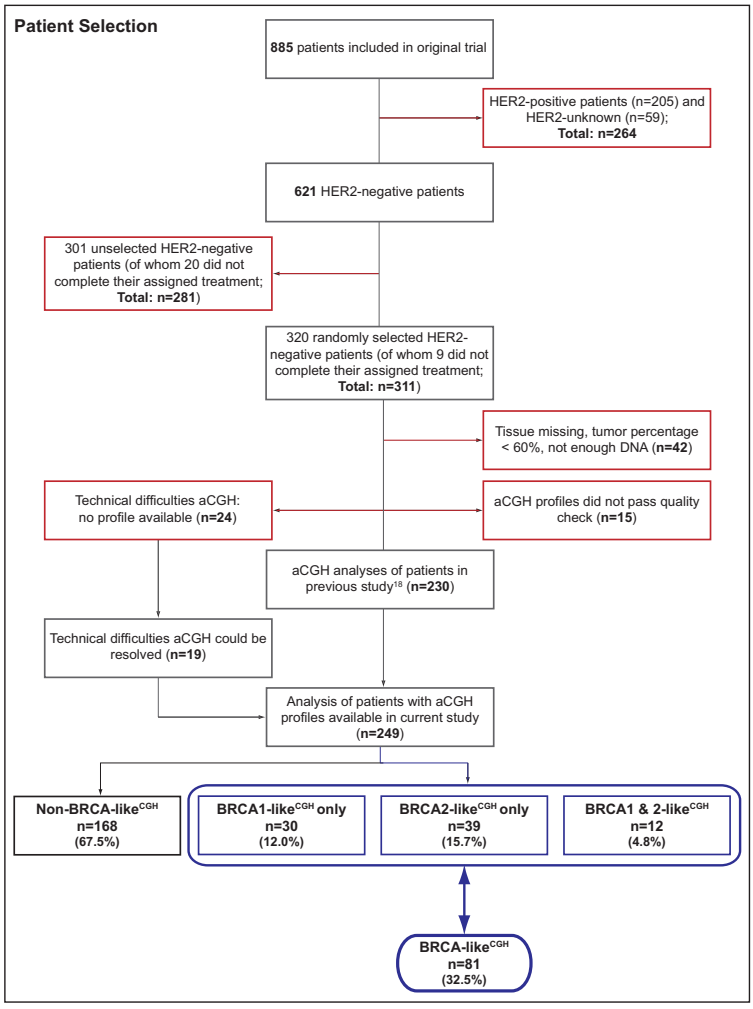

Figure 1. Flow diagram of patient selection in the study. Flow diagram depicting the motivations for removing patients and the number of patients remaining after each adjustment step. Reasons for dropout are listed. Tumors of 249 patients could be evaluated for the presence of the BRCA1-like^{CGH} and BRCA2-like^{CGH} pattern. The blue boxes at the bottom indicate the number of patients assigned to the BRCA1-like^{CGH}, BRCA2-like^{CGH} and BRCA-like^{CGH} categories. *Abbreviations:* aCGH, array comparative genomic hybridization.

1,600 mg/m^2 carboplatin [19]. Patients in both treatment-arms received radiotherapy and tamoxifen as described earlier[19].

Array comparative genomic hybridization

aCGH patterns of 230 patients generated previously on a 3.5k Human BAC array (NCBI's Gene Expression Omnibus platform number: GPL4560) platform were used in this study[18]. Tumors of 19 patients could additionally be analyzed

(Figure 1). In short, genomic DNA was extracted from FFPE primary tumors [21]. For seven of these 19 additional patients, only lymph-node tissue containing primary tumor tissue, removed at first diagnosis, was available. Three of these 19 samples had DNA concentrations too low for direct aCGH-analysis and were amplified with the BioScore™ Screening and Amplification Kit (42440, Enzo Life Sciences). Tumor and reference DNA was labelled according to the manufacturers' instructions (Kreatech Biotechnology, Amsterdam, http://www.kreatech.com/) and used for aCGH on the same 3.5k Human BAC array platform, as previously described [22]. Quality of each aCGH pattern was determined using a profile-quality and hybridization quality score, as published previously [18].

BRCA-like^{CGH} status

Each aCGH profile was classified as either BRCA1-like or Non-BRCA1-like and as either BRCA2-like or Non-BRCA2-like as previously published based on respectively the evaluation of the BRCA1-like [18], and the BRCA2-like CGH pattern [17].

The BRCA-like^{CGH} class contained tumors with a BRCA1-like and/or a BRCA2-like CGH pattern; all other tumors were assigned to the Non-BRCA-like^{CGH} class. The reproducibility of the BRCA-like^{CGH} status was tested by hybridizing 21 tumor DNA samples in duplicate. In total two tumors switched classes from BRCA-like^{CGH} score upon second hybridization (in the analysis the first aCGH hybridization was used).

Histopathology

Hematoxylin & eosin slides were scored for tumor percentages by a breast cancer pathologist (JW). ER, PR, P53, and HER2 status were determined by immunohistochemistry as described previously [19,23].

Statistical analyses

Groups of interest were tested for differences using Fisher's exact tests and Chi square tests for trend. Recurrence-free survival (RFS) was defined as the time between randomization and appearance of local or regional recurrence, metastases or death from any cause, whichever came first [19]. Overall survival (OS) was calculated from randomization to death from any cause, or end of follow-up. Patients alive at last follow-up were censored at that time. Median RFS and OS were 7.7 and 8.3 years, respectively, for all 249 patients. Survival curves were computed using the Kaplan-Meier method and compared using log-rank tests; Cox regression was used to calculate hazard ratios (HR). To ensure a direct correlation between aCGH pattern and treatment received, only patients who completed their assigned treatment were analyzed (per-protocol analysis).

This study was designed according to the predictive marker trial design 'Indirect assessment: Marker by treatment interaction design, test of interaction' (design 2) [24]. With this design the hypothesis can be tested whether the treatment effect (i.e. HD-CTC versus $FE_{90}C$) on survival in the presence of the marker (i.e. BRCA-like^{CGH}) is significantly different from that in the absence of the marker (i.e. Non-BRCA-like^{CGH}) with a statistical test for interaction.

Evidence for non-proportional hazards was found; all multivariate Cox regression models were therefore stratified for number of lymph nodes (4-9 vs.

Table 1. Patient characteristics by BRCA-like^{CGH} status

	Patients with Non-BRCA-like^{CGH} tumors		Patients with BRCA-like^{CGH} tumors		
Variable	**n**	**%**	**n**	**%**	**p values**
Total	168	67.5	81	32.5	
Treatment					
$FE_{90}C$ chemotherapy	81	48.2	41	50.6	0.787
HD-CTC Chemotherapy	87	51.8	40	49.4	
Type of surgery					
Breast conserving therapy	33	19.6	18	22.2	0.620
Mastectomy	135	80.4	63	77.8	
Age in categories					
< 40 years	34	20.2	27	33.3	
40 - 49 years	91	54.2	39	48.1	0.032*
≥ 50 years	43	25.6	15	18.5	
Tumor classification					
T1	32	19.0	15	18.5	
T2	112	66.7	51	63.0	0.642*
T3	23	13.7	14	17.3	
Unknown	1	0.6	1	1.2	
No. of positive lymph nodes					
4-9	109	64.9	54	66.7	0.887
≥ 10	59	35.1	27	33.3	
Histologic grade					
I	51	30.4	4	4.9	
II	70	41.7	23	28.4	<0.001*
III	42	25.0	50	61.7	
Not determined	5	3.0	4	4.9	
Estrogen receptor status					
Negative (<10%)	25	14.9	40	49.4	<0.001
Positive (≥10%)	143	85.1	41	50.6	
Progesterone receptor status					
Negative (<10%)	50	29.8	51	63.0	<0.001
Positive (≥10%)	118	70.2	28	34.6	
Unknown	0	0.0	2	2.5	
Triple-negative status					
Triple-negative	22	13.1	38	46.9	<0.001
ER or PgR positive (≥ 10%)	146	86.9	41	50.6	
Unknown	0	0.0	2	2.5	
P53 status					
< 10%	99	58.9	43	53.1	
10 – 50%	48	28.6	11	13.6	0.087*
> 50%	16	9.5	19	23.5	
Unknown	5	3.0	8	9.9	

p values: patients with unknown values were omitted. p values were calculated using the Fisher's exact test, except for *Chi square test for trend. *Abbreviations:* $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin; ER, estrogen-receptor; PgR, progesterone-receptor.

≥10) and TN status (ER<10% and PR<10% vs. other) which ensured hazards were proportional.

All calculations were performed using the statistical package SPSS 15.0 (for Windows). Supplementary figure 1 was generated using the ggplot2 package in R version 2.12.1.

RESULTS

Frequency of BRCA-like^{CGH} status and patient characteristics

aCGH profiles could be obtained from 249 tumors. Thirty tumors were classified as BRCA1-like (BRCA1-like^{CGH}), 39 tumors as BRCA2-like (BRCA2-like^{CGH}) and 12 tumors as both BRCA1- and BRCA2-like^{CGH} (Figure 1); thereby assigning 81 patients to the BRCA-like^{CGH} class (81/249, 32%; Figure 1). Patients with BRCA-like^{CGH} tumors were generally younger and their tumors were more often ER-negative, PR-negative and poorly differentiated compared to patients with Non-BRCA-like^{CGH} tumors (Table 1). BRCA1-like^{CGH} tumors were more often ER-negative (36/42, 86%; Supplementary Table 2) than BRCA2-like^{CGH} tumors (16/51, 31%; Supplementary Table 3). Supplementary figure 1 summarizes grade, receptor and BRCA-like^{CGH} status per patient.

Patient characteristics did not differ by treatment arm within the patient subgroups (with either BRCA- or Non-BRCA-like^{CGH} tumor; Supplementary Table 4). In univariate Cox regression analyses, large pathological tumor size according to TNM classification, high number of positive lymph nodes, poor Bloom Richardson grade (BR-grade), TN status and conventional $FE_{90}C$ treatment were significantly associated with decreased OS (Supplementary Table 5). All further Cox regression analyses were therefore stratified for triple-negativity and number of positive lymph nodes, and adjusted for pathological tumor size, BR-grade, and treatment.

Different treatment effects on survival between patients with BRCA-like^{CGH} and Non-BRCA-like^{CGH} tumors

Patients with a BRCA-like^{CGH} tumor had a significantly better OS after HD-CTC compared with conventional $FE_{90}C$ (adjusted HR 0.19, 95%CI: 0.08-0.48, Table 2, Figure 2A), while there was no survival difference between treatment-arms among patients with Non-BRCA-like^{CGH} tumors (adjusted HR 0.90, 95%CI: 0.53-1.54, Table 2, Figure 2B). The effect of HD-CTC over conventional $FE_{90}C$ chemotherapy was significantly different between patients with BRCA-like^{CGH} tumors and Non-BRCA-like^{CGH} tumors (test for interaction p=0.004, Table 2). Similar results were obtained for RFS (Supplementary Figure 2A and 2B, test for interaction p=0.003). The BRCA-like^{CGH} status retained its predictive capacity within the following subgroups: ER-positive patients, TN patients, patients younger than 45 years, and patients with histological grade-III tumors only (Supplementary Figure 3 and 4).

Analyzing the BRCA1-like^{CGH} pattern and BRCA2-like^{CGH} pattern separately produced generally similar but weaker results with regard to OS (test for interaction p=0.083 and p=0.033, respectively; Table 2, Figure 2C-F,) and RFS (Supplementary Figure 2C-F).

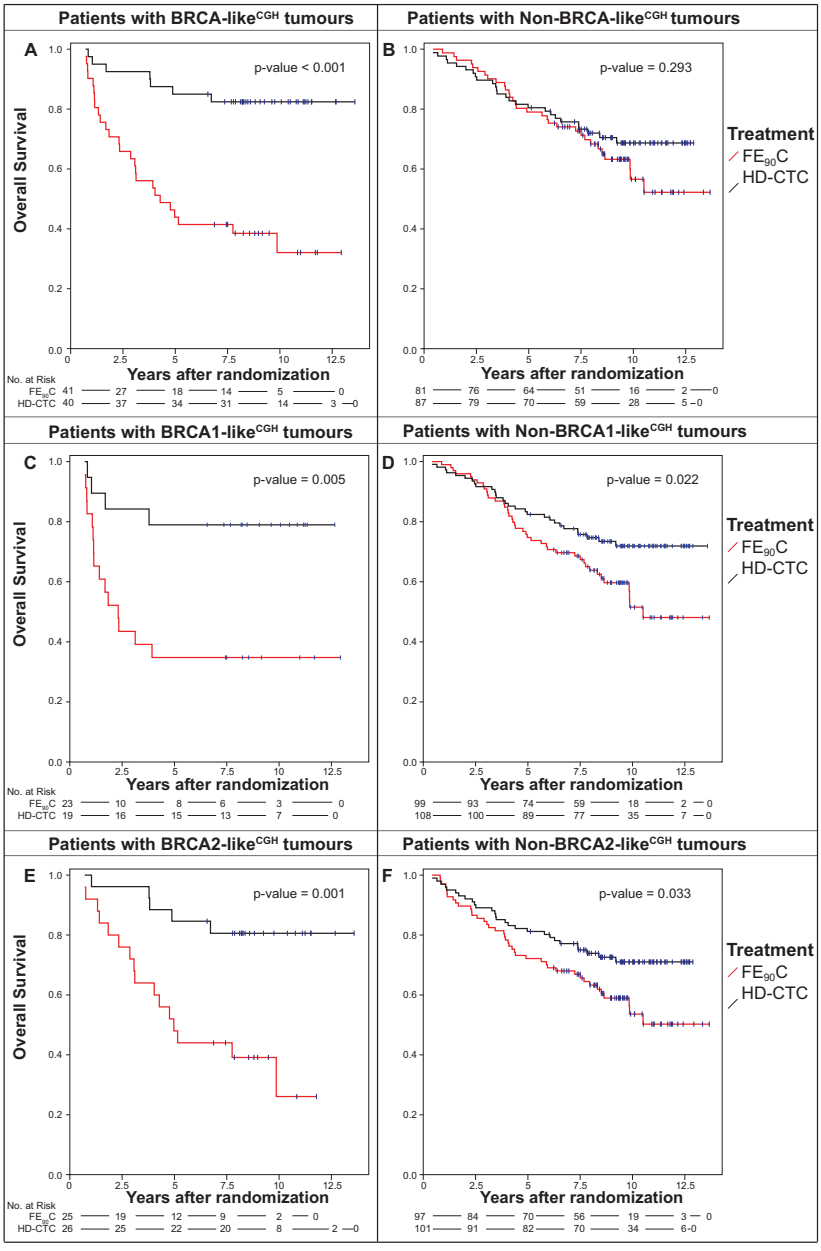

Figure 2. Association of the BRCA-like^{CGH} status with overall survival after HD-CTC and conventional chemotherapy. Kaplan-Meier survival curves for OS were generated separately for patients with BRCA-like^{CGH} (A) and with Non-BRCA-like^{CGH} (B) tumors; for patients with BRCA1-like^{CGH} (C) and with Non-BRCA1-like^{CGH} (D) tumors; and for patients with BRCA2-like^{CGH} (E) and with Non-BRCA2-like^{CGH} tumors; who had been randomly assigned between HD-CTC-chemotherapy and conventional chemotherapy. *Abbreviations:* $FE_{90}C$, 5-fluorouracil, epirubicin, cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.

Table 2. Multivariate Cox proportional-hazard analysis of the risk of death (OS) and the BRCA1-like^{CGH} pattern, BRCA2-like^{CGH} pattern and BRCA-like^{CGH} status

Variable	BRCA-like^{CGH} status†			
	No. Events / No. patients	Hazard Ratio	95% CI	p value
p T-stage				
pT1	14 / 43	1.00		
pT2	51 / 157	0.95	0.52 – 1.74	0.870
pT3	22 /37	1.85	0.93 – 3.68	0.082
Histologic grade				
I	15 /55	1.00		
II	33 /92	1.18	0.62 – 2.24	0.606
III	39 / 90	1.52	0.76 – 3.03	0.236
aCGH pattern*				
Non-BRCA /1- /2-like^{CGH} tumor	56 / 162	1.00		
BRCA /1- /2-Like^{CGH} tumor	31 / 75	1.75	0.96 – 3.20	0.067
BRCA /1- /2-Like^{CGH} tumor*				
$FE_{90}C$ chemotherapy	25 / 40	1.00		
HD-CTC chemotherapy	6 / 35	0.19†	0.08 – 0.48	<0.001
Non-BRCA /1- /2-like^{CGH} tumor*			† Homogeneity: p=0.004	
$FE_{90}C$ chemotherapy	30 / 79	1.00		
HD-CTC chemotherapy	26 / 83	0.90†	0.53 – 1.54	0.708

Three separate multivariate Cox regression models were run with the aCGH variable of interest (BRCA-like^{CGH} status, BRCA1-like^{CGH} pattern and BRCA2-like^{CGH} pattern; see top row) and an interaction term with treatment; all models were stratified for number of lymph nodes (4-9 vs. ≥10) and triple-negative status (ER<10% and PR<10% vs. other) and based on 237 patients (12 patients contributing 3 events were excluded due to missing values for at least one of the variables shown). Test of homogeneity of both treatment-specific hazard ratios based on an interaction term: p=0.004 (†), p= 0.033 (§) and p=0.083 (‡).
Abbreviations: p T-stage, pathological Tumor size; aCGH, array Comparative Genomic Hybridization; $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.

Effect of technical parameters on survival data

To determine the influence of the empirically chosen thresholds of the aCGH quality (profile-quality score) and the minimal tumor percentage required for inclusion, and the influence of the previously determined thresholds defining the BRCA-like^{CGH} score (i.e. the threshold of the BRCA1-like^{CGH} and BRCA2-like^{CGH} patterns[17,18]), we varied the cut-offs around these thresholds and evaluated the influence of these changes on survival analyses. No substantial modification of the HRs of treatment among patients with BRCA-like^{CGH} or Non-BRCA-like^{CGH} tumors (Figure 3), or of the tests for interaction (all p-values remained significant, Figure 3), was observed.

	BRCA1-like^{CGH} pattern ‡				BRCA2-like^{CGH} pattern§			
	No. Events / No. patients	Hazard Ratio	95% CI	p value	No. Events / No. patients	Hazard Ratio	95% CI	p value
	14 / 43	1.00			14 / 43	1.00		
	51 / 157	0.93	0.51 – 1.70	0.813	51 / 157	0.97	0.53 – 1.78	0.924
	22 /37	1.89	0.94 – 3.81	0.074	22 /37	1.91	0.96 – 3.81	0.067
	15 /55	1.00			15 /55	1.00		
	33 /92	1.10	0.59 – 2.07	0.765	33 /92	1.13	0.60 – 2.14	0.706
	39 / 90	1.55	0.80 – 3.01	0.193	39 / 90	1.43	0.73 – 2.79	0.297
	70 / 199	1.00			67 / 189	1.00		
	17 / 38	1.56	0.74 – 3.30	0.245	20 / 48	1.73	0.93 – 3.21	0.084
	14 / 22	1.00			16 / 25	1.00		
	3 / 16	0.20‡	0.06 – 0.72	0.013	4 /23	0.19§	0.06 – 0.58	0.004
			‡ Homogeneity: p=0.083				§ Homogeneity: p=0.033	
	41 / 97	1.00			39 / 94	1.00		
	29 / 102	0.67‡	0.42 – 1.09	0.105	28 / 95	0.72§	0.44 – 1.19	0.200

DISCUSSION

In this study we investigated whether we could identify a subgroup of HER2-negative patients with substantial benefit from an intensified DNA-crosslinking regimen, cyclophosphamide-thiotepa-carboplatin, with autologous stem cell support when compared to conventional $FE_{90}C$ [19]. We hypothesized that the aCGH patterns characteristic for *BRCA1*- or *-2*-mutated breast cancers (BRCA-like^{CGH} status) would identify a subgroup of not only TN, but also ER-positive, HER2-negative breast cancer patients with tumors exquisitely sensitive to DNA-crosslinking agents. Supporting evidence had come from a case report describing a patient with *BRCA2*-mutated metastatic breast cancer, who had been in continuous complete remission for 11 years after HD DNA-crosslinking chemotherapy with autologous stem cell support [25] and from our own metastatic series [18]. Indeed, in the current study breast cancer patients with a BRCA-like^{CGH} tumor had a markedly better overall survival after adjuvant HD-CTC than after conventional chemotherapy; this selective benefit was not present in patients with a Non-BRCA-like^{CGH} tumor, as confirmed by a highly significant test for interaction. A substantial proportion of HER2-negative breast cancer patients had a BRCA-like^{CGH} tumor (81/249, 32%) of which 51% (41/81) were ER-positive.

The following strengths of this study should be emphasized; (i) the BRCA-like^{CGH} status has been tested in the context of a RCT with long-term follow-up; (ii) the combination of the BRCA1-like and BRCA2-like classifiers identified few overlapping cases resulting in half of the BRCA-like^{CGH} cases being ER-positive, making this the first study reporting on a potential marker for sensitivity to DSB-inducing agents within the sporadic ER-positive breast cancer population; (iii) the results are robust with regard to choice of several technical parameters; (iv) the aCGH assay requires limited amounts of DNA isolated from FFPE tissue; and (v) the biomarker has molecular merit based on preclinical and early clinical work [4-8,12].

Early studies of PARP-inhibitors in *BRCA1*- and *BRCA2*-mutated breast cancer patients have shown promising results [8,11]. There is evidence that similar abnormalities in homologous recombination repair may also be present in sporadic

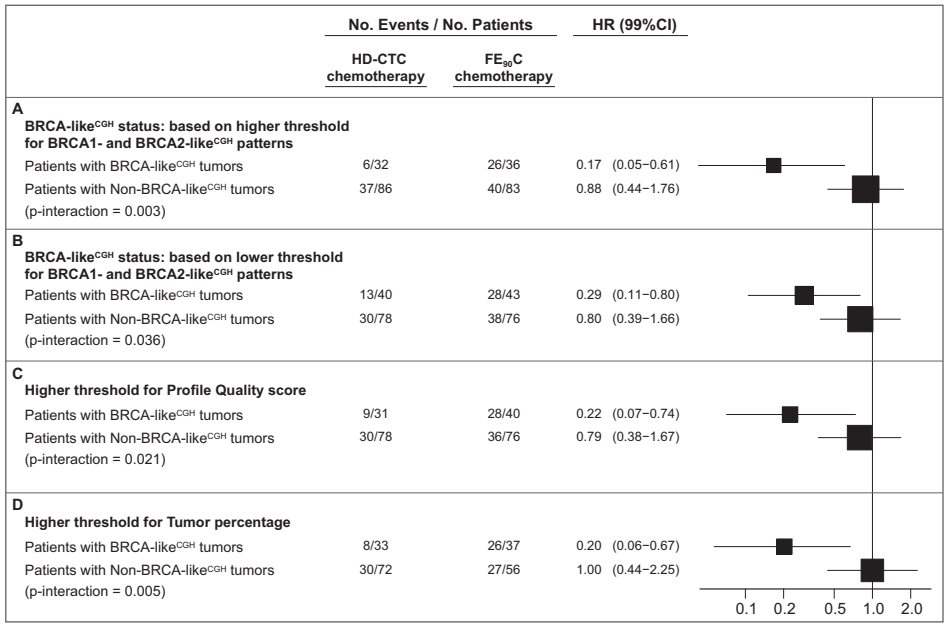

Figure 3. Sensitivity analyses of the BRCA-like^{CGH} status. We varied the previously determined thresholds defining the BRCA-like^{CGH} status (i.e. the threshold of the BRCA1-like^{CGH} and BRCA2-like^{CGH} patterns ([17, 18])) and the empirically chosen thresholds of the aCGH quality (profile-quality status) and the tumor percentage, and evaluated the influence on results for overall survival. A) The thresholds of the BRCA1- and BRCA2-like^{CGH} patterns, on which the BRCA-like^{CGH} status is based, were increased by 0.1 (from 0.63 to 0.73 and from 0.5 to 0.6, respectively ([17, 18])); B) similarly, thresholds were decreased by 0.1; C) The threshold determining aCGH quality was increased (from 0.85 to 0.95 ([18])), resulting in a subgroup of 225 patients; D) The threshold of tumor percentage was increased from 60% to 70% resulting in a subgroup of 198 patients. All analyses were stratified for number of lymph nodes (4-9 vs. ≥10) and double negative ER/PR status (ER<10% and PR<10% vs. other) and adjusted for pathologic tumor size (T1 vs T2 vs T3), histologic grade (I vs II vs III) and BRCA-like^{CGH} status. *Abbreviations:* $FE_{90}C$, 5-fluorouracil, epirubicin, cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.

breast cancers [12]. The presence of this phenotype, also referred to as 'BRCAness', is not easy to establish in clinical samples since a gold standard for BRCAness does not exist. Studies have used RAD51 staining [26], gene expression profiling [27], *BRCA1*-promoter methylation and *BRCA1* gene expression measurements [10]. In contrast to our study, these studies lacked a controlled study set-up which is essential to assess the predictive capacity of a biomarker. The performance of predictive biomarkers can only be studied in two comparable groups of patients where only one group has received the treatment of interest, thereby dissecting general chemosensitivity and/or prognosis from selective sensitivity to a particular treatment strategy [28].

A method selecting sporadic ER-positive patients benefitting from DSB-inducing agents has not been published to date. *BRCA2*-mutated tumors, however, are often ER-positive and have been shown to benefit from DNA DSB-inducing agents [7,8,11,15,29]. We therefore employed, in addition to the BRCA1-like^{CGH} pattern, the BRCA2-like^{CGH} pattern, which was previously shown to be able to identify *BRCA2*-mutated ER-positive tumors [17]. Combining the two patterns into a single BRCA-like^{CGH} status resulted in the designation of a substantial proportion of patients (32%) who had either ER-positive/HER2-negative or TN breast cancer. Interestingly, this percentage supports the earlier estimated percentage of BRCAness among sporadic breast cancers (30%) [12]. The ER status among BRCA1-like^{CGH} and BRCA2-like^{CGH} breast cancers was similar to that reported for *BRCA1*-mutated and *BRCA2*-mutated breast cancers, respectively [12,15].

We performed sensitivity analyses to assess if our test was robust with regard to choice of thresholds since some of them were previously trained on relatively small series. The results of these analyses showed that the association between HD-CTC benefit and the BRCA-like^{CGH} status remained significant with relatively few patients switching classes.

Our study had also some limitations; (i) a low resolution aCGH platform was used; and (ii) the DNA-crosslinking regimen consisted of a high-dose chemotherapy regimen with autologous stem cell support, which is currently regarded as obsolete.

A technical restriction of this study was the use of an aCGH platform with a lower resolution than currently used platforms. The reason is that we had to employ the same platform for validation as we had used to build the classifiers. Of note, the low resolution regions employed in the BRCA-like^{CGH} classifier will, of course, not disappear when a higher resolution platform is employed. Clearly, it is not the platform, but rather the chromosomal regions which are important and our findings should be applicable on data generated by any technology which identifies DNA gains and losses.

Unfortunately, we could not determine whether a lower, non-myeloablative dose of CTC would also have resulted in a similarly improved outcome. This is important, since high-dose myeloablative chemotherapy has been abandoned in breast cancer treatment[3,30,31], although a small survival advantage has been reported for HER2-negative breast cancer in a recent meta-analysis [32]. There is evidence that an intensified, but non-myeloablative dose might suffice [33]. In the neoadjuvant setting, cT3-4 breast cancer patients with a *TP53*-mutation had a

higher likelihood of pathological complete remission (pCR) and an 80% 6-years RFS after intensified cyclophosphamide-based chemotherapy, but only a 50% 6-years RFS after FEC-docetaxel [33,34]. The *TP53*-mutations were determined using a yeast-based screen for functional *TP53*-mutations [35]. Interestingly, these specific types of *TP53* mutations have been found in high frequencies in *BRCA1*-mutated breast cancers[36] and could possibly function as an alternative marker for sensitivity to DNA-crosslinking agents. In line with this reasoning, protein-truncating nonsense or frameshift *TP53* mutations were also found to predict good response (Miller-Payne score 3,4,5) to neoadjuvant cisplatin in cT2, TN breast cancer patients [10]. At present the optimal intensified cyclophosphamide dose is unclear. In the B-25 trial breast cancer patients under 50 years of age with 4-9 positive axillary lymph nodes benefitted significantly more from highly intensified (4*2,400 mg/m2 q 3 weeks) than from moderately intensified cyclophosphamide-based chemotherapy (4*1,200 mg/m2 q 3 weeks) [2]; similarly in the CONSORT study (breast cancer patients with $\geq$ 4 axillary involved lymph nodes), intense dose-dense sequential epirubicin, paclitaxel and cyclophosphamide (3*2,500 mg/m2 q 2 weeks) significantly improved survival outcome compared with conventional chemotherapy [37]. Of course, in these two latter studies, no stratification based on a BRCAness marker was made, which might have resulted in a much more pronounced benefit in the BRCAness group.

In conclusion, we showed in a subset of 249 patients from a randomized controlled trial that a BRCA-like^{CGH} classification was able to identify both ER-positive and TN breast cancer patients who derived a marked benefit of intensified DNA-crosslinking chemotherapy. Patients with BRCA-like^{CGH} tumors had about a five times lower risk of death after HD-CTC compared to $FE_{90}C$ chemotherapy, while no significant benefit was observed among patients with Non-BRCA1-like^{CGH} tumors. This finding strongly suggests the existence of breast cancer subtypes, defined by distinct CGH patterns that have a markedly improved outcome after treatment with an intensified DNA-crosslinking regimen and may explain why high-dose chemotherapy trials carried out in the general breast cancer population have remained negative in the past. This study should be considered as an explorative study, as it tested a hypothesis in a retrospective series. Therefore, before these results can be introduced into daily clinical practice, they should be validated in other controlled studies in which intensified alkylating regimens and/or PARP-inhibitors have been employed.

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CONFLICT OF INTEREST

SCL, PMN, MAV and EHL have a non-financial interest that may be relevant to the submitted work in the form of a provisional patent application for the aCGH

BRCA1-like^{CGH} and BRCA2-like^{CGH} classifier of which they are name inventor or contributor (EHL).

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Supplementary Table 1. Distribution of clinicopathological variables between randomly selected HER2-negative patients included in this study and HER2-negative patients not in the current analysis (all completed their assigned treatment).

	Total		In analysis with aCGH classifier		Not in current analysis		
Variable	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**p values**
Total	592	100.0	249	42.1	343	57.9	
Treatment							
$FE_{90}C$ chemotherapy	298	50.3	122	49.0	176	51.3	0.618
HD-CTC chemotherapy	294	49.7	127	51.0	167	48.7	
Age in categories							
< 40 years	133	22.5	61	24.5	72	21.0	
40 - 49 years	324	54.7	130	52.2	194	56.6	0.665*
≥ 50 years	135	22.8	58	23.3	77	22.4	
Type of surgery							
Breast conserving therapy	135	22.8	51	20.5	84	24.5	0.276
Mastectomy	457	77.2	198	79.5	259	75.5	
Tumor classification							
T1	136	23.0	47	18.9	89	25.9	
T2	357	60.3	163	65.5	194	56.6	0.222*
T3	90	15.2	37	14.9	53	15.5	
Unknown	9	1.5	2	0.8	7	2.0	
No. of positive lymph nodes							
4-9	384	64.9	163	65.5	221	64.4	0.862
≥ 10	208	35.1	86	34.5	122	35.6	
Histologic grade							
I	137	23.1	55	22.1	82	23.9	
II	221	37.3	93	37.3	128	37.3	0.702*
III	217	36.7	92	36.9	125	36.4	
Not determined	17	2.9	9	3.6	8	2.3	
Estrogen receptor status							
Negative (<10%)	140	23.6	65	26.1	75	21.9	0.242
Positive (≥10%)	451	76.2	184	73.9	267	77.8	
Unknown	1	0.2	0	0.0	1	0.3	
Progesterone receptor status							
Negative (<10%)	213	36.0	101	40.6	112	32.7	0.081
Positive (≥10%)	368	62.2	146	58.6	222	64.7	
Unknown	11	1.9	2	0.8	9	2.6	
P53 status							
< 10%	329	55.6	142	57.0	187	54.5	
10 – 50%	134	22.6	59	23.7	75	21.9	0.534*
> 50%	91	15.4	35	14.1	56	16.3	
Unknown	38	6.4	13	5.2	25	7.3	

p values: patients with unknown values were omitted; p values were calculated using the Fisher exact, except for *Chi square test for trend.
Abbreviations: aCGH, array Comparative Genomic Hybridization; $_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.

Supplementary Table 2. Patient characteristics by BRCA1-like^{CGH} status.

	Patients with Non-BRCA1-like^{CGH} tumors		Patients with BRCA1-like^{CGH} tumors		
Variable	**n**	**%**	**n**	**%**	**p values**
Total	207	83.1	42	16.9	
Treatment					
$FE_{90}C$ chemotherapy	99	47.8	23	54.8	0.499
HD-CTC Chemotherapy	108	52.2	19	45.2	
Type of surgery					
Breast conserving therapy	37	17.9	14	33.3	0.035
Mastectomy	170	82.1	28	66.7	
Age in categories					
< 40 years	44	21.3	17	40.5	0.007*
40 - 49 years	110	53.1	20	47.6	
≥ 50 years	53	25.6	5	11.9	
Tumor classification					
T1	35	16.9	12	28.6	0.146*
T2	138	66.7	25	59.5	
T3	32	15.5	5	11.9	
Unknown	2	1.0	0	0.0	
No. of positive lymph nodes					
4-9	136	65.7	27	64.3	0.860
≥ 10	71	34.3	15	35.7	
Histologic grade					
I	55	26.6	0	0.0	<0.001*
II	88	42.5	5	11.9	
III	58	28.0	34	81.0	
Not determined	6	2.9	3	7.1	
Estrogen receptor status					
Negative (<10%)	29	14.0	36	85.7	<0.001*
Positive (≥10%)	178	86.0	6	14.3	
Progesterone receptor status					
Negative (<10%)	63	30.4	38	90.5	<0.001*
Positive (≥10%)	144	69.6	2	4.8	
Unknown	0	0.0	2	4.8	
Triple-negative status					
Triple-negative	26	12.6	34	81.0	<0.001*
ER or PR positive (≥ 10%)	181	87.4	6	14.3	
Unknown	0	0.0	2	4.8	
P53 status					
< 10%	127	61.4	15	35.7	<0.001*
10 – 50%	54	26.1	5	11.9	
> 50%	19	9.2	16	38.1	
Unknown	7	3.4	6	14.3	

p values: patients with unknown values were omitted. p values were calculated using the Fisher's exact test, except for *Chi square test for trend.
Abbreviations: $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin; ER, estrogen-receptor; PgR, progesterone-receptor.

Supplementary Table 3. Patient characteristics by BRCA2-like^{CGH} status.

	Patients with Non-BRCA2-like^{CGH} tumors		Patients with BRCA2-like^{CGH} tumors		
Variable	**n**	**%**	**n**	**%**	**p values**
Total	198	79.5	51	20.5	
Treatment					
$FE_{90}C$ chemotherapy	97	49.0	25	49.0	1.000
HD-CTC Chemotherapy	101	51.0	26	51.0	
Type of surgery					
Breast conserving therapy	43	21.7	8	15.7	0.437
Mastectomy	155	78.3	43	84.3	
Age in categories					
< 40 years	43	21.7	18	35.3	0.173
40 - 49 years	108	54.5	22	43.1	
≥ 50 years	47	23.7	11	21.6	
Tumor classification					
T1	39	19.7	8	15.7	0.281*
T2	131	66.2	32	62.7	
T3	27	13.6	10	19.6	
Unknown	1	0.5	1	2.0	
No. of positive lymph nodes					
4-9	129	65.2	34	66.7	0.871
≥ 10	69	34.8	17	33.3	
Histologic grade					
I	51	25.8	4	7.8	0.006*
II	73	36.9	20	39.2	
III	67	33.8	25	49.0	
Not determined	7	3.5	2	3.9	
Estrogen receptor status					
Negative (<10%)	49	24.7	16	31.4	0.372
Positive (≥10%)	149	75.3	35	68.6	
Progesterone receptor status					
Negative (<10%)	76	38.4	25	49.0	0.203
Positive (≥10%)	120	60.6	26	51.0	
Unknown	2	1.0	0	0.0	
Triple-negative status					
Triple-negative	44	22.2	16	31.4	0.202
ER or PR positive (≥ 10%)	152	76.8	35	68.6	
Unknown	2	1.0	0	0.0	
P53 status					
< 10%	109	55.1	33	64.7	0.269*
10 – 50%	53	26.8	6	11.8	
> 50%	28	14.1	7	13.7	
Unknown	8	4.0	5	9.8	

p values: patients with unknown values were omitted. p values were calculated using the Fisher's exact test, except for *Chi square test for trend.
Abbreviations: $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin; ER, estrogen-receptor; PgR, progesterone-receptor.

Supplementary Table 4. Patient characteristics by treatment arm and BRCA-like^{CGH} status.

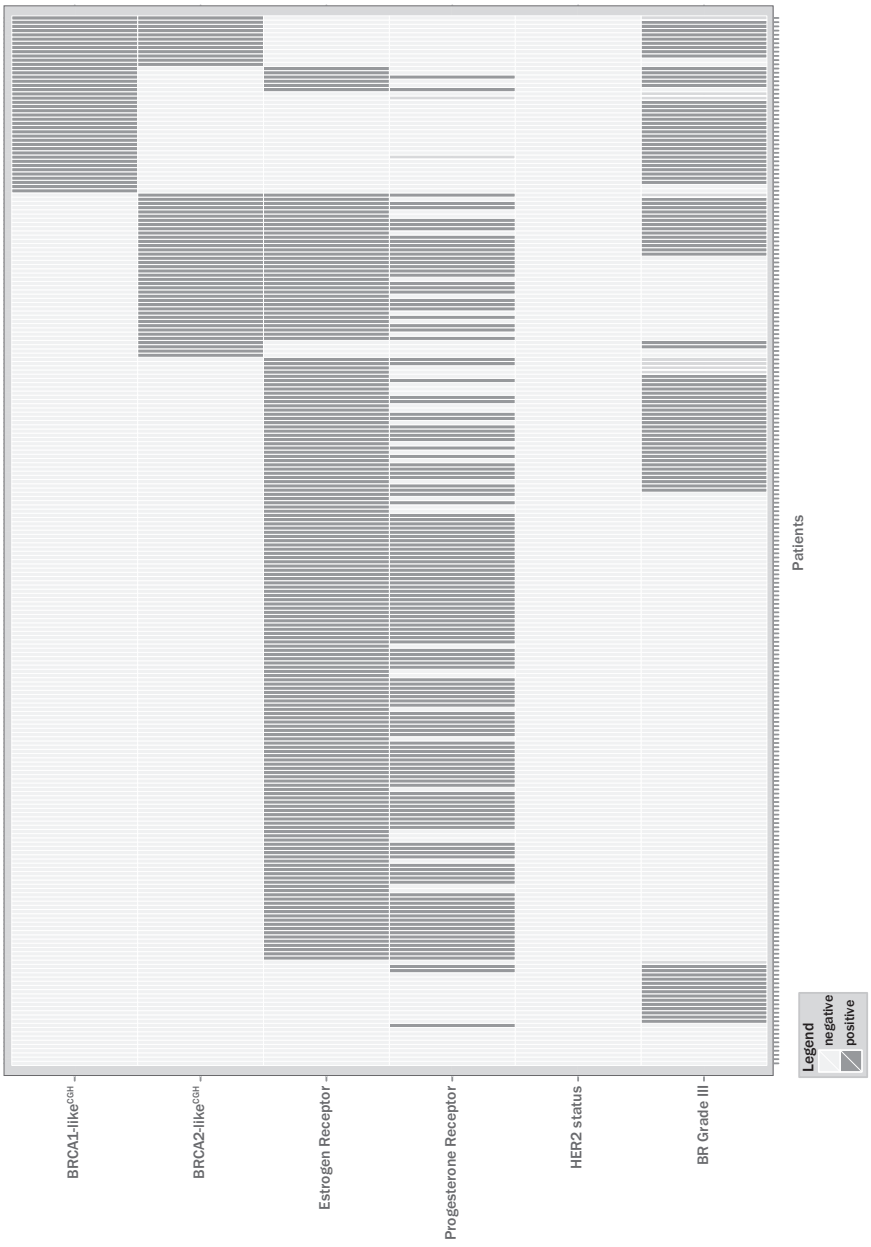
	Patients with Non-BRCA-like^{CGH} tumors					Patients with BRCA-like^{CGH} tumors				
	$FE_{90}C$ Chemotherapy		HD-CTC chemotherapy		p	$FE_{90}C$ Chemotherapy		HD-CTC chemotherapy		p
Variable	**n**	**%**	**n**	**%**	**values**	**n**	**%**	**n**	**%**	**values**
Total	81	48.2	87	51.8		41	50.6	40	49.4	
Age in categories										
< 40 years	13	16.0	21	24.1		15	36.6	12	30.0	
40 - 49 years	50	61.7	41	47.1	0.910*	18	43.9	21	52.5	0.876*
≥ 50 years	18	22.2	25	28.7		8	19.5	7	17.5	
Type of surgery										
Breast conserving therapy	15	18.5	18	20.7	0.846	10	24.4	8	20.0	0.790
Mastectomy	66	81.5	69	79.3		31	75.6	32	80.0	
Tumor classification										
T1	14	17.3	18	20.7		8	19.5	7	17.5	
T2	56	69.1	56	64.4	0.787*	24	58.5	27	67.5	0.587*
T3	11	13.6	12	13.8		9	22.0	5	12.5	
Unknown	0	0.0	1	1.1		0	0.0	1	2.5	
No. of positive lymph nodes										
4-9	51	63.0	58	66.7	0.631	31	75.6	23	57.5	0.102
≥ 10	30	37.0	29	33.3		10	24.4	17	42.5	
Histologic grade										
I	21	25.9	30	34.5		2	4.9	2	5.0	
II	37	45.7	33	37.9	0.407*	11	26.8	12	30.0	0.570*
III	21	25.9	21	24.1		28	68.3	22	55.0	
Not determined	2	2.5	3	3.4		0	0.0	4	10.0	
Estrogen receptor status										
Negative (<10%)	12	14.8	13	14.9	1.000	21	51.2	19	47.5	0.825
Positive (≥10%)	69	85.2	74	85.1		20	48.8	21	52.5	
Progesterone receptor status										
Negative (<10%)	24	29.6	26	29.9	1.000	28	68.3	23	57.5	0.352
Positive (≥10%)	57	70.4	61	70.1		12	29.3	16	40.0	
Unknown	0	0.0	0	0.0		1	2.4	1	2.5	
Triple-negative status										
ER or PR positive (≥ 10%)	11	13.6	11	12.6	0.823	20	48.8	18	45.0	1.000
Triple-negative	70	86.4	76	87.4		20	48.8	21	52.5	
Unknown	0	0.0	0	0.0		1	2.4	1	2.5	
P53 status										
< 10%	41	50.6	58	66.7		19	46.3	24	60.0	
10 – 50%	28	34.6	20	23.0	0.079*	7	17.1	4	10.0	0.282*
> 50%	9	11.1	7	8.0		11	26.8	8	20.0	
Unknown	3	3.7	2	2.3		4	9.8	4	10.0	

p values: patients with unknown values were omitted; p values were calculated using the Fisher's exact test, except for *Chi square test for trend. *Abbreviations:* $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin; ER, estrogen-receptor; PgR, progesterone-receptor.

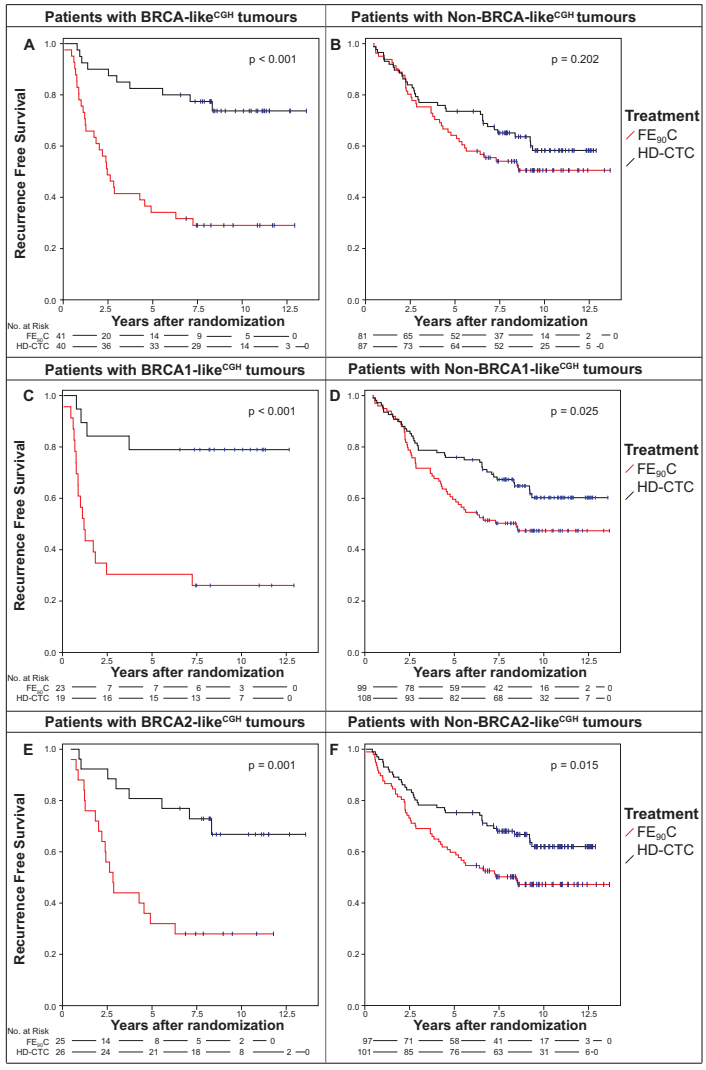
Supplementary Table 5. Univariate Cox proportional-hazard regression analysis of the risk of death (OS) after randomization.

Variable	No. Events / No. patients	Hazard Ratio	95% CI	p values
Age				
Continuously	90 / 249	0.99	0.96 – 1.02	0.545
Type of surgery				
Breast conserving therapy	19 / 51	1.00		
Mastectomy	71 / 198	0.94	0.57 – 1.57	0.821
Pathological tumor classification				
T1	16 / 47	1.00		
T2	52 / 163	0.91	0.52 – 1.59	0.734
T3	22 / 37	2.27	1.19 – 4.32	0.013
No. of positive lymph nodes				
4-9	50 / 163	1.00		
≥ 10	40 / 86	1.73	1.14 – 2.62	0.010
Histologic grade				
I	15 / 55	1.00		
II	33 / 93	1.40	0.76 – 2.58	0.280
III	40 / 92	1.98	1.09 – 3.58	0.024
Estrogen receptor status				
Negative (<10%)	31 / 65	1.00		
Positive (≥10%)	59 / 184	0.48	0.31 – 0.75	0.001
Progesterone receptor status				
Negative (<10%)	45 / 101	1.00		
Positive (≥10%)	44 / 146	0.56	0.37 – 0.85	0.006
Triple-negative status				
Triple-negative	29 / 60	1.00		
ER or PR positive (≥ 10%)	60 / 187	0.48	0.31 – 0.75	0.001
P53 status				
< 10%	52 / 142	1.00		
10 – 50%	18 / 59	0.73	0.43 – 1.25	0.246
> 50%	14 / 35	1.15	0.64 – 2.08	0.640
Treatment				
$FE_{90}C$ Chemotherapy	57 / 122	1.00		
HD-CTC Chemotherapy	33 / 127	0.48	0.31 – 0.74	0.001
aCGH BRCA-like status				
Non-BRCA-like^{CGH} tumor	57 / 168	1.00		
BRCA-like^{CGH} tumor	33 / 81	1.40	0.91 – 2.15	0.127
aCGH BRCA1-pattern				
Non-BRCA1-like^{CGH} tumor	71 / 207	1.00		
BRCA1-like^{CGH} tumor	19 / 42	1.79	1.08 – 2.98	0.024
aCGH BRCA2-pattern				
Non-BRCA2-like^{CGH} tumor	69 / 198	1.00		
BRCA2-like^{CGH} tumor	21 / 51	1.28	0.79 – 2.09	0.319

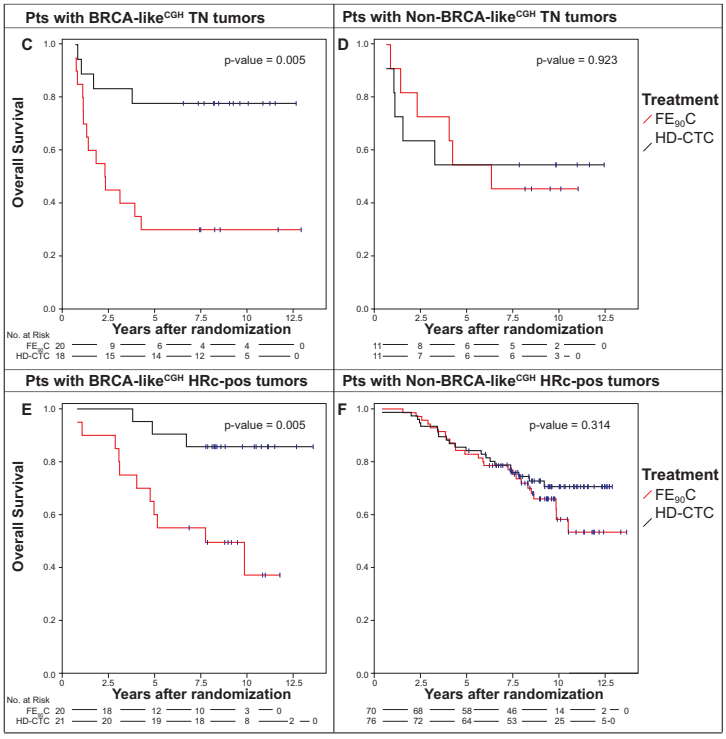
Number of events is not equal for all variables, since some patients have missing data; maximum missing variables (i.e. events) is 6 /112. *Abbreviations:* CI, confidence interval; ER, estrogen-receptor; PgR, progesterone-receptor; $FE_{90}C$, 5-fluorouracil-epirubicin-cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin; CGH, comparative genomic hybridization; HD-CTC, high-dose.



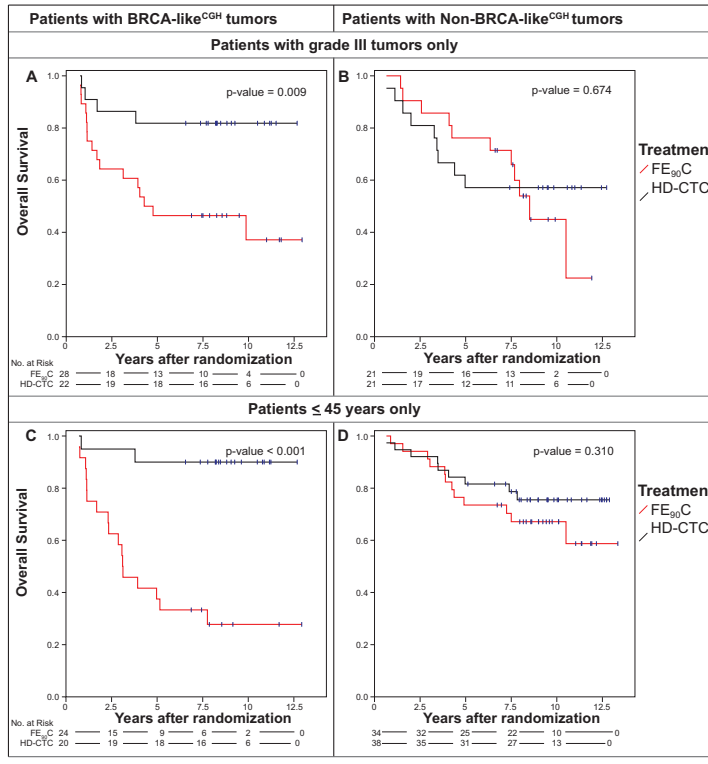
Supplementary Figure 1. Overview of histological patient characteristics and the aCGH classification per patient. Subdivision of characteristics studied: Estrogen-receptor negative (<10%) vs. positive (≥10%); Progesterone-receptor negative (<10%) vs. positive (≥10%); HER2 status negative vs. positive (the study comprised only HER2 negative patients); Bloom Richardson Grade I+II (negative) vs. Grade III (positive); non-BRCA1-like^{CGH} (negative) vs. BRCA1-like^{CGH} (positive); non-BRCA2-like^{CGH} (negative) vs. BRCA2-like^{CGH} (positive). Legends consist of lightgray: negative cases; darkgray: positive cases for above-mentioned characteristic; black: cases unknown for above-mentioned characteristic.



Supplementary Figure 2. Association of the BRCA-like^{CGH} status with Recurrence-Free survival after HD-CTC and conventional $FE_{90}C$ chemotherapy. Kaplan-Meier survival curves for RFS by randomly assigned treatment-arms were generated separately for patients with BRCA-like^{CGH} (A; adjusted HR (HD-CTC versus $FE_{90}C$ chemotherapy) 0.23, 95%CI: 0.11 – 0.50, p<0.001) and with Non-BRCA-like^{CGH} (B; adjusted HR 0.90, 95%CI: 0.56 – 1.45, p=0.67) tumors (test for interaction p=0.003); for patients with BRCA1-like^{CGH} (C; adjusted HR 0.16, 0.05 – 0.53, p=0.003) and with Non-BRCA1-like^{CGH} (D; adjusted HR 0.73, 95%CI: 0.48 – 1.11, p=0.14) tumors (test for interaction p=0.021); and for patients with BRCA2-like^{CGH} (E; adjusted HR 0.26, 95%CI: 0.11 – 0.64, p=0.003) and with Non-BRCA2-like^{CGH} (F; adjusted HR 0.72, 95%CI: 0.47 – 1.12, p=0.144) tumors (test for interaction p=0.046); All adjusted HRs were based on Cox regression analyses stratified for number of lymph nodes (4-9 vs. >=10) and triple-negative status (ER<10% and PR<10% vs. other) and adjusted for pathological tumor size (pT1 vs. pT2 vs. pT3) and Bloom and Richardson grade (Grade I vs. Grade II vs. Grade III). *Abbreviations:* $FE_{90}C$, 5-fluorouracil, epirubicin, cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.



Supplementary Figure 3. Association of the BRCA-like^{CGH} status with overall survival after HD-CTC and conventional chemotherapy in TN patients only and hormone-receptor positive patients only. Kaplan-Meier survival curves for OS were generated separately for patients with TN, BRCA-like^{CGH} (A) and with TN, Non-BRCA-like^{CGH} (B) tumors; for patients with hormone-receptor-positive (ER- and, or PR-positive), BRCA-like^{CGH} (C) and with hormone-receptor-positive, Non-BRCA-like^{CGH} (D) tumors; who had been randomly assigned between HD-PB-chemotherapy and conventional chemotherapy. *Abbreviations:* Pts, patients, TN, triple-negative; HRc-pos, hormone-receptor positive; $FE_{90}C$, 5-fluorouracil, epirubicin, cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.



Supplementary Figure 4. Association of the BRCA-like^{CGH} status with overall survival after HD-CTC and conventional chemotherapy in patients with grade-III tumors only and in patients younger or equal to 45 years only. Kaplan-Meier survival curves for OS were generated separately for patients with histological grade-III, BRCA-like^{CGH} (A) and with grade-III, Non-BRCA-like^{CGH} (B) tumors; for patients younger or equal to 45 years and with a BRCA-like^{CGH} (C) or with Non-BRCA-like^{CGH} (D) tumors; who had been randomly assigned between HD-PB-chemotherapy and conventional chemotherapy. *Abbreviations:* $FE_{90}C$, 5-fluorouracil, epirubicin, cyclophosphamide; HD-CTC, high-dose cyclophosphamide-thiotepa-carboplatin.

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5

IMPACT OF INTERTUMORAL HETEROGENEITY ON PREDICTING CHEMOTHERAPY RESPONSE OF BRCA1-DEFICIENT MAMMARY TUMORS

ABSTRACT

Lack of markers that predict chemotherapy response is a handicap in cancer treatment. We have searched for gene expression patterns that correlate with docetaxel or cisplatin response using mammary tumors generated in a mouse model for breast cancer associated with BRCA1 deficiency. Array-based expression profiling did not identify a single marker gene predicting docetaxel response, despite an increase in *Abcb1* (P-glycoprotein) gene expression sufficient to explain resistance in several poor responders. Our results demonstrate how intertumoral heterogeneity impairs the identification of predictive gene expression signatures for docetaxel. Only a special algorithm designed to detect differential gene expression in a subgroup of the poor responders was able to identify tumors with increased *Abcb1* transcript levels. In contrast, standard analytical tools, such as Significance Analysis of Microarrays (SAM), will detect a marker only if it correlates with response in a substantial fraction of tumors. As an example, we found low expression of the *Xist* gene in most mouse tumors with cisplatin hypersensitivity. This marker also predicted long recurrence-free survival of HER2-negative, stage-III breast cancer patients treated with intensive platinum-based chemotherapy. Our findings in the mouse model may therefore be useful to select patients with high risk breast cancer who could benefit from platinum-based therapy.

INTRODUCTION

Most forms of cytotoxic cancer chemotherapy also hit normal tissues. This is acceptable when the tumor responds, but frustrating when the tumor is intrinsically resistant and the patient only suffers from the side effects of an unsuccessful treatment. A major goal of molecular oncology is therefore to identify biomarkers that predict the response of tumors before treatment is started. Such predictive markers have been found for some targeted therapies in which the target and its interaction with drugs are well defined. Examples are the presence of a functional estrogen receptor predicting response to anti-estrogens [1]; the requirement of *HER2* amplification for a therapeutic effect of trastuzumab [2]; defects in homologous DNA recombination making the tumor susceptible to inhibitors of Poly (ADP-ribose) polymerase [3]; and several examples in which alterations in drug targets or oncogenes prevent drug action [4-7]. For classical cytotoxic chemotherapy with DNA damaging drugs or antimitotics, however, predictive biomarkers have been harder to find.

In an attempt to find new biomarkers many investigators have turned to the analysis of genome-wide gene expression profiles. These profiles have been successful for predicting prognosis, *i.e.* whether patients will require adjuvant chemotherapy after tumor removal [8]. Prognostic and predictive biomarkers are fundamentally different, however [9]. To detect predictive markers, considerable effort and money has been invested in the analysis of human breast cancer samples [10]. In particular the neoadjuvant setting seemed attractive to correlate gene expression profiles with therapy outcome. No clear response profile was obtained, however [11,12]. Other studies have gathered a number of unrelated signatures [9]. These profiles either still await validation in an independent study; or the sensitivity and specificity was inadequate for clinical decision making; and some were based on flawed data [13-15]. Moreover, cell line-based approaches to identify biomarkers suffer from the complication that the multidrug resistance transcriptome has been substantially altered during the long-term culture of these cell lines *in vitro* [16].

As progress in defining useful biomarkers using human tumor material has been limited, we have turned to a mouse model. In recent years chemotherapy responses have been investigated in a new generation of genetically engineered mouse models (GEMMs) [17]. These models employ conditional, tissue-specific activation of oncogenes and/or deletion of tumor suppressor genes in a stochastic fashion. The resulting tumors closely mimic the epithelial cancers in humans. Using the *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* model for hereditary breast cancer [18] we have shown that these tumors acquire resistance to classical and novel targeted anti-cancer drugs such as the topoisomerase I-targeting camptothecin topotecan, the topoisomerase II-inhibiting anthracycline doxorubicin, and the PARP inhibitor olaparib [19-22]. We have observed that the initial response of these tumors is variable, as in human tumors, thus providing an attractive opportunity to correlate drug response with gene expression. The tumors are similar, as they start out with the ablation of the *Brca1* and the *p53* genes. Differences between tumors should make it comparatively easy to sort out which genes determine whether a tumor responds to drug or not.

An advantage of this model is that tumors can be orthotopically transplanted into syngeneic, immunocompetent animals without losing their molecular fingerprint, morphologic phenotype or drug sensitivity [19]. For the identification of underlying resistance mechanisms, gene expression analysis of matched samples of untreated and resistant tumors derived from the same individual tumor was employed successfully [19-22]. Using this orthotopically transplantable mouse model, we set out to find predictive markers of cisplatin or docetaxel response.

MATERIALS AND METHODS

Mice and drug treatments

KB1P mammary tumors were generated, genotyped, orthotopically transplanted and treated as described [18,19]. Additional details including the generation of KB1PM mammary tumors can be found in Supplementary Materials. All experimental procedures on animals were approved by the Animal Ethics Committee of the Netherlands Cancer Institute.

Genome-wide expression profiling

RNA extraction, amplification, microarray hybridization using MEEBO arrays (Illumina BV, Eindhoven), data processing and statistical analyses were performed as described [19,22,23]. For the gene expression analysis using 45K MouseWG-6 v2.0 BeadChips (Illumina, Eindhoven, The Netherlands) total RNA was processed according to the manufacture's instructions (http://www.illumina.com/products/mousewg_6_expression_beadchip_kits_v2.ilmn). All probes with significant changes in expression (log2ratio >1 or <−1) in <10% of the samples were filtered out. Also probes with missing data points in more than 10% of the hybridizations were excluded. The microarray data reported in this article have been deposited in the Array Express database, www.ebi.ac.uk/arrayexpress (accession no. E-MTAB-413 [Illumina] and E-MTAB-415 [MEEBO]).

Quantitative RNA analyses using Reverse Transcription-Multiplex ligation-dependent probe amplification (RT-MLPA) or TaqMan low density arrays (TLDA)

These procedures were carried out as reported previously [20,21,24]. Additional details are presented in Supplementary Materials.

ArrayCGH

ArrayCGH data was available from a recent study [25]. Segmentation of the CGH profiles was performed with the CGHseg package [26]. The CGHcall R package (v 2.12.0) was used to call aberrations in CGH profiles.

Histology

Tissues were fixed in 4% formaldehyde overnight, embedded in paraffin, and cut in 4μm sections. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin according to standard procedures.

FISH

Three samples per individual tumor were investigated in a blinded fashion using tissue microarrays of the trial cohort. At least 100 nuclei per sample were assessed. Sections were deparaffinized with xylene and treated with 1M sodium thiocyanate for 10 minutes at 80°C to remove crosslinks. Cells were permeabilized with 750 U/ml pepsin (Sigma-Aldrich) for 8 minutes. RNF12-specific DNA FISH probes were prepared from RP11 BAC clones. BAC DNA was digested and labeled using random primer labeling (Invitrogen) with Bio-16-dUTP or (Roche). Probes were validated using control metaphases. *XIST* RNA FISH probe was prepared from a plasmid containing 12kb of the *XIST* sequence and labeled with DIG-11-dUTP (Roche). Digoxigenin- or biotin-labeled probes were detected using a FITC-labeled mouse anti-DIG antibody or Alexa 594-labeled streptavidin, respectively. If the number of cells with no *XIST* RNA clouds was >60%, the sample was classified 0 for "XIST RNA cloud". In the presence of one X chromosome detected by the RNF12 DNA probe, XIST RNA was usually absent (loss of Xi). In the presence of two X chromosomes, loss of Xi and a XaXa configuration was defined as a more than 50% reduction in the number of expected *XIST* RNA clouds based on the *RNF12* DNA FISH.

Patients

In a previous study stage-III HER2-negative breast cancer patients were randomly selected from a large randomized controlled trial (RCT) performed in the Netherlands between 1993 and 1999 [27] and analyzed for aCGH classification [25]. Further details on these patients are presented in Supplementary Materials. All trials described in this manuscript were approved by the Institutional Review Board of the Netherlands Cancer Institute.

RESULTS

Brca1$^{-/-}$*;p53*$^{-/-}$ (KB1P) mammary tumors show individual and reproducible differences in docetaxel or cisplatin sensitivity

We have previously shown that individual KB1P mammary tumors differ substantially in their response to docetaxel [19]. The response to cisplatin varied as well: although all tumors were sensitive to cisplatin, the time until relapse differed between tumors [19]. To exploit these inter-tumoral differences, we analyzed docetaxel or cisplatin responses of 43 individual tumors. Orthotopic transplantation into syngeneic mice allowed us to collect response data for docetaxel, cisplatin and doxorubicin for 36 individual tumors (Supplementary Fig. S1).

The correlation of drug sensitivities with characteristics of a particular tumor is only possible if the responses are reproducible. We therefore explored the heterogeneity within a single tumor by orthotopic transplantation of multiple tumor fragments (Fig. 1). For this purpose, 3 animals carrying orthotopically transplanted fragments of the same spontaneous tumor were treated with the maximum tolerable dose of docetaxel on days 0, 7 and 14. Fig. 1A shows that the docetaxel response was consistent for all 3 fragments derived from one

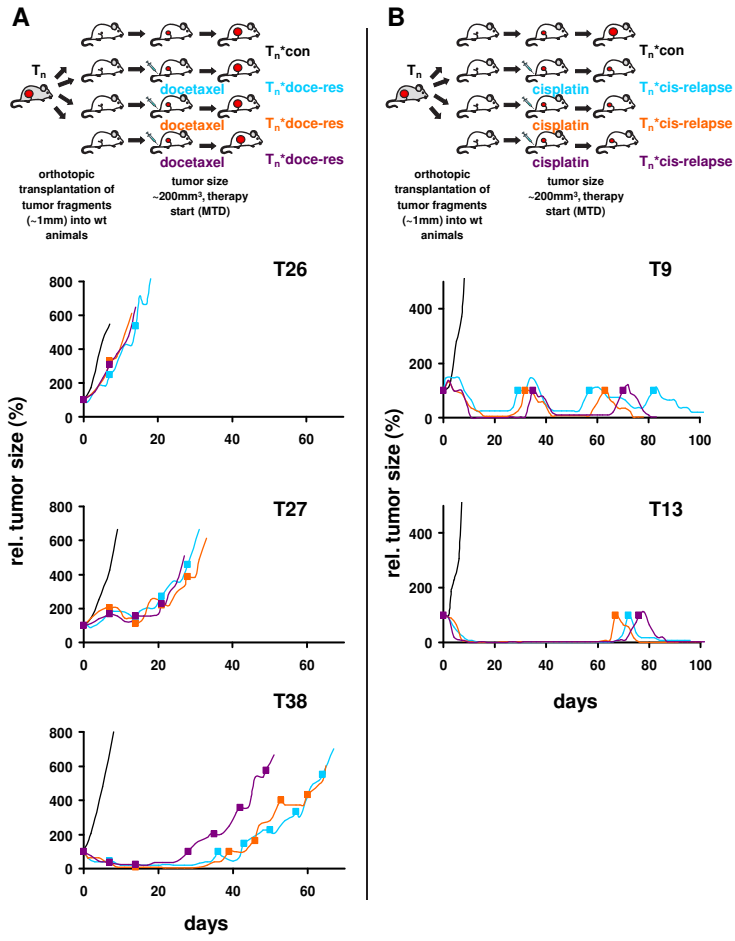


Figure 1. Reproducibility of docetaxel or cisplatin sensitivity of individual KB1P tumors using orthotopic transplantation. Small tumor fragments of spontaneous mammary tumors were transplanted orthotopically into syngeneic wild-type female mice as shown in (A) for docetaxel and (B) for cisplatin. After a mean latency of about 4 weeks, when tumors reached a size of 150–250mm^3 (V = length × width^2 × 0.5), animals were treated with 25mg of docetaxel per kg i.v. on days 0, 7 and 14 (A, filled squares) or 6mg cisplatin per kg i.v. (B, filled squares). Treatment of tumors was resumed once the tumor relapsed to its original size (100%).

tumor (T26 was consistently poor; T38 responded well; T27 fragments all had an intermediate response). The rate at which the tumors eventually become completely resistant to docetaxel differs somewhat between fragments from the same tumor (T38*docetaxel 3 vs. 1 or 2), as previously observed for doxorubicin [19]. The initial drug response is reproducible, however. Also for cisplatin we confirmed that the time to relapse was reproducible (Fig. 1B; T9 tumor fragments all relapsed early, T13 fragments all relapsed late). Hence, this tumor model can be used to

correlate initial docetaxel or cisplatin responses with other tumor characteristics, such as gene expression levels.

Supervised gene expression profiling using ordered arrays does not yield a general signature that correlates with docetaxel response

In our model we used the tumor volume as the basis for a response classifier. We found that after completion of the initial docetaxel treatment on day 14, 22 tumors did not shrink below their original size when treatment was started (100%), and eventually continued growing ('poor response'). In contrast, 21 tumors regressed to a volume below 50% of the original size ('good response'), and took on average 28 days (SD 11d) after the last docetaxel treatment to grow back to 100% (Fig. 2A and Supplementary Table S1). With such an obvious separation, we expected to identify genes that are differentially expressed between these 2 groups. To detect these, RNA of all 43 untreated tumors was analyzed using 39K Mouse Exonic Evidence Based Oligonucleotide (MEEBO) gene expression microarrays, which we have used previously to find genes involved in topotecan resistance [22], and 45K Illumina Sentrix mouse V6 single-channel bead arrays. Unsupervised hierarchical cluster analysis did not separate good from poor responders (Supplementary Fig. S2). For the supervised analysis we used Significance Analysis of Microarrays (SAM) [28], which is frequently applied to detect differential gene expression. SAM uses non-parametric statistics to compute for each gene a delta that measures the strength of the relationship between gene expression and docetaxel sensitivity. Permutations of repeated measurements are employed to estimate the false discovery rate (FDR). Using this approach we expected to find several differentially expressed genes between good and poor docetaxel responders with a delta>0.8 (FDR<5%). Remarkably, this analysis did not detect a single gene that correlated with drug sensitivity with a meaningful delta (Fig. 2B).

This negative result might be due to the lack of sensitivity of the gene expression platforms used for genes that are relevant for drug resistance. This is exemplified by the work of Orina et al [29] on drug transporters of the ATP-binding cassette (ABC) family. Using the NCI-60 panel of cell lines, they showed that TaqMan low density arrays (TLDA) are more precise and more sensitive in measuring the expression of these transporter genes than oligonucleotide arrays [29]. Within this ABC family, a number of genes has been associated with docetaxel resistance, including ABCB1/P-glycoprotein (P-gp) [30], ABCC2 [31] and ABCC10 [32]. We therefore examined whether the more quantitative TLDA analysis of the 49 genes that encode mouse ABC proteins would reveal differences between poor and good docetaxel responders. As shown in Fig. 2C and Supplementary Table S2, none was found at a significance level of $P<0.01$ (Mann-Whitney U test).

We note in passing that on both platforms used to analyze gene expression (Supplementary Fig. S2) two poor responders (T26*con and T41*con) form a separate branch which correlates with the sarcomatoid morphology (carcinosarcoma) of these tumors (Supplementary Table S1). Most likely, these 2 tumors have undergone an epithelial-mesenchymal transition (EMT), since in the *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* model the Cre-mediated deletion of the *Brca1* and *p53* genes selectively occurs in epithelial cells driven by the *K14* promoter.

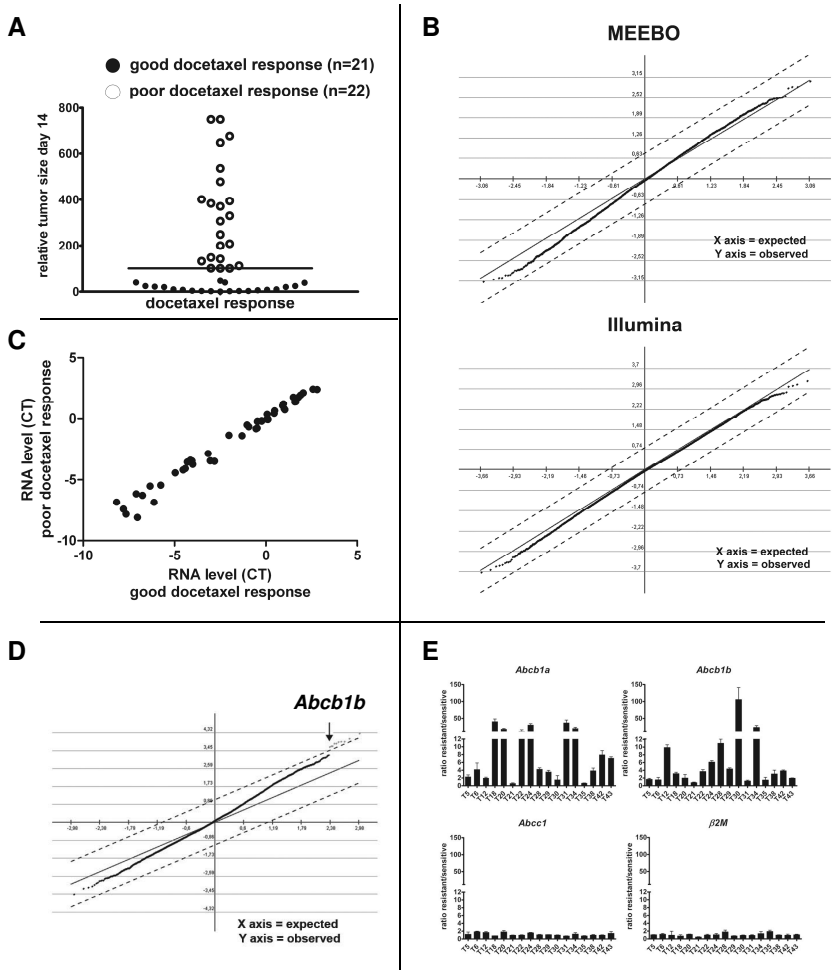


Figure 2. Analysis of intrinsic (A, B, C) or acquired (D, E) docetaxel resistance of KB1P tumors using gene expression profiling. A, Relative tumor size of 43 individual tumors after completion of initial treatment using 25mg docetaxel per kg i.v. on days 0, 7 and 14. Tumors with a relative volume below 100% (bar) were classified as good responders, the remaining as poor responders. B, significance analysis of microarrays (SAM) of untreated tumors of good vs. poor docetaxel responders (Δ=0.8) using the MEEBO or Illumina platform. C, the average of median-normalized cycle threshold (CT) values determined by quantitative TLDA of 46 genes encoding ABC proteins is shown for good versus poor docetaxel responders. For *Abca14, Abca15, Abca16, Abca17, Abcb5, Abcb11* and *Abcg8* no expression was detected in the tumors. D, SAM of tumors that acquired docetaxel resistance (T5doce-res, T6doce-res, T12*doce-res, T18*doce-res, T20*doce-res, T21*doce-res, T22*doce-res, T24*doce-res, T28*doce-res, T29*doce-res, T30*doce-res, T31*doce-res, T34*doce-res, T35*doce-res, T38*doce-res) in comparison to the corresponding docetaxel-sensitive controls (Δ=1.1, number of false significant genes = 0). T42 and T43 were not included because docetaxel-resistant tumors of these were not available at the time of analysis. E, ratios of gene expression of *Abcb1a* or *Abcb1b* of docetaxel-resistant tumors and samples from the matched drug-sensitive control tumors. Shown are RT-MLPA analyses of 17 docetaxel-resistant tumors. Error bars indicate standard deviation of three independent reactions.

Whether such a morphologic change correlates with drug resistance is under investigation.

Increased gene expression of the *Abcb1a* and *Abcb1b* genes is frequently found in acquired docetaxel resistance

Since our analysis of gene expression did not turn up a single gene that correlated with intrinsic docetaxel resistance, we tested tumors with acquired docetaxel resistance. Genes responsible for the acquired resistance might also cause intrinsic docetaxel resistance. We therefore compared RNA from samples of the same tumor before treatment and after they had become resistant to docetaxel. Unsupervised hierarchical cluster analysis did not separate sensitive from resistant tumors. Instead, tumors derived from the same original tumor were found in close proximity (Supplementary Fig. S3A). Exceptions are tumors T20 and T38, but the docetaxel-resistant versions of these tumors (T20*doce-res or T38*doce-res) had a high content of stromal tissue (Supplementary Fig. S3B), explaining the unusual distance between resistant tumor and docetaxel-sensitive control.

The SAM analysis of docetaxel-resistant tumors versus matched docetaxel-sensitive control tumors (Fig. 2D) yielded 9 genes that were significantly increased in docetaxel-resistant tumors (in red, see also Supplementary Table S3). Of these, only the *Abcb1b* gene -which encodes the mouse drug efflux transporter P-gp- can functionally explain docetaxel resistance. The other 8 genes (Gnγ*10, Gp49a, lysozyme, Lzp-s, CD18, Trem2, Lilrb4, Slc11a1*) appear to be linked to macrophages infiltrating drug-treated tumors to remove dead cells, as we have found previously for doxorubicin- or topotecan-resistant tumors [19,22]. More precise quantification of the *Abcb1a* and *Abcb1b* transcripts that encode mouse P-gp by RT-Multiplex Ligation-dependent Probe Amplification (RT-MLPA) confirmed that one or both of the *Abcb1* genes were upregulated at least 3-fold in 14 of the 17 tumors that acquired docetaxel resistance (Fig. 2E). We also investigated mouse *Abcc1*, which is a poor taxane transporter [33]. Expression of this control gene was not altered in any of the docetaxel-resistant tumors.

Since the expression of *Abcb1a* was frequently found to be increased by RT-MLPA in the resistant tumors, it is surprising that it was not identified by the SAM analysis shown in Fig. 2D. This proved to be due to the poor sensitivity of the *Abcb1a* probe. When we investigated T18, T20, T22, T24, T31 and T34, the 6 tumors with a more than 10-fold increase in *Abcb1a* transcripts in the resistant tumors, as determined by RT-MLPA, *Abcb1a* was the top hit by SAM (Supplementary Fig. S4A). However, when we added 4 tumors with only a ~4-fold increase in *Abcb1a* expression by RT-MLPA (T6, T28, T29 and T38), *Abcb1a* was lost as a significant gene (Supplementary Fig. S4B). This shows that the sensitivity of the *Abcb1a* probe is low in the MEEBO arrays.

Increased expression of the *Abcb1a* and *Abcb1b* genes can explain poor docetaxel response of 5/22 non-responders

In addition to conventional SAM analyses we also tested an algorithm designed to specifically detect differential gene expression that only occurs in a subgroup

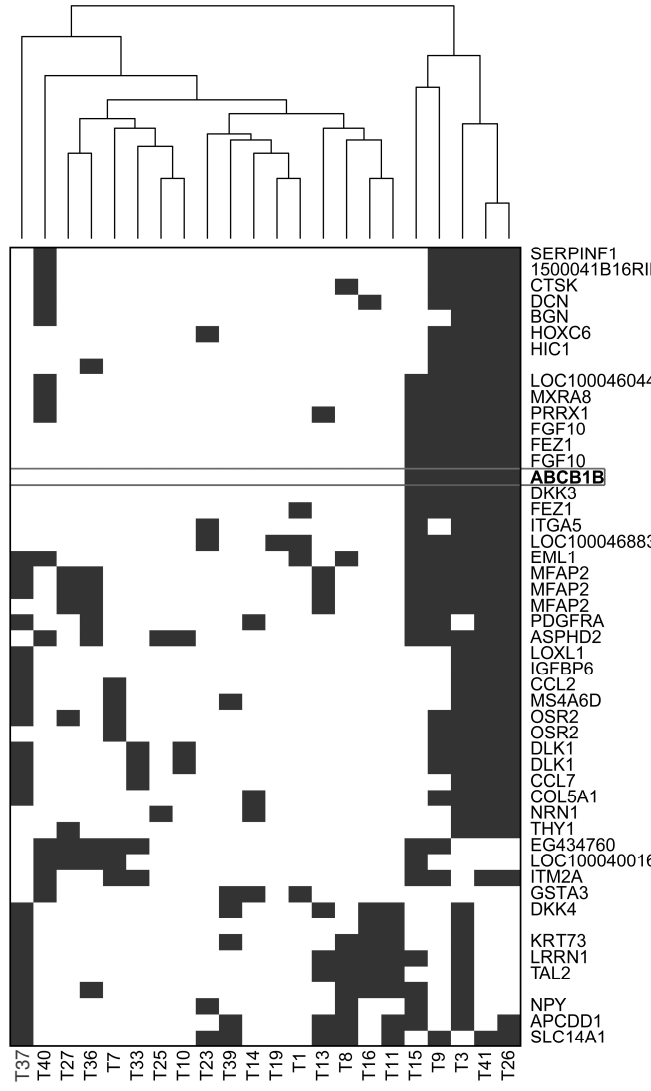


Figure 3. Identification of outliers present in subgroups of poor docetaxel responders using an algorithm developed by de Ronde *et al.* [34]. Clustering analysis of top 50 ranked genes is shown. Each black block represents a tumor with higher expression than the maximum of the expression of that particular gene in the responder group.

of tumors within the non-responding group [34]. This algorithm places a threshold on the gene expression corresponding to the highest expression level in the docetaxel responder group. For the docetaxel poor responders that exceed this threshold the sum of the differences of the expression is then calculated. When this algorithm was applied to the gene expression data, we found that *Abcb1b* was among the top outliers and formed a cluster with several other genes (Fig.

3 and Supplementary Table S4). This suggests that *Abcb1b* is not only involved in acquired docetaxel resistance, but may also contribute to upfront docetaxel resistance of some tumors. To further investigate whether an increased expression of the *Abcb1a/b* genes can explain the poor intrinsic docetaxel response of some of the 22 poor responder tumors (Fig. 2A), we quantified the RNA levels in the untreated tumors by RT-MLPA (Fig. 4A). In 5/22 tumors we found a 7- to 9-fold

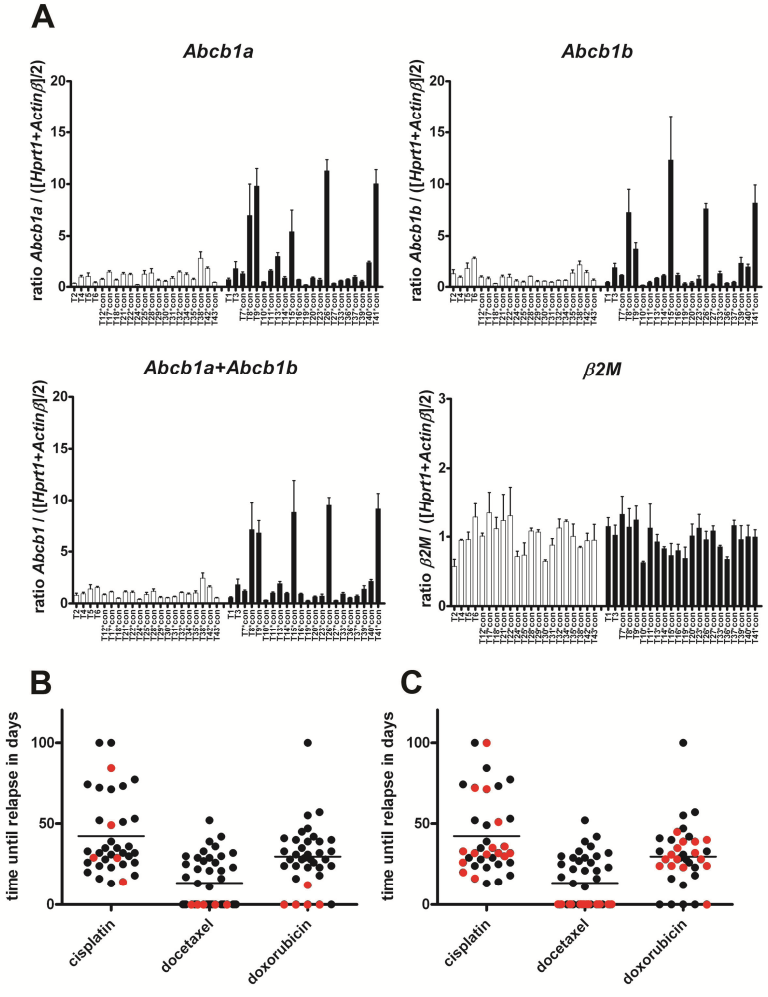


Figure 4. Quantification of the expression of the mouse *Abcb1* genes in untreated mouse mammary tumors. A, RT-MLPA analyses of *Abcb1a* or *Abcb1b* transcript levels of 43 individual KB1P tumors. Error bars indicate standard deviation of three independent reactions. B, time until relapse of KB1P tumors treated with the MTD of cisplatin, docetaxel or doxorubicin. The 5 tumors that showed increased *Abcb1* gene expression are highlighted in red. In panel C the remaining 15 poor docetaxel responders that were also treated with cisplatin and doxorubicin are indicated in red.

increase in *Abcb1* RNA above the average level of the good responders. *Abcb1a* RNA was elevated as well in these 5 tumors (Fig. 4A). We have previously shown that a modest upregulation of *Abcb1* by a factor 7-9 is sufficient to cause drug resistance in these tumors [21]. Indeed, we found that the 5 tumors with increased *Abcb1* gene expression also did not respond to the P-gp substrate doxorubicin (Fig. 4B and Supplementary Fig. S1), whereas the poor docetaxel responders without increased *Abcb1* RNA usually shrank below 50% with doxorubicin (Fig. 4C and Supplementary Fig. S1). As expected, there was no correlation of *Abcb1* transcript levels with cisplatin relapse-free survival (Fig. 4B,C), since cisplatin is not a substrate of P-gp.

P-gp-deficient mammary tumors are docetaxel hypersensitive

To improve our ability to detect P-gp-independent mechanisms of docetaxel resistance, we introduced the *Abcb1a/b* null alleles into the *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* model. Alleles were bred to homozygosity in FVB/N mice. The lack of functional P-gp did not affect mammary tumor latency or morphology of the female *K14cre;Brca1$^{F/F}$;p53$^{F/F}$;Abcb1a/b$^{-/-}$* mice (data not shown). P-gp-deficient mice carrying spontaneous mammary tumors cannot be treated with the docetaxel MTD, because P-gp contributes to the normal disposition of docetaxel in the mouse. We therefore grafted *Brca1$^{-/-}$;p53$^{-/-}$;Abcb1a/b$^{-/-}$* tumors (KB1PM) orthotopically into syngeneic wild-type mice (Fig. 5A). In sharp contrast to *Abcb1a/b* wt tumors (KB1P), tumors unable to make P-gp were hypersensitive to the docetaxel MTD: only 1 out of 11 individual KB1PM tumors responded poorly to docetaxel and the mouse had to be sacrificed 40 days after the start of treatment (Fig. 5B). The median recurrence-free survival time increased significantly (P<0.001) from 14 (T7-T43) to 51 days (KB1PM-1 and KB1PM-3 to -11), and for 1 tumor (KB1PM-2) no relapse occurred within 250 days, suggesting that this tumor was even eradicated (Fig. 5B,C). We never observed this outcome in the P-gp-proficient KB1P tumors. With the exception of KB1PM-5 none of the P-gp-deficient tumors acquired docetaxel resistance, and eventually the mice had to be killed due to cumulative docetaxel toxicity. The median survival of animals carrying orthotopically transplanted P-gp-deficient tumors increased significantly (P<0.001) to 164 days (+/- 69 SD, n=11) compared with 45 days (+/- 28 SD, n=37) of animals with P-gp-proficient tumors (Fig. 5D). These data show that P-gp is a major contributor to docetaxel resistance of KB1P mammary tumors *in vivo*. Obviously, this high docetaxel sensitivity makes it impossible to identify P-gp-independent resistance mechanisms, if mice are treated with the MTD of docetaxel, but we have recently observed acquired resistance to lower drug doses (unpublished results).

Low expression of the *Xist* gene correlates with high cisplatin sensitivity of KB1P tumors and predicts benefit of platinum-based chemotherapy in patients with high risk primary breast cancer

Since we found variation in the response to drug, not only for docetaxel, but also for cisplatin (Fig. 6A), we wondered whether standard gene expression analyses would also fail to identify predictive markers for this treatment. All KB1P tumors were cisplatin sensitive, but 23 tumors relapsed already within 39 days, whereas

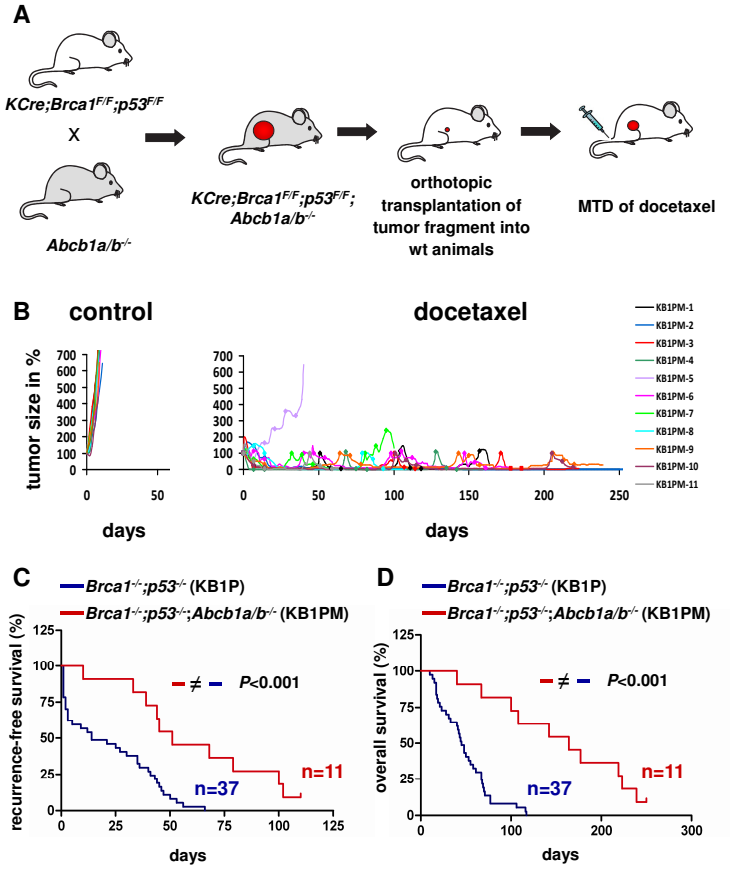


Figure 5. Docetaxel responses of P-gp;BRCA1;p53-deficient (KB1PM) mammary tumors. A, *Abcb1a/1b*$^{-/-}$ alleles were crossed to homozygosity into the *KCre;Brca1*$^{F/F}$*;p53*$^{F/F}$ model in a FVB/N background. KB1PM tumors which developed in this model were then orthotopically transplanted into female FVB/N animals. Once the tumors reached a volume of 150-250mm^3 animals were treated with the docetaxel MTD. B, 11 orthotopically transplanted KB1PM tumors were left untreated or received 25mg of docetaxel per kg i.v. on days 0, 7 and 14 (rhombi). When tumors relapsed back to ~200mm^3 or showed progressive growth (tumor size ≥ 50%) after a recovery time of 7 days following the day 14 injection, treatment was resumed as indicated by the rhombi. Comparison of the time until tumors relapsed back to the original size of treatment start (C) or survival (D) of KB1PM-1 to -11 with the orthotopically transplanted P-gp-proficient KB1P tumors T7-T43. *P* values were determined using the logrank test.

12 tumors only grew back to 100% after 49 days. When we stratified the gene expression profiles of the untreated tumors based on their cisplatin sensitivity (above or below the mean time to relapse), we found a low expression of the *Xist* gene to correlate significantly with cisplatin hypersensitivity on 2 independent gene expression platforms (Fig. 6B).

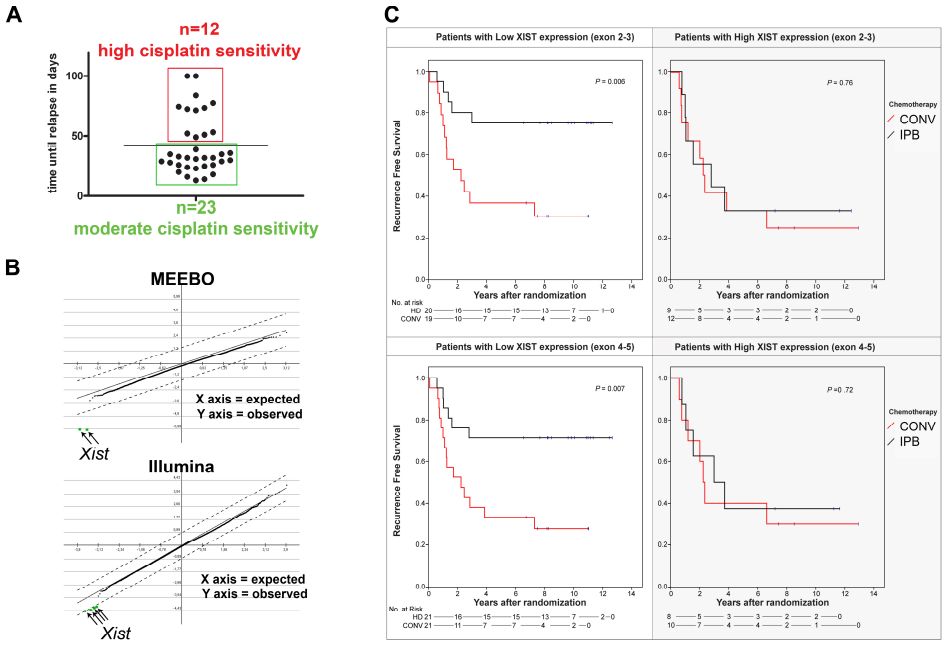


Figure 6. Correlation of gene expression with the response to platinum drugs. A, 35 mice with KB1P tumors were treated with 6mg of cisplatin per kg i.v. on day 0. The time until relapse is the time required for the tumors to grow back to 100%. The bar indicates the mean. B, SAM of highly vs. moderately cisplatin-sensitive KB1P tumors using the MEEBO (Δ=1.5; FDR=0) or Illumina (Δ=0.85; FDR=0) platform. C, Kaplan Meier survival curves according to *XIST* gene expression levels of patients who had been randomized between conventional (CONV, red) and intensive platinum-based chemotherapy (IPB, black). *P* values were calculated using the logrank test.

The physiological role of the non-coding RNA *Xist* is to coat one X chromosome of female cells in *cis* and subsequently trigger chromatin remodeling to form the heterochromatic Barr body (condensed inactivated X chromosome [Xi]). *XIST* is transcribed exclusively from the Xi to achieve equal X-linked gene dosage between the sexes. The analysis of genes correlating with a low *Xist* expression in our tumor model revealed a reduced expression of 3 other X-linked genes: *Utx*, *Jarid1c*, and *Eif2s3x* (Supplementary Fig. S5). As all of these genes are known to escape X inactivation [35], they are independent markers for the loss of the Xi.

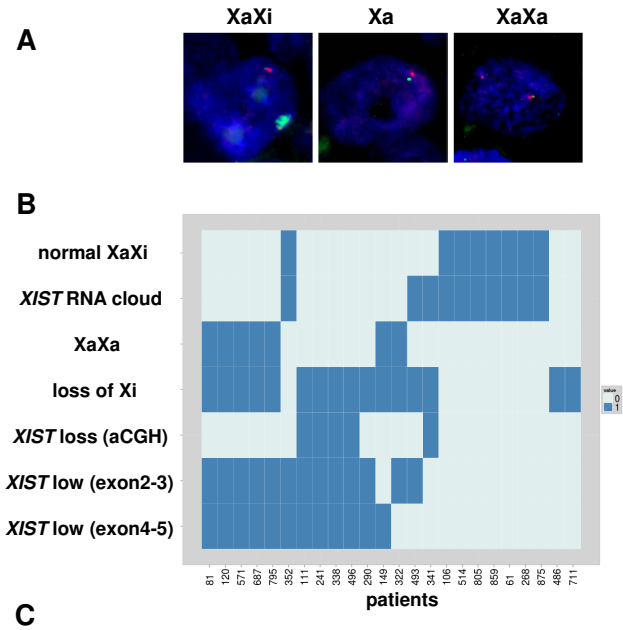
Given the high frequency of reduced *Xist* expression in cisplatin hypersensitive mouse tumors, we tested whether *XIST* expression could serve as a biomarker to predict response to platinum-based chemotherapy in human breast cancer. For this purpose we took tumor samples of 60 stage-III, HER2-negative breast cancer patients who had been randomized between two treatment arms: intensive platinum-based chemotherapy, or a standard anthracycline-based regimen (conventional chemotherapy) [27]. The patient information is summarized in Supplementary Table S5. To quantify *XIST* gene expression levels of FFPE

material (>60% tumor cells), we used RT-MLPA including 2 independent probes hybridizing to the exon 2-3 or exon 4-5 boundary of *XIST* cDNA (Supplementary Table S6). The cut-off to determine low *XIST* expression was defined as 2 times the SD below the average expression of normal breast tissue (Supplementary Fig. S6). Analysis of the recurrence-free survival (RFS) showed that patients with a low *XIST* expression significantly benefited from the intensive platinum-based therapy compared to conventional chemotherapy: the 5-year RFS increased from 37% to 75% (Fig. 6C, adjusted hazard-ratio: 0.30, 95%CI: 0.11-0.82 for the probe of exon 4-5, Supplementary Table S5B). In patients with *XIST* gene expression above the cut-off no significant survival benefit was observed of platinum-based chemotherapy (5-year RFS 33% both treatment arms, Fig. 6C; adjusted hazard-ratio: 0.81, 95%CI: 0.23-2.89 for the exon 4-5 probe, Supplementary Table S5B). Analyses with the exon 2-3 probe confirmed those obtained with the exon 4-5 probe (Fig. 6C and Supplementary Table S5B). Intriguingly, low *XIST* gene expression was associated with *BRCA1* promoter methylation, BRCA1-like status on array CGH, but not *BRCA1* mutated breast cancer (Figure S8).

To determine whether loss of the *XIST* gene could explain the low *XIST* gene expression detected with both RT-MLPA probes, we analyzed the DNA of 37 patients with arrayCGH using probes flanking the *XIST* locus. Indeed, a *XIST* gene was lost in 16 out of 37 patients. Loss of *XIST* DNA correlated significantly with low RNA expression for all 60 patients ($P<0.017$ for the exon 2-3 probe, Fisher's exact test). For 24 of the 60 samples we also managed to perform *XIST* RNA FISH analyses on the available FFPE material (Fig. 7). A DNA probe targeting RNF12 was taken along as X chromosome marker (Fig. 7A). RNA FISH confirmed that patients with low *XIST* gene expression had significantly fewer *XIST* clouds (Fig. 7B,C). Moreover, the combination of RNA and DNA FISH showed for all cases with aCGH-based *XIST* deletion that only the Xa was present (Fig. 7B). In several patients with low *XIST* gene expression, but no *XIST* gene deletion detectable by aCGH, we found two active X chromosomes and loss of Xi (Fig. 7B). Together, our data show that loss of Xi is the main cause of low *XIST* gene expression ($P< 0.027$, Fig. 7C).

High prevalence of a predictive marker is required for its detection

Since *Xist* was readily identified as predictive marker for cisplatin sensitivity in our mouse model by SAM, it remains remarkable that our initial search to detect predictive markers for docetaxel sensitivity (Fig. 2) failed. Although sensitivity of our ordered arrays for *Abcb1a* is low, *Abcb1b* transcripts are readily detected. So, why did the SAM analysis of Fig. 2B not identify *Abcb1b* as predictive marker for docetaxel response? When we analyzed only the tumors with an intrinsically high *Abcb1* expression (T8*con, T9*con, T15*con, T26*con and T41*con) versus the 21 docetaxel-sensitive tumors as defined in Fig. 2A, *Abcb1b* was one of the most significantly increased genes on both the MEEBO and Illumina gene expression platforms (Supplementary Fig. S4C,D). Also the TLDA expression data showed a significant difference for *Abcb1a* and *Abcb1b* when only the 5 poor responders T8*con, T9*con, T15*con, T26*con and T41*con were compared with the docetaxel-sensitive tumors (*Abcb1a*: $P < 0.006$; *Abcb1b*: $P < 0.004$; Mann-Whitney



	XIST exon 2-3 probe low			XIST exon 4-5 probe low		
	OR	95%CI	P	OR	95%CI	P
Presence *XIST* RNA cloud (FISH)	0.04	0.001-0.423	0.003	0.08	0.005-0.668	0.011
XaXa (FISH)	9.91	0.89-548.68	0.068	7.85	0.71-431.54	0.078
Loss of Xi (FISH)	13.56	1.23-751.28	0.027	17.94	1.59-1014.8	0.008

Figure 7. X chromosome aberrations investigated by FISH. A, examples of normal XaXi or abnormal cells with Xa or XaXa configuration (green: XIST RNA probe; red: RNF12 DNA). B, overview of 24 patients for which FISH results were obtained and their correlation to *XIST* RNA expression (0: *XIST* low, 1: *XIST* high) or aCGH (0: normal copy number, 1: *XIST* lost). Normal XiXa, XaXa and loss of Xi are also indicated categorically (0= no, 1= yes). *XIST* RNA cloud describes whether a normal *XIST* cloud is absent in >60% of cells (0) or not (1). (C) Associations between low *XIST* gene expression and aberrations identified by FISH (n=24, Fisher's exact test). OR: Odds ratio, CI: Confidence interval.

U test). However, since increased expression of the *Abcb1* genes is only found in a subgroup of the poor docetaxel responders, this significance is lost when samples with other docetaxel resistance mechanisms are added (Supplementary Fig. S7). In fact, addition of 5 samples without *Abcb1* upregulation suffices to dilute the *Abcb1* signal below significance.

In contrast to *Abcb1b* in the case of docetaxel treatment, the prevalence of low *Xist* expression was high in cisplatin hypersensitive tumors: 11 (MEEBO platform) or 10 (Illumina platform) out of the 12 showed *Xist* gene expression below the median (Supplementary Fig. S7). It is therefore not surprising that *Xist* was picked up by SAM.

DISCUSSION

We have investigated whether predictive markers for chemotherapy benefit can be identified in a GEMM using genome-wide expression profiling. GEMMs should be ideal for this purpose, as they lack the profound genetic heterogeneity of tumors from human patients. The mice are inbred, all tumors originate from the targeted deletion of *Brca1* and *p53*, and all differences between tumors originate from a limited number of random mutations in the period between the initiating deletions of *Brca1* and *p53* and the development of a mammary tumor. These additional mutations are responsible for the marked and stable differences in sensitivity to docetaxel and cisplatin that we find in individual tumors.

Even in this genetically homogeneous tumor system, we did not find a signature predicting docetaxel response, using genome-wide expression profiling. This negative result is instructive, however, because it has allowed us to delineate what is required to get useful predictive signatures. In our collection of 22 poor docetaxel responders, 5 tumors contained a substantial increase in *Abcb1* RNA, known to be sufficient to cause drug resistance [21]. Nevertheless, this increase in *Abcb1* RNA was completely missed by 2 independent platforms measuring gene expression profiles. The *Abcb1b* transcript was readily detected in the 5 tumors with elevated transcript levels, as long as these tumors were analyzed as a group. However, when the results were pooled with those from only 5 tumors without elevated *Abcb1b* RNA, the positive result was completely lost. Results leading to the same conclusion were obtained in a study analyzing the multidrug resistance transcriptome of 32 unpaired ovarian serous carcinoma effusion samples obtained at diagnosis or at disease recurrence following chemotherapy [24]. High *ABCB1* transcript levels were detected in four patients who had received chemotherapy, even though *ABCB1* was not statistically correlated with progression-free survival in the samples as a whole. This shows why it is difficult to develop predictive markers, based on genome-wide expression arrays: only if the response to a drug is primarily determined by the expression level of a gene in most tumors, one can expect that gene to show up in the array-based gene expression analyses.

We found such a gene in analyzing the response of the mouse tumors to cisplatin. The low *Xist* expression associated with tumors hypersensitive to cisplatin was present in 10 out of 12 tumors and therefore detectable in our array analysis. Our results show that standard statistical tests will only detect predictive markers if they can explain therapy response in a high proportion of the samples analyzed. The detection sensitivity can only be increased by the use of special algorithms that can identify subgroups within the samples. We show that such an algorithm is able to identify *Abcb1b* as outlier within the poor docetaxel responders. However, even with a more sophisticated analysis the problem remains that probes on the arrays are not sensitive enough to detect all relevant expression differences of genes causing therapy resistance. Gillet and co-workers found in a panel of cancer cell lines that the expression of the 380 "resistance–relevant" genes could only be reliably measured by quantitative PCR. For many genes the results obtained by microarrays were useless because of low sensitivity [29,36].

Given all these hurdles in finding predictive markers for chemotherapy, it is gratifying that we identified a gene that correlated with cisplatin response. It is encouraging that the low expression of *XIST* predicting high sensitivity to cisplatin in drug-naïve mouse tumors, also predicted an increased recurrence-free survival of high risk, primary breast cancer patients treated with intensive platinum-based chemotherapy. Although detected in a rather small group of 60 patients, the effect found is considerable. Intensive chemotherapy has largely been abandoned for the treatment of breast cancer, because for many patients the therapeutic benefit is limited [37]. Nevertheless, several studies suggest that there are subgroups of patients that do benefit from this therapy, but the predictive tests to identify them are lacking [38,39]. Hence, the analysis of *XIST* gene expression may be a useful tool to decide whether intensive platinum-based chemotherapy should be considered as alternative therapy for patients with HER2-negative, high risk breast cancer. Not all patients with a low *XIST* expression that we investigated benefited from the platinum-based therapy. An optimized cut-off for the level of *XIST* expression, validated in prospective clinical trials, may increase the positive predictive value, as may a combination with other classifiers, such as BRCA1-like CGH profiles [25].

In 2002 a low *XIST* expression was reported in a recurrent tumor of an ovarian cancer patient [40]. Subsequent work with a few cell lines suggested that a low *XIST* expression predicts paclitaxel resistance of ovarian cancer. This result was never validated in clinical trials and in our mouse model we do not find it.

Why tumors with a low expression of *XIST* are platinum hypersensitive is under investigation. Low *XIST* gene expression may be a flag for genomic instability as we found loss of Xi as the main cause underlying low transcript levels. The loss of Xi is most likely the consequence of chromosome segregation errors, which may be enhanced in BRCA1-defective cells due to a compromised spindle checkpoint [41]. It has recently been found that mis-segregation stress induces a DNA damage response [42] and it was observed that aneuploid cells are more sensitive to anti-proliferative drugs [43]. Mammary tumor cells with defects in DNA repair which are additionally stressed by improper execution of mitosis may therefore be hypersensitive to intensive platinum-based therapy.

The precise mechanism of *XIST*-mediated X inactivation is still under debate [44]. It was suggested that BRCA1 supports the localization of XIST RNA to the Xi, as the BRCA1-deficient cells or tumors examined had lost localized *XIST* RNA [45-47]. However, this hypothesis was challenged by others [48,49]. The recent finding that BRCA1 maintains heterochromatin integrity [50] supports the idea that BRCA1 contributes to X inactivation after *XIST*-induced chromatin condensation. The contribution is not a simple one, however. Despite the large *Brca1* deletion present in all mammary tumors of our mouse model, *Xist* gene expression varies considerably. Variability of *XIST* expression was also present in those human breast cancers in which a *BRCA1* mutation was found, or which were classified as BRCA1-like by aCGH [25].

Our study shows that GEMMs that resemble breast cancer in humans are useful to investigate chemotherapy response prediction. Tools to identify predictive markers can be tested under controlled conditions, and targeted ablation of genes helps to dissect mechanisms of resistance. Ultimately, predictive markers

identified in GEMMs may improve the clinical success rate for chemotherapy in humans.

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CONFLICT OF INTEREST

M.J. and M.L. are employees of MRC-Holland BV which markets the RT-MLPA tests used in this article.

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SUPPLEMENTARY MATERIALS AND METHODS

Mice

KB1P mammary tumors were generated and genotyped as described (Liu ***et al.*** Proc Natl Acad Sci U S A 2007;104:12111-6). To produce KB1PM mammary tumors on a FVB/N genetic background, ***FVB.129P2-Abcb1a^{tm1Bor}Abcb1b^{tm1Bor}***, ***FVB-Tg(KRT14-cre)8Brn***, ***FVB.129P2-Trp53^{tm1Brn}***, or ***FVB.129P2-Brca1^{tm1Brn}*** mice were backcrossed on FVB/N animals for at least 8 generations (the first 5 generations using marker-assisted breeding) and eventually crossed to generate ***FVB.Cg- Abcb1a^{tm1Bor}Abcb1b^{tm1Bor} Trp53^{tm1Brn} Brca1^{tm1Brn} Tg(KRT14-cre)8Brn/A*** compound mice. ***Abcb1a$^{\Delta 6\text{-}7/\Delta 6\text{-}7}$*** and ***Abcb1b$^{\Delta 3\text{-}4/\Delta 3\text{-}4}$*** genotypes were confirmed by PCR with specific primers (forward-***Abcb1a***: 5′- GTGCATAGACCACCCTCAAGG-3′; forward-***Abcb1b***: 5′-AAGCTGTGCATGATTCTGGG-3′) for wildtype (reverse-***Abcb1a***: 5′-GTCATGCACATCAAACCAGCC-3′; reverse-***Abcb1b:*** 5′-GAGAAACGATGTCCTTCCAG-3′) and deleted alleles (reverse-***Abcb1a***: 5′-GGAGCAAAGCTGCTATTGGC-3′). Orthotopic transplantations, mammary tumor measurements and sampling were performed as explained previously (Rottenberg ***et al***. Proc Natl Acad Sci U S A 2007;104:12117-22), and allowed us to collect response data for docetaxel, cisplatin and doxorubicin for 36 individual KB1P tumors. In addition, KB1PM mammary tumors were transplanted into FVB/N animals. Deletion of ***Brca1*** and ***p53*** of orthotopically transplanted tumors was confirmed by PCR (absence of ***Brca1$^{F5\text{-}13/F5\text{-}13}$;p53$^{F2\text{-}10/F2\text{-}10}$*** alleles and presence of ***Brca1$^{\Delta 5\text{-}13/\Delta 5\text{-}13}$;p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*** alleles) as described (Liu ***et al.*** Proc Natl Acad Sci U S A 2007;104:12111-6).

Drugs and treatment of tumor-bearing animals

Docetaxel (Taxotere, 10 mg/ml in Tween80/ethanol/saline 20:13:67 vol/vol/vol; Aventis, Antony Cedex, France) was diluted with saline to 5 mg/ml before injection. Cisplatin (1 mg/ml in saline-mannitol) originated from Mayne Pharma (Brussels, Belgium). Doxorubicin (Adriblastina; Amersham Pharmacia Netherlands, Woerden, The Netherlands) was diluted to 1 mg/ml in saline (Braun, Emmer-Compascuum, The Netherlands). When mammary tumors reached a volume of 150-250mm^3 (0.5 × length × width^2) 25mg docetaxel per kg (days 0, 7, 14), 6mg cisplatin per kg (day 0) or 5mg doxorubicin per kg (day 0) were injected i.v. as initial treatment. To avoid accumulating toxicity of repeated injections, an additional treatment was not given during the recovery time of 7 days in case the tumor responded to the treatment (tumor size <50% of the original volume, partial response). Treatment was continued once the tumor relapsed to its original size (100%). For tumors with a volume ≥50% after the recovery time, an additional treatment with the same dose as mentioned above was given.

Histology

Tissues were fixed in 4% formaldehyde overnight, embedded in paraffin, and cut in 4μm sections. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin according to standard procedures.

Processing and Analysis of the Microarray Data

Data normalization of the dual channel MEEBO arrays was carried out as described (Yang et al. Nucleic Acids Res 30:e15, 2002). We then used a modified Rosetta error model (Weng et al. Bioinformatics 22:1111–1121, 2006) on the fluorochrome-reversed two-color duplicates to calculate the average ratio per gene and a *P* value indicating the chance a gene is falsely classified. For the single channel Illumina arrays background correction was performed using the bg.adjust method from the Bioconductor affy package (Gautier et al. Bioinformatics 20: 307-315, 2004). For normalization between arrays the robust spline method was applied. Filtering of probes on the single channel arrays was performed using the detection p-value (significantly different from background in at least one sample), which reduced the number of reporters from 45,281 to 26,352. For filtering probes of the dual channel arrays the p-value from the rosetta error model was used (fold change significantly different from 0 in at least one sample). In addition, probes with missing data points in more than 10% of the hybridizations were excluded. This reduced the number of reporters from 38,784 to 21,791. The TIGR Multiexperiment Viewer 4.6 software (TMV4.6, www.tm4.org/mev.html) was used to perform the SAM analysis. Unsupervised hierachical clustering analysis was carried out using the pvclust software (www.is.titech.ac.jp/~shimo/prog/pvclust/). The hierachical clustering algorithm was based on Euclidean distance and average linkage was applied to group tumor samples according to similarity in the pattern of gene expression. For the SAM in Figure 2D T5doce-res, T6doce-res, T12*doce-res, T18*doce-res, T20*doce-res, T21*doce-res, T22*doce-res, T24*doce-res, T28*doce-res, T29*doce-res, T30*doce-res, T31*doce-res, T34*doce-res, T35*doce-res, and T38*doce-res were used. These tumors with acquired docetaxel resistance were compared to the corresponding docetaxel-sensitive controls. T42 and T43 were not included, because docetaxel-resistant tumors of these were not available at the time of analysis. The microarray data reported in this article have been deposited in the Array Express database, www.ebi.ac.uk/arrayexpress (accession no. E-MTAB-413 [Illumina] and E-MTAB-415 [MEEBO]).

Reverse Transcription-Multiplex ligation-dependent probe amplification (RT-MLPA)

From snap frozen mouse tumors total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. RNA from FFPE human breast cancers was isolated using the High Pure RNA Paraffin Kit (Roche, Woerden, The Netherlands) as described in the instruction manual. Reverse transcription, hybridization, ligation, PCR amplification and fragment analysis by capillary electrophoresis were carried out as reported previously [20,21]. The ***Abcb1*** gene expression levels were normalized to the internal reference genes ***Actin***β and ***Hprt1***. The average expression of ***Abcb1a*** or ***Abcb1b*** was lower than that of (***Hprt1***+***Actin***β)/2. To simplify Fig. 4A all ***Abcb1a/***([***Hprt1***+***Actin***β]/2) ratios were multiplied with the factor 24.8, those of ***Abcb1b/***([***Hprt1***+***Actin***β]/2) with a factor of 25.8 and those of (***Abcb1a+Abcb1b)/***([***Hprt1***+***Actin***β]/2) with a factor of 12.6. A list of the human-specific RT-MLPA probes is provided in Supplementary

Table S4. For normalization of ***XIST*** gene expression, we used the mean of 8 reference probes detecting β***2M***, ***GAPDH*** (2x), ***LDH***, ***FAU***, ***OAZ1***, ***BIRC2*** and ***ARHGDIA*** gene expression. The cut-off to determine low ***XIST*** expression was defined as 2 times the SD below the average expression of normal breast tissue (Supplementary Fig. S6).

TaqMan low density arrays (TLDA)

Synthesis of cDNA from 1 µg total RNA in a 20 µl reaction volume was carried out using the High Capacity cDNA kit with RNAse inhibitor (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. The reverse transcription conditions were as follows: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C. Following reverse transcription, cDNA was stored at 4°C. Expression levels of 49 murine Abc transporter genes were measured using custom-made Taqman Low Density Arrays (Applied Biosystems, Foster City, CA, USA). cDNA was mixed with 2X Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), loaded on the TLDA card (125ng per port), and run on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions**.** For *Abca14*, *Abca15*, *Abca16*, *Abca17*, *Abcb5*, *Abcb11* and *Abcg8* no expression was detected in the mouse tumors.

FISH

Sections were deparaffinized with xylene and treated with 1M sodium thiocyanate for 10 minutes at 80°C to remove crosslinks. Cells were permeabilized with 750 U/ml pepsin (Sigma-Aldrich) for 8 minutes. RNF12-specific DNA FISH probes were prepared from RP11 BAC clones. BAC DNA was digested and labeled using random primer labeling (Invitrogen) with Bio-16-dUTP or (Roche). Probes were validated using control metaphases. *XIST* RNA FISH probe was prepared from a plasmid containing 12kb of the *XIST* sequence and labeled with DIG-11-dUTP (Roche). Digoxigenin- or biotin- labeled probes were detected using a FITC-labeled mouse anti-DIG antibody or Alexa 594-labeled streptavidin, respectively.

Patients

In a previous study stage-III HER2-negative breast cancer patients were randomly selected from a large randomized controlled trial (RCT) performed in the Netherlands between 1993 and 1999 [27] and analyzed for aCGH classification [25]. Patients were randomized between conventional chemotherapy (5*FEC: 5-fluorouracil 500mg/m^2, epirubicin 90mg/m^2, cyclophosphamide 500mg/m^2) and intensive platinum-based chemotherapy (4*FEC, followed by 1*CTC: cyclophosphamide 6000mg/m^2, thiotepa 480mg/m^2 and carboplatin 1600mg/m^2). From the aCGH study we selected those patients of whom *BRCA1*-mutation status was available (n=60 from the previous study [25] and 13 additionally analyzed). Of 13 patients no fragments could be visualized upon electrophoresis due to poor FFPE RNA quality, resulting in a subset of 60 patients of whom RT-MLPA data was available. For statistical analyses recurrence-free survival (RFS) was calculated from randomization to appearance of local or regional recurrence, metastases or

to death from any cause. All other events were censored. Differences between groups of interest were tested using Fisher's exact tests. Survival curves were generated using the Kaplan-Meier method and compared using log-rank tests. Hazard ratios (HR) were calculated using Cox-proportional hazards regression.

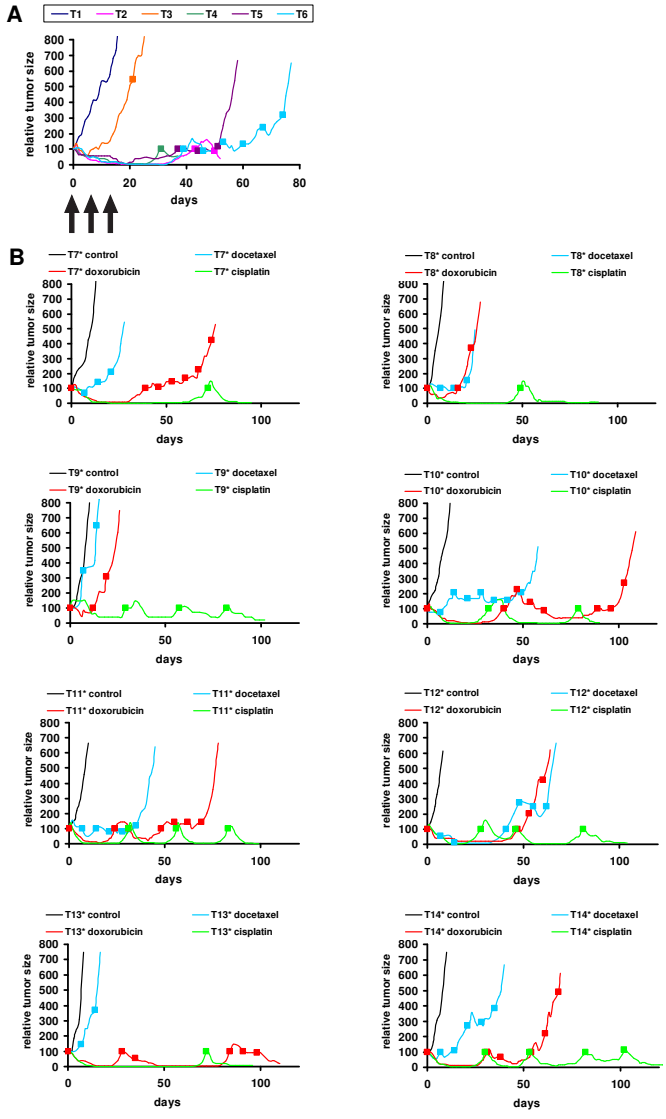
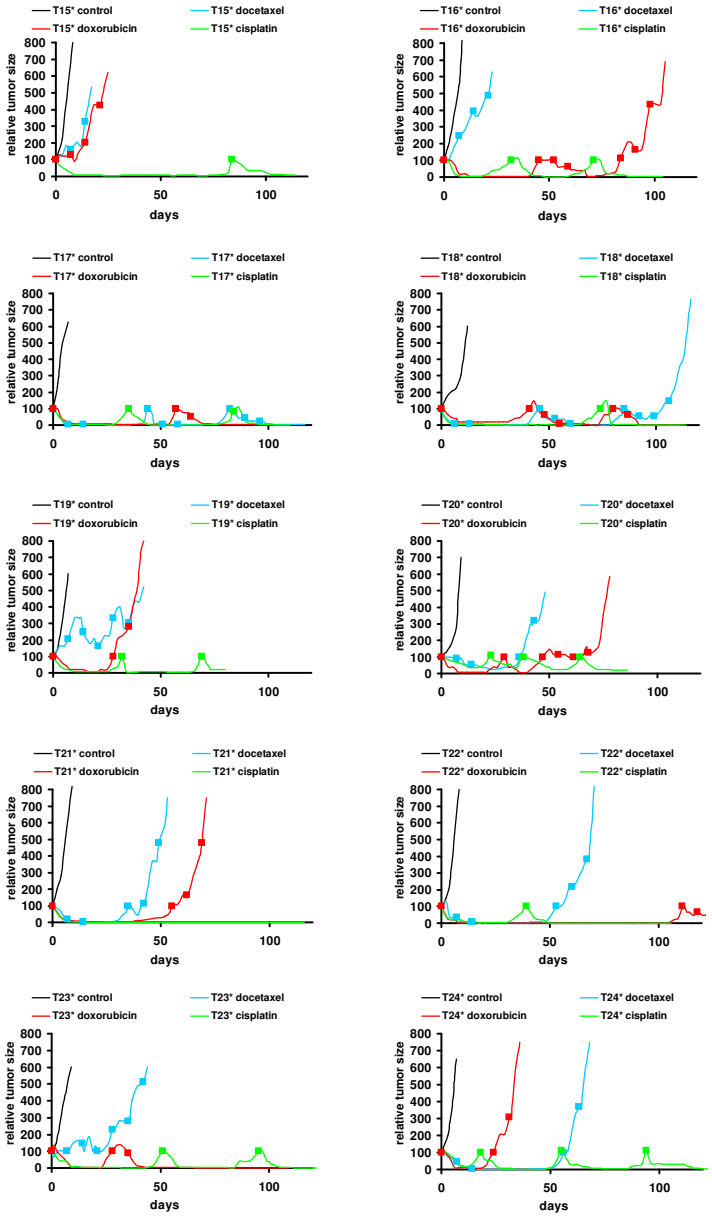


Figure S1. Response of BRCA1;p53-deficient tumors to the maximum tolerable dose of docetaxel, doxorubicin or cisplatin. A, 6 individual K14cre,Brca1F/F,p53F/F animals carrying a spontaneous mammary tumor of ~200 mm3 (T1-T6) were treated with 25 mg docetaxel per kg mouse i.v. on days 0, 7 and 14 (arrows). When tumors relapsed back to ~200 mm3 or showed progressive growth (tumor size ≥ 50%) after a recovery time of 7 days following the day 14 injection, treatment was resumed as indicated by the filled squares. Animals carrying T2 or T4 had to be sacrificed before full docetaxel resistance developed due to the presence of a squamous cell carcinoma of the lip (T2) or ear (T4). **B**, animals with

Figure S1B continued



37 individual orthotopically transplanted BRCA1;p53-deficient mammary tumors (volume ~200 mm^3) were left untreated (black line) or treated with 25 mg docetaxel per kg i.v. on days 0, 7 and 14, 5 mg doxorubicin per kg i.v. on day 0, or 6 mg cisplatin per kg i.v. on day 0. When tumors relapsed or showed progressive growth (tumor size ≥ 50%) after a recovery time of 7 days (docetaxel, doxorubicin) or 14 days (cisplatin), treatment was resumed as indicated by the filled squares. The responses of T1, T3, T7, T10, T11 and T14 have already been published in Rottenberg et al. PNAS 104:12117-22, 2007.

Figure S1B continued

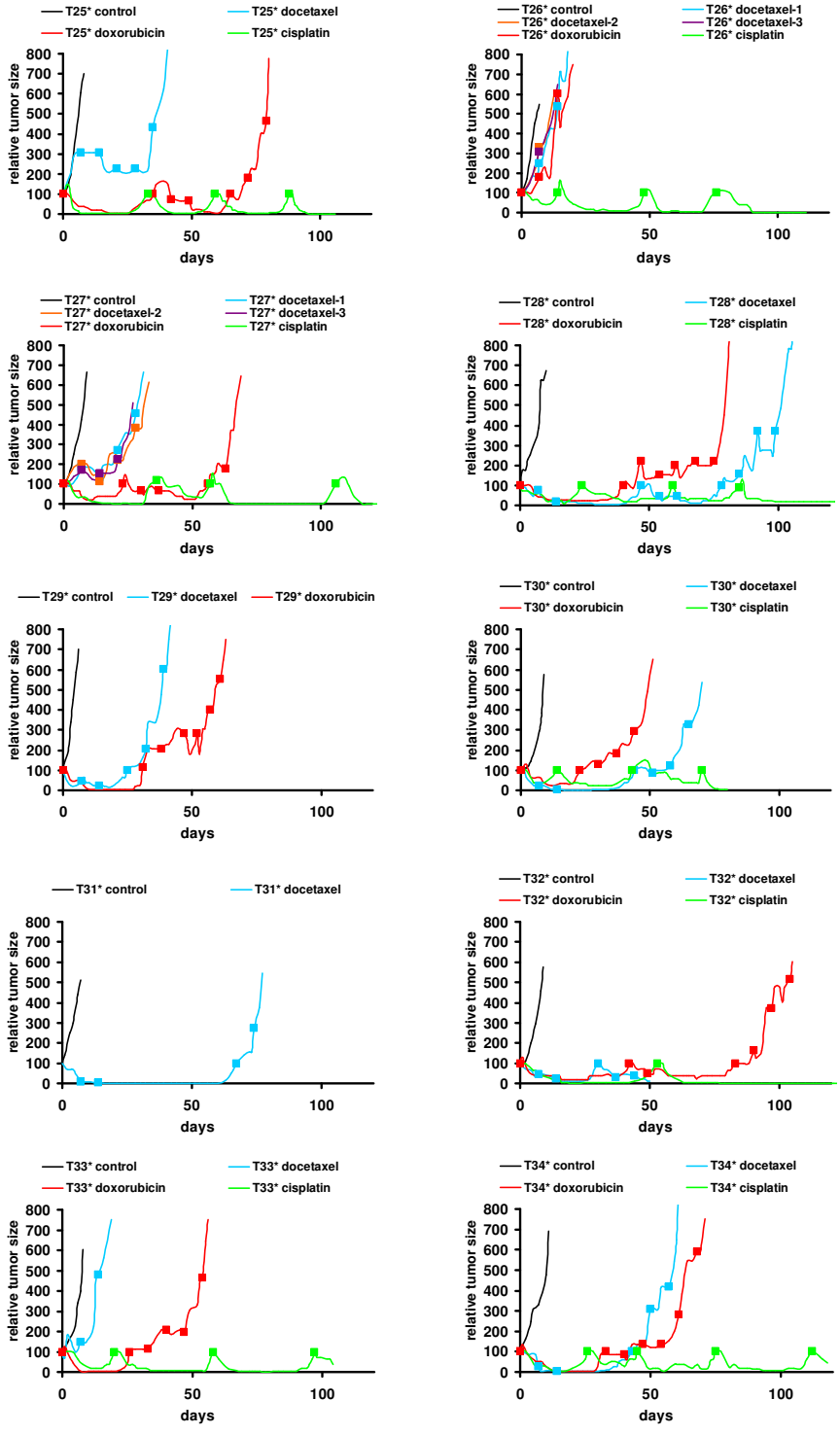
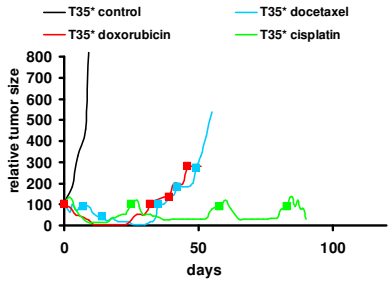
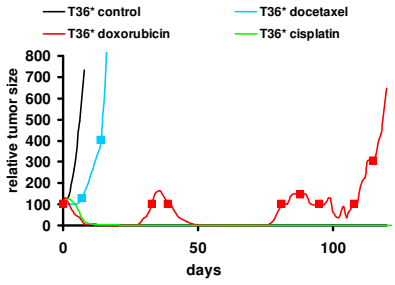
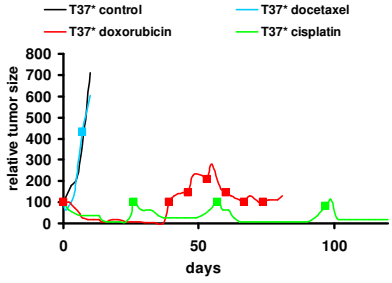
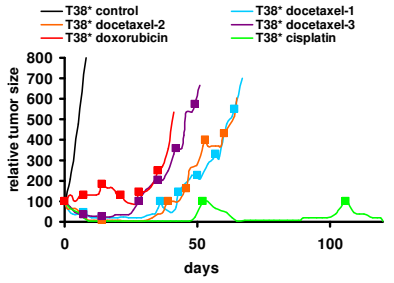


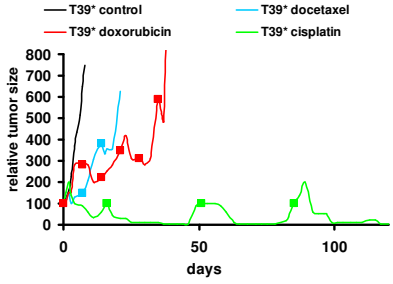
Figure S1B continued

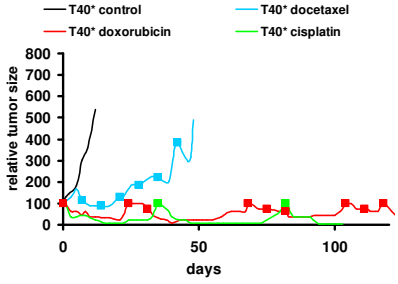


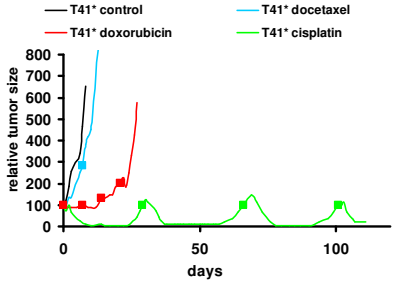


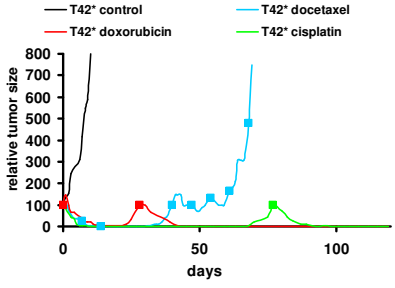


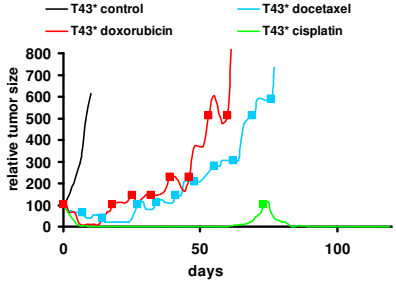


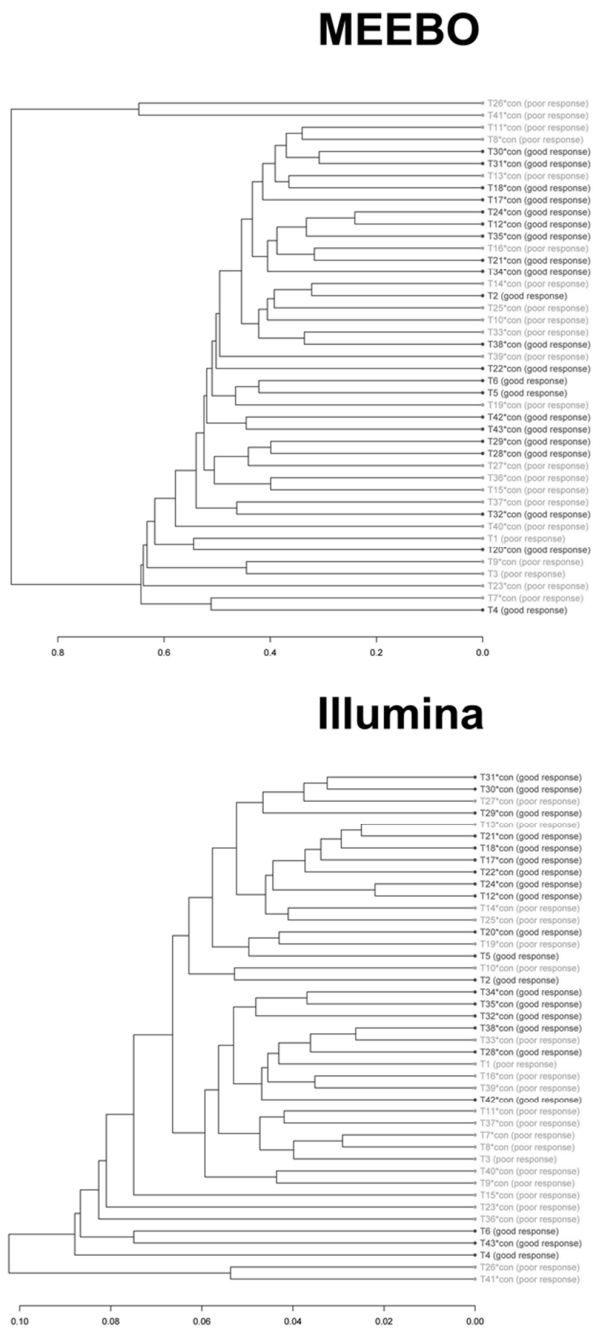




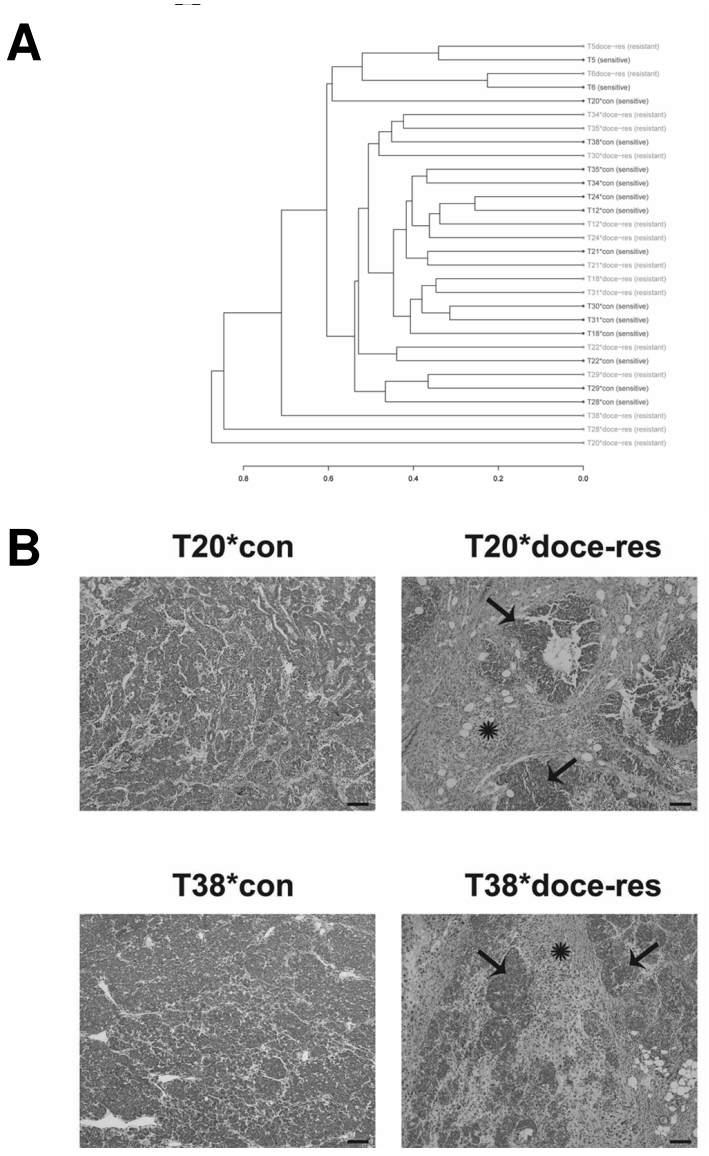




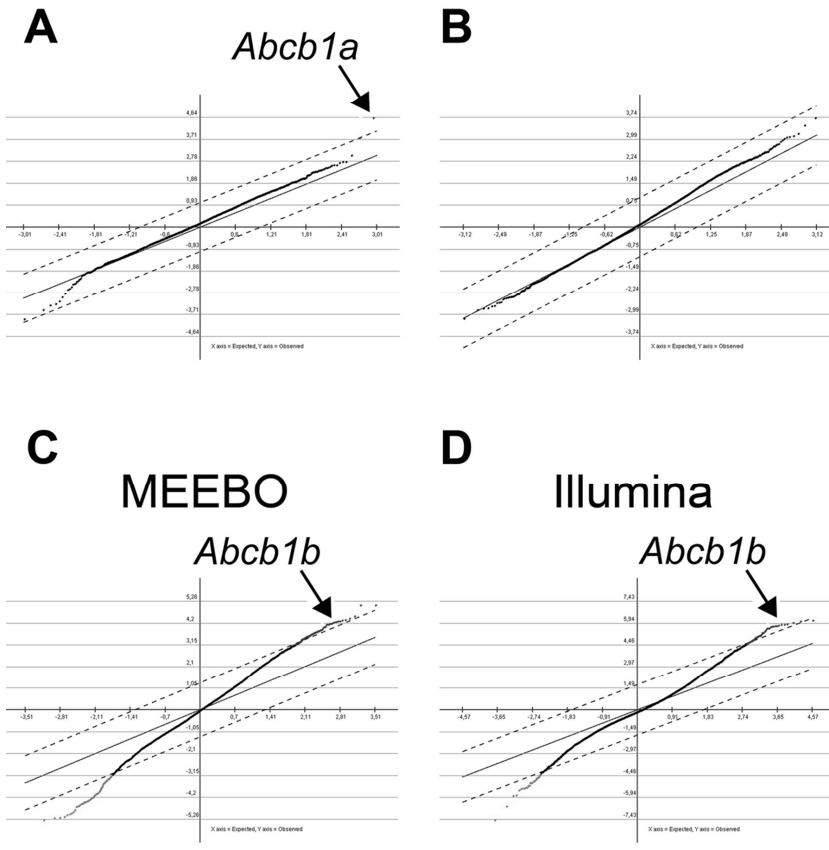




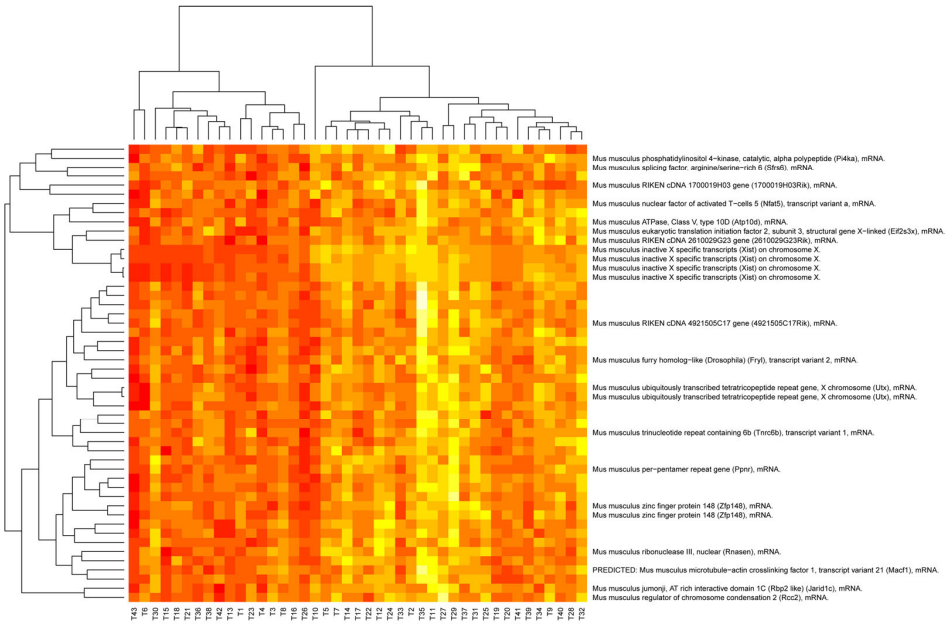
Supplementary Figure 2. Unsupervised hierarchical cluster analysis (average linkage) of gene expression data generated using the MEEBO or Illumina platform. Tumors with good docetaxel response (as defined in Figure 2A) are in black, those with a poor response in gray.



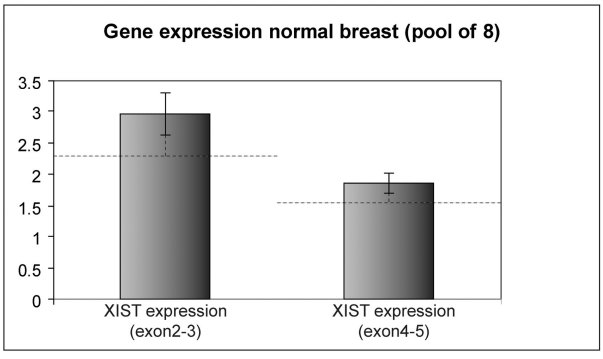
Supplementary Figure 3. (A) Unsupervised hierarchical cluster analysis of gene expression data of 15 tumors that were initially sensitive to docetaxel and eventually acquired resistance. MEEBO microarrays were used to analyze untreated tumors before treatment (sensitive, black) and the corresponding resistant (gray) tumors. (**B)** HE stainings of T20*con, T20*doce-res, T38*con and T38*doce-res carcinomas. For T20*con and T38*con dense lobules of undifferentiated polyploid tumor cells separated by a fine-vascular stroma can be seen. In contrast, T20*doce-res and T38*doce-res show islands of tumor cells (arrows) separated by a thick stroma (star) containing macrophages, collagen-producing fibroblasts, lymphocytes, plasma cells and some neutrophils. Bar represents 100mm.



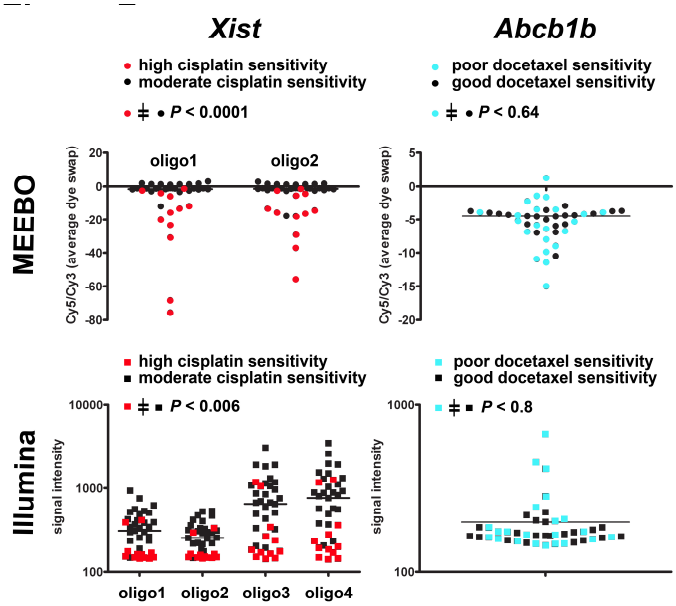
Supplementary Figure 4. SAM of docetaxel-resistant tumors with increased *Abcb1* **expression by RT-MLPA (see Figure 2E).** (**A)** Comparison of T18*con, T20*con, T22*con, T24*con, T31*con and T34*con versus T18*doce-sens, T20*doce-sens, T22*doce-sens, T24*doce-sens, T31*doce-sens and T34*doce-sens. Δ=1.0, FDR=0. (**B)** Comparison of T6*con, T18*con, T20*con, T22*con, T24*con, T28*con, T29*con, T31*con, T34*con and T38*con versus T6*doce-sens, T18*doce-sens, T20*doce-sens, T22*doce-sens, T24*doce-sens, T28*doce-sens, T29*doce-sens, T31*doce-sens T34*doce-sens and T38*doce-sens. Δ=1.0. (**C)** SAM (MEEBO platform) of T8*con, T9*con, T15*con, T26*con and T41*con versus the drug-naïve samples of the 21 docetaxel-sensitive controls (T2, T4, T5, T6, T12*con, T17*con, T18*con, T21*con, T22*con, T24*con, T25*con, T28*con, T29*con, T30*con, T31*con, T32*con, T34*con, T35*con, T38*con, T42*con, T43*con). Δ=1.3, number of significant genes=220, FDR=0.6%. (**D)** SAM of the same samples as shown in panel (**C)**, but analyzed on the Illumina platform. Δ=1.7, number of significant genes=91, FDR=0.9%.



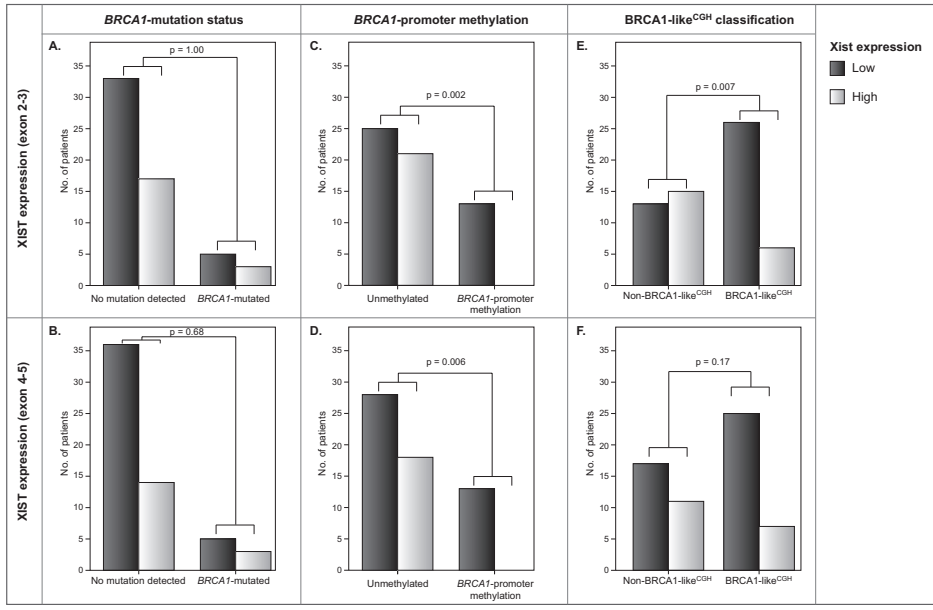
Supplementary Figure 5. Heatmap of the top 50 genes that correlate with the *Xist* expression pattern using the Illumina platform (r > 0.53).



Supplementary Figure 6. Quantification of the expression of the human *XIST* gene using two independent probes. RT-MLPA analyses of a pool of RNA isolated from FFPE normal breast tissue of 8 different patients. The XIST gene expression levels were normalized to the internal reference genes present in the RT-MLPA mix (*LDHA*, 2 probes for *GAPDH*, *B2M*, *ARHGDIA*, *FAU*, *OAZ1* and *BIRC2*). Depicted is the average gene expression of 8 independent measurements (one measurement is the average of 3 independent MLPA reactions); the error bars indicate the standard deviation of these 8 measurements; the dashed line represent two times the standard deviation and was used as the cut-off for further analyses.



Supplementary Figure 7. Distribution of gene expression levels of untreated KB1P mouse tumors using the probes for *Xist* or *Abcb1b* present on the MEEBO and Illumina platforms. *P* values were determined by the Kruskal-Wallis and post-hoc Mann-Whitney U tests (*Xist* probes, highest *P* value shown) or the Mann-Whitney U test only (*Abcb1b*).



Supplementary Figure 8. Association of Xist gene expression with BRCA1-status. RT-MLPA analyses were performed on FFPE RNA from patients included in the randomized controlled trial. The Xist gene expression levels were normalized to the internal reference genes present in the RT-MLPA mix (LDHA, 2 probes for GAPDH, B2M, ARHGDIA, FAU, OAZ1 and BIRC2). Xist gene expression levels were divided into high and low based on Xist gene expression in normal breast tissue (Figure S5.); this division was made on relative Xist gene expression levels which was the average of three independent MLPA reactions per patient. (**A + B**) Tumors of patients present in our study were previously screened for the presence of a BRCA1-mutation using a method that identifies ~73% of the types of BRCA1-mutations prevalent in the Netherlands were determined. BRCA1-mutated tumors and non-mutated tumors had equally low and high expressions of Xist when analyzed with Xist probe exon 2-3 (**A**) and Xist probe exon 4-5 (**B**). **(B + C)** BRCA1-promoter methylation data acquired by MS-MLPA analyses were available from the previous study. BRCA1-methylated tumors were highly associated with low Xist expression when analyzed with Xist probe exon 2-3 **(C)** and Xist probe exon 4-5 **(D)**. **(E+F)** Using an aCGH-classifier derived from BRCA1-mutated tumors (not discussed in this manuscript) tumors could be classified as BRCA1-likeCGH or Non-BRCA1-likeCGH based on their specific copy number aberrations present in the tumors. Tumors with a BRCA1-likeCGH tumor generally had a low Xist expression (26/32, 70% BRCA1-likeCGH tumors versus 13/28, 28% Non-BRCA1-likeCGH tumor for exon 2-3 **(E)**; respectively 25/32, 60% versus 17/11, 40% for exon 4-5 **(F)**)

Table S1, related to Figure 1. Overview of treatment responses of KB1P tumors.

	time until relapse			classification drug sensitivity		
no	docetaxel (d)	doxorubicin (d)	cisplatin (d)	docetaxel	cisplatin	morphology
T1	0	N/A	N/A	poor	N/A	undifferentiated carcinoma
T2	29	N/A	N/A	good	N/A	undifferentiated carcinoma
T3	0	N/A	N/A	poor	N/A	undifferentiated carcinoma
T4	17	N/A	N/A	good	N/A	undifferentiated carcinoma
T5	23	N/A	N/A	good	N/A	undifferentiated carcinoma
T6	25	N/A	N/A	good	N/A	undifferentiated carcinoma
T*7con	0	39	71	poor	high	undifferentiated carcinoma
T*8con	0	16	49	poor	high	undifferentiated carcinoma
T*9con	0	12	29	poor	moderate	undifferentiated carcinoma
T*10con	0	40	32	poor	moderate	undifferentiated carcinoma
T*11con	0	24	31	poor	moderate	undifferentiated carcinoma
T*12con	27	47	28	good	moderate	undifferentiated carcinoma
T*13con	0	28	72	poor	high	undifferentiated carcinoma
T*14con	0	31	30	poor	moderate	undifferentiated carcinoma
T*15con	0	0	84	poor	high	undifferentiated carcinoma
T*16con	0	45	32	poor	moderate	undifferentiated carcinoma
T*17con	30	57	35	good	moderate	undifferentiated carcinoma
T*18con	32	41	74	good	high	undifferentiated carcinoma
T*19con	0	28	32	poor	moderate	undifferentiated carcinoma
T*20con	22	29	23	good	moderate	undifferentiated carcinoma
T*21con	21	55	>100	good	high	undifferentiated carcinoma
T*22con	39	>100	39	good	moderate	undifferentiated carcinoma
T*23con	0	28	51	poor	high	undifferentiated carcinoma
T*24con	42	24	18	good	moderate	undifferentiated carcinoma
T*25con	0	35	33	poor	moderate	undifferentiated carcinoma
T*26con	0	0	14	poor	moderate	carcinosarcoma
T*27con	0	23	36	poor	moderate	undifferentiated carcinoma
T*28con	33	40	24	good	moderate	undifferentiated carcinoma
T*29con	11	31	N/A	good	N/A	undifferentiated carcinoma
T*30con	30	23	13	good	moderate	undifferentiated carcinoma
T*31con	52	N/A	N/A	good	N/A	undifferentiated carcinoma
T*32con	16	42	53	good	high	undifferentiated carcinoma
T*33con	0	26	20	poor	moderate	undifferentiated carcinoma
T*34con	29	33	26	good	moderate	undifferentiated carcinoma
T*35con	21	32	25	good	moderate	undifferentiated carcinoma
T*36con	0	33	>100	poor	high	undifferentiated carcinoma
T*37con	0	39	26	poor	moderate	undifferentiated carcinoma
T*38con	36	0	52	good	high	undifferentiated carcinoma
T*39con	0	0	16	poor	moderate	undifferentiated carcinoma
T*40con	0	24	35	poor	moderate	undifferentiated carcinoma
T*41con	0	0	29	poor	moderate	carcinosarcoma
T*42con	26	28	77	good	high	undifferentiated carcinoma
T*43con	13	18	73	good	high	undifferentiated carcinoma

Table S2. Summary of the TLDA data.

good docetaxel responders

Median Normalized cycle threshold values *-1	T2con	T4con	T5con	T6con	T*12con	T*17con	T*18con	T*20con	T*21con	T*22con	T*24con	T*28con	T*29con	T*30con	T*31con	T*32con	T*34con	T*35con	T*38con	T*42con	T*43con
Abca1-Mm00442646_m1	1,051306	3,137418	1,445298	1,096705	1,153979	1,546138	2,050434	2,939046	0,993671	1,931336	1,313312	1,910521	1,072666	1,622119	1,884148	4,256464	3,160832	2,03711	2,235042	0,972675	1,918664
Abca2-Mm00431553_m1	0,926542	1,796944	0,340588	-0,63886	0,066496	0,627594	-0,29795	0,766783	0,472936	-0,36454	0,371534	-0,07271	0,790393	1,071748	0,628783	1,367316	0,793652	0,292227	0,031491	0,698849	0,435293
Abca3-Mm00550501_m1	-0,46001	3,120449	0,994788	1,698455	0,01252	-0,06671	-0,00389	2,580139	0,276358	1,123921	-0,33945	0,808233	2,619228	0,844456	0,616747	0,50405	-0,07877	1,568777	1,647772	0,204999	1,635632
Abca4-Mm00492004_m1	N/A	N/A	-7,78049	-5,61766	-8,95324	N/A	-8,04739	-6,3152	N/A	N/A	N/A	-6,9832	-6,62928	N/A	-9,513	-7,63202	-6,76767	N/A	N/A	N/A	-7,183484
Abca5-Mm00461656_m1	-0,43322	-0,34447	-1,07053	-1,44718	-1,94109	-2,11489	-1,10831	-1,50226	-1,11992	-1,9886	-1,44139	-0,16557	-0,88047	-0,96434	-1,66036	-0,36678	0,427356	-1,024334	-0,726806	-0,876352	-0,788799
Abca6-Mm00461636_m1	N/A	-6,63799	-7,98542	-7,47378	N/A	-8,34282	N/A	-7,57926	N/A	-6,4173	-7,71446	-7,09403	-8,48497	-7,60207	-7,37051	-6,22011	-4,12952	N/A	-3,935873	-6,232926	N/A
Abca7-Mm00497010_m1	0,334731	2,953392	0,499307	-0,88789	1,33936	2,812068	2,718971	0,522044	1,445046	1,133247	1,461776	1,46111	5,04003	2,449415	1,069547	2,124799	2,101068	1,6444	0,493468	1,262469	1,306698
Abca8a-Mm00462440_m1	-4,95628	-7,67522	-5,13609	-7,1705	-4,83208	-3,29884	-5,4354	-4,97687	-6,32264	-4,32271	-5,09189	-2,96987	-6,85959	-4,47931	-3,66038	-2,09858	-0,28939	-4,759624	-2,266593	-2,920183	-0,777786
Abca8b-Mm00457361_m1	-4,37358	-6,35043	-5,16236	-2,86471	-4,92892	-5,63523	-6,89645	-5,17288	-3,57649	N/A	-4,83614	-5,05827	-7,1622	-3,77278	-6,75587	-1,73116	-2,31206	-6,304268	-1,972919	-2,801371	-2,933023
Abca9-Mm00461704_m1	-4,26069	-0,61556	-1,99442	-4,67113	-2,6795	-0,74834	-2,18097	-1,4072	-2,47433	-1,84238	-2,05672	-1,15933	-3,29313	-2,41719	-1,59991	-0,18142	-0,26515	-2,311742	-1,475444	-1,095089	-3,768595
Abca12-Mm00613683_m1	-3,88407	-7,88789	-4,541	-7,37978	-1,32794	-3,04079	-0,59173	-4,56899	-0,17127	-0,99096	-1,11763	0,532436	-7,75362	-3,67608	-3,58526	-0,91693	0,667988	-0,805479	-1,617175	-2,898354	-3,471657
Abca13-Mm00624342_m1	N/A	-4,037	-8,75388	-8,49331	-6,11285	N/A	N/A	-4,67861	-6,69607	-9,75311	-4,43221	-6,20203	N/A	N/A	N/A	-5,30647	-5,47754	-5,897206	-7,997599	-6,698227	N/A
Abcb1a-Mm00440761_m1	-5,79042	-3,1579	-3,55257	-5,83913	-4,77267	-3,5261	-4,29398	-4,00707	-3,27214	-3,94942	-5,32913	-2,94219	-4,86705	-4,07031	-4,22371	-2,17662	-2,87942	-5,105138	-3,584393	-2,939017	-4,732963
Abcb1b-Mm00440736_m1	-2,44447	-1,9428	-2,53155	-1,55023	-3,45203	-3,54172	-4,41479	-3,81086	-2,59468	-2,98551	-3,27507	-2,57718	-4,17761	-3,40921	-4,59148	-2,81269	-3,86284	-2,972974	-3,178347	-2,73502	-3,490291
Tap1-Mm00443188_m1	0,508935	3,851225	0,803635	0,800829	1,499785	1,455819	1,875762	2,131613	2,449244	2,807837	0,889583	1,694931	1,759965	0,662767	0,671334	2,892072	0,407889	1,666968	0,899893	2,408244	2,330677
Tap2-Mm00441668_m1	0,519771	3,483129	0,557861	0,031792	0,586206	1,676304	1,244994	1,027214	1,354666	1,693089	-0,27864	1,261545	1,509089	0,575981	0,312166	2,077067	-0,46464	0,098639	0,585032	1,941702	2,069535
Abcb4-Mm00435630_m1	N/A	-4,39885	N/A	-2,94208	-7,13784	-6,87149	-7,22515	-8,70564	-7,75212	N/A	-9,35219	-5,92619	N/A	-6,13992	N/A	-5,97517	-5,92312	N/A	-5,951954	-6,74737	-6,71209
Abcb6-Mm00470049_m1	-0,39651	-0,22869	0,05175	1,17155	-1,07846	-0,7238	-0,83967	-0,63952	-0,40096	0,112656	-0,7629	0,403854	-0,8238	0,042344	-0,67751	-0,17582	0,348088	-0,903636	0,508216	-0,796886	0,912147
Abcb8-Mm00472410_m1	-0,07999	0,654635	0,098363	0,264156	0,431407	0,07718	0,255076	-0,05251	0,006926	-0,7286	0,441271	-0,21369	0,050286	0,433854	0,032237	0,626754	0,225355	-0,530807	-0,285279	-0,094494	0,751034
Abcb9-Mm00498197_m1	-4,9891	-3,52911	-4,7108	-1,69232	-5,89719	-4,49397	-4,30912	-4,19338	-4,72549	-5,71881	-5,23429	-4,06494	-4,00288	-5,21497	-5,7261	-3,70662	-4,13324	-4,208156	-4,607163	-3,760018	-3,753041
Abcb10-Mm00497927_m1	0,774314	0,6809	-0,16099	0,550113	-0,19689	0,608995	0,755676	0,461817	0,91025	-0,05983	0,837104	0,273827	0,040667	0,817348	0,486808	0,608985	-0,05467	0,566807	0,645552	0,269697	0,657159
Abcc1-Mm00456156_m1	2,421147	2,42097	0,887515	1,00221	2,661649	1,967306	1,854198	0,264339	2,152044	1,757493	2,884622	1,831981	1,472239	1,36897	1,910705	2,035559	2,457951	2,326315	1,399458	2,239496	0,002731
Abcc2-Mm00496899_m1	N/A	-5,72953	-4,5978	-7,85586	N/A	N/A	N/A	N/A	N/A	N/A	-8,26572	N/A	N/A	-9,09228	N/A	-8,50881	N/A	N/A	-8,084723	N/A	N/A
Abcc3-Mm00551550_m1	-0,93837	0,113262	-2,34959	-2,58563	-2,46922	0,042989	-1,52544	-2,32937	-1,4096	-1,7937	-2,25963	-1,141	-0,84468	-1,90893	-0,05184	0,393531	-1,26212	-1,815037	-2,118095	-0,316129	-0,737452
Abcc5-Mm00443360_m1	0,879996	-0,73695	-0,72885	0,373278	-1,40134	-0,27368	0,571392	-0,86285	-0,50906	-1,37727	-0,05309	-0,60209	0,135628	-0,64154	-0,52825	-0,94326	-0,79445	-0,627163	-0,780772	0,014601	-1,115853
Abcc6-Mm00497685_m1	N/A	-5,78488	N/A	N/A	N/A	N/A	-6,23167	N/A	-5,95812	N/A	N/A	N/A	N/A	N/A	-7,03494	N/A	N/A	N/A	-7,576392	-4,995294	N/A
Cftr-Mm00445197_m1	-5,27855	0,89602	-6,30222	-3,08959	-0,85162	-0,83469	-4,25309	-2,38219	-1,88961	-2,14326	-1,61838	-2,05555	-7,17314	-4,23126	-5,29107	-2,20446	-0,86268	-1,975967	-5,147027	-1,923758	-5,367772
Abcc8-Mm00803450_m1	N/A	N/A	N/A	N/A	N/A	-8,37212	-5,29265	N/A	-3,97978	N/A	-7,49426	-3,48917	-1,20169	-6,45284	-6,06836	-1,05532	N/A	N/A	-6,260771	-0,917177	N/A
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Abcc10-Mm00467403_m1	0,774314	0,6809	-0,16099	0,550113	-0,19689	0,608995	0,755676	0,461817	0,91025	-0,05983	0,837104	0,273827	0,040667	0,817348	0,486808	0,608985	-0,05467	0,566807	0,645552	0,269697	0,657159
Abcc12-Mm00556685_m1	-2,59126	-8,86748	-7,03421	-10,0189	-3,12634	-5,14392	-4,62015	-5,43759	-2,47136	-2,90377	-3,02285	-3,27462	-3,40238	-4,67385	-6,73701	-5,08318	-4,87751	-5,841203	-1,559323	-7,412171	-6,083143
Abcd1-Mm00431749_m1	1,075191	2,328355	1,813214	-0,73373	0,568669	0,84353	0,663115	1,244402	1,014365	0,011903	0,728194	0,765011	1,58274	0,880873	0,657815	1,590264	0,002529	0,302168	1,813246	1,19076	1,816734
Abcd2-Mm00496455_m1	-6,35311	-1,82539	-4,10563	-4,83672	-5,38813	-3,39938	-4,27168	-4,54521	-5,29656	-4,12563	-4,15425	-2,62586	-5,51836	-4,4151	-4,95079	-2,94773	-2,47773	-4,538154	-3,553994	-3,576766	-3,723356
Abcd3-Mm00436150_m1	1,344432	3,214703	1,769688	2,615036	1,671986	1,608338	1,837035	2,487002	2,222484	1,761518	2,026577	2,347863	1,276947	1,406377	1,04336	2,603034	2,256697	1,710587	2,179772	2,367294	3,294041
Abcd4-Mm00436180_m1	-0,88072	-0,60852	-0,31314	0,466141	-0,55808	-1,5814	-1,27005	-0,23894	-1,09544	0,414837	-0,01325	-0,07073	-0,46802	-0,43665	-0,46655	-0,46975	-0,25808	-0,44561	-0,612776	-0,771241	-0,103943
Abce1-Mm00649858_m1	2,911753	3,373748	2,077256	4,265078	3,483063	2,281492	2,80367	2,092795	3,032773	2,326144	3,187584	2,662189	1,572072	3,492544	1,476711	2,563668	5,181653	2,52456	2,285664	2,6598	2,581712
Abcf2-Mm00457400_g1	0,917353	1,901733	1,889917	2,735961	1,862687	1,069854	1,420221	1,664081	1,7586	1,036152	1,96458	1,586508	0,828731	1,853115	0,850014	1,878966	2,531821	0,768611	0,659727	0,639935	1,756328
Abcf3-Mm00658695_m1	0,588335	-0,03617	-0,42159	1,009074	0,26619	-0,87863	0,480998	0,474976	0,258644	0,007254	0,818534	0,360651	-0,55608	-0,37846	-0,19588	0,219771	0,003906	0,155338	-0,582376	0,102152	-0,127022
Abcg1-Mm00437390_m1	0,891421	3,022233	2,29669	0,380045	1,4892	1,693885	0,787265	3,019162	1,252686	2,199541	1,12751	2,152766	0,835937	0,482813	1,170831	3,349148	1,09451	2,215939	1,865742	1,507938	2,009647
Abcg2-Mm00496364_m1	-1,99469	-0,54236	1,48771	2,070874	-0,29319	-2,1341	-3,51867	0,437043	-0,66541	-0,56663	-0,58915	-0,86619	-2,304	-0,58727	-2,88626	-2,14867	-0,22775	-1,896791	0,268486	-1,273979	-1,471103
Abcg3-Mm00446072_m1	-5,42548	-4,92587	-4,44224	-6,68361	-4,95675	-2,00212	-3,64743	-3,98971	-2,81185	-3,72669	-4,04626	-3,32184	-4,40059	-5,29823	-2,734	-2,63606	-1,93696	-5,063552	-4,860498	-2,844577	-4,295092
Abcg4-Mm00507250_m1	-2,44512	-6,91927	-4,13875	-5,05352	-5,43475	-6,59352	-5,57223	-7,28264	-5,1657	-8,54765	-3,75345	-5,68406	-3,47848	-6,79404	-7,25369	-5,31981	-7,13783	-5,167888	-5,666431	-5,429438	-8,017371
Abcg5-Mm00446249_m1	N/A	-7,35849	-5,59845	-5,21231	N/A	N/A	-3,28056	-9,39542	-5,37983	-7,15011		-5,42043	-2,74837	-4,4548	-6,45352	-7,31942	-4,86901	N/A	-8,717291	N/A	-5,769369
Abcb7-Mm01235258_m1	0,853394	2,1587	1,436411	0,782331	1,277517	0,088407	0,809541	0,902305	0,92468	0,258004	1,110126	0,540918	0,12226	1,201957	0,474965	1,653279	2,932692	0,096709	1,035546	0,682436	1,744428
Abcc4-Mm01226380_m1	-0,16543	-0,59488	-2,64321	-1,7159	0,123422	0,494403	-0,00713	-0,07939	-0,51981	1,397962	0,025022	-1,77943	0,264193	-1,51676	-1,06815	-0,05578	-1,11888	0,223425	-1,456449	-0,521894	-0,536125
Abcf1-Mm01275245_m1	2,783458	3,148457	2,644323	3,509463	2,964563	2,525274	2,240664	2,191724	2,581581	2,074206	2,792036	2,530767	1,816643	2,676645	1,825955	2,649924	3,066194	2,511285	2,040652	2,538693	3,05131

poor docetaxel responders

Median Normalized cycle threshold values *-1	T1con	T3con	T*7con	T*8con	T*9con	T*10con	T*11con	T*13con	T*14con	T*15con	T*16con	T*19con	T*23con	T*25con	T*26con	T*27con	T*33con	T*36con	T*37con	T*39con	T*40con	T*41con	average good docetaxel response	average poor docetaxel response	*P* value (Mann Whitney U test)
Abca1-Mm00442646_m1	0,996779	2,043997	2,72533	1,341243	1,320006	1,39287	2,569683	1,874227	1,380667	2,695654	1,26713	0,651993	2,985465	2,22694	2,755381	2,794633	1,837323	0,823965	2,333711	1,473469	2,150344	3,54694	1,891851611	1,963079477	0,6357
Abca2-Mm00431553_m1	0,279259	0,595549	0,556881	1,030322	0,490244	1,370085	1,113449	0,678738	0,560893	1,061451	-0,00829	0,320358	2,058704	1,364342	-0,4497	1,030195	-0,49284	1,047143	0,870949	-0,14087	0,783505	0,204447	0,481196151	0,651128114	0,3752
Abca3-Mm00550501_m1	3,164605	0,38932	1,50567	0,931012	-0,55055	2,138662	0,299454	0,363441	0,359788	1,399145	0,043222	0,387264	3,160699	1,251761	0,585378	1,302939	3,445356	1,049222	0,183364	0,728018	0,43886	1,200815	0,919414008	1,080792674	0,568
Abca4-Mm00492004_m1	-10,08024	-8,05453	N/A	N/A	-2,0029	N/A	-7,94121	-8,72437	-8,65461	-7,22572	N/A	N/A	-8,49862	-7,63323	-5,82057	-3,44443	-7,72043	-4,76272	-7,14612	-8,91874	N/A	-5,55745	-7,402057591	-7,011618656	0,941
Abca5-Mm00461656_m1	-1,014864	0,139604	-1,464784	-0,943627	0,097405	-0,29873	-1,0806	-0,6002	-0,53892	0,886001	-0,11904	-1,26878	-2,27382	-0,12125	1,17411	-0,49748	-0,14327	-2,70423	-0,96742	0,310779	-0,43115	1,035733	-1,025633786	-0,492024523	0,0279
Abca6-Mm00461636_m1	N/A	-4,836547	N/A	-8,221748	-2,62743	-7,68726	-6,58699	-6,83839	-7,33721	-4,53856	-6,84908	N/A	-7,82672	-6,7099	-3,313	-6,09005	-7,30417	-7,84978	-6,19312	-6,83118	-4,68614	-4,11343	-6,881402056	-6,128458096	0,1653
Abca7-Mm00497010_m1	0,796114	2,429373	2,430903	2,035166	0,049211	1,788552	2,39744	0,930488	0,509563	1,256591	1,723964	1,726909	1,906424	1,45488	1,864372	2,672011	0,085185	-0,14316	1,107791	1,302689	0,466935	1,810134	1,585002802	1,390979083	0,6885
Abca8a-Mm00462440_m1	-4,211473	-2,674458	-4,515391	-4,093325	0,105245	-4,19754	-4,07443	-3,55154	-3,70622	-3,59883	-4,01126	-4,76484	-7,30776	-3,20468	-0,4094	-2,68301	-4,08455	-5,13448	-4,43755	-3,93055	-1,08625	-2,45073	-4,299992262	-3,546500038	0,1062
Abca8b-Mm00457361_m1	-5,158636	-3,66376	-6,610755	-5,817837	-2,17536	-4,121	-5,10216	-4,50823	-3,9869	-2,27577	-2,18171	-3,99308	-5,00257	-2,68806	-1,72459	-4,40415	-4,78957	-6,91598	-6,43379	-4,43994	-2,94303	-3,41811	-4,530055308	-4,197953553	0,4729
Abca9-Mm00461704_m1	-1,760681	0,010127	-0,41447	-0,576803	0,1188	-2,60503	-0,57982	-1,09209	-2,07319	-1,97578	-2,50759	-4,75093	-2,49017	-2,33651	0,212611	-0,76927	-1,08718	-2,48758	-0,77554	-2,12204	-0,11244	0,718109	-2,023725611	-1,33897525	0,1062
Abca12-Mm00613683_m1	-7,992327	-2,291567	-3,114	-1,364647	0,639366	-1,49582	-2,33808	-2,39997	-3,1847	-7,87274	-2,13655	-5,86722	-5,69967	-0,55121	-7,81563	-3,4597	-3,83449	-6,15535	-4,88688	0,061066	1,259761	-5,977	-2,810770437	-3,476244326	0,4019
Abca13-Mm00624342_m1	N/A	-6,395517	N/A	N/A	N/A	N/A	-7,98255	N/A	-7,3796	-8,45342	-5,11385	N/A	-4,44948	-7,41121	-8,89595	-7,62133	N/A	N/A	N/A	-7,67114	N/A	N/A	-6,46686606	-7,137404683	0,1444
Abcb1a-Mm00440761_m1	-4,646652	-2,987668	-3,077992	-3,961842	-1,43469	-4,35926	-3,36292	-3,18015	-4,19894	-2,07993	-4,83371	-6,52197	-5,13614	-3,37112	-0,05824	-4,27054	-3,92793	-3,76004	-3,40595	-4,63593	-2,60548	-0,27352	-4,048159627	-3,458663826	0,2387
Abcb1b-Mm00440736_m1	-4,466453	-1,774492	-2,198287	-2,694298	-2,00346	-4,48017	-4,0514	-3,4959	-3,42012	0,028682	-3,46672	-4,77483	-3,59523	-4,58069	0,333954	-4,24075	-1,65839	-4,16051	-3,65203	-1,84166	-2,06418	-0,20951	-3,15958804	-2,839383023	0,8937
Tap1-Mm00443188_m1	1,290569	1,268035	3,148451	2,881394	0,390874	2,535463	1,730971	2,418007	1,0961	-0,40445	0,930496	-0,0155	2,725952	0,786869	-0,07002	2,304624	2,932692	0,663845	2,097856	0,876794	0,958499	0,449455	1,641381214	1,408953386	0,568
Tap2-Mm00441668_m1	0,985794	0,457428	2,794789	2,579735	0,531587	-3,05621	2,113129	2,030221	0,674899	-1,25004	0,665889	0,459869	1,689289	0,262662	-0,14773	1,631842	1,829359	-0,02689	1,757847	0,030815	0,362783	0,061903	1,041071278	0,747226083	0,568
Abcb4-Mm00435630_m1	N/A	-7,232788	-4,566971	-4,32597	-7,25169	N/A	-6,22054	N/A	N/A	-3,66672	-8,45294	N/A	N/A	-6,72177	-3,5204	N/A	-5,96719	-9,90208	N/A	-7,6132	-4,14508	-3,63025	-6,517411178	-5,944113726	0,5557
Abcb6-Mm00470049_m1	0,532031	0,489714	0,585198	0,085674	-2,36942	-1,75669	-1,16163	-0,19107	-0,01201	1,358599	0,167862	-0,4278	-0,42181	0,632964	-0,76628	0,195918	0,636324	0,757948	-0,24114	0,224524	-0,50273	-1,89913	-0,233217246	-0,185589341	0,4157
Abcb8-Mm00472410_m1	-0,368223	-0,215936	-0,005463	-0,200532	-1,13437	-1,87017	0,369309	0,268717	-0,22689	1,116672	0,303086	0,001205	0,588277	0,351613	-0,39056	0,60598	0,022229	1,202723	-0,04941	-0,49586	-0,11129	-0,77589	0,112531992	-0,046126492	0,2906
Abcb9-Mm00498197_m1	-4,793565	-4,852083	-4,079382	-4,52705	-4,10475	-4,94009	-3,95401	-4,22922	-3,595	-3,76486	-5,91706	-4,14615	-4,50965	-3,52977	-3,78621	-1,9002	-4,6877	-3,94709	-4,31781	-4,23726	-2,10438	-3,74188	-4,412891167	-4,075689523	0,269
Abcb10-Mm00497927_m1	0,093226	0,257919	0,450988	0,155085	-0,30151	1,272454	-0,19606	0,809875	1,182174	0,628149	0,239291	0,911297	0,026281	-0,25601	-0,33477	0,901017	1,269252	1,633045	1,408716	-0,07459	0,171205	-0,62605	0,451125421	0,437317871	0,7246
Abcc1-Mm00456156_m1	0,710537	1,496921	2,162126	2,359935	1,544211	1,056011	2,194167	1,899886	2,157295	1,87298	1,394966	1,604027	2,322937	0,411716	2,373996	0,873491	1,804242	2,410973	1,980362	2,42502	2,181796	2,310339	1,777090341	1,797633432	0,9516
Abcc2-Mm00496899_m1	-5,146976	-7,377042	N/A	N/A	N/A	-4,94775	N/A	N/A	N/A	N/A	-9,11968	-8,17844	N/A	N/A	N/A	N/A	-6,09739	N/A	N/A	N/A	N/A	-5,68057	-7,447818595	-6,649691381	0,4557
Abcc3-Mm00551550_m1	-1,884762	-0,453605	-1,1144	-1,639902	-2,2427	-0,95192	-0,38303	-1,12687	-1,83933	-2,65715	-1,64647	-2,81113	-0,22607	-1,93764	-0,71073	-1,00788	-1,64562	-2,42638	-0,80998	-1,55625	-1,42657	0,019162	-1,300287992	-1,385419538	0,8554
Abcc5-Mm00443360_m1	-3,062764	-1,026488	-0,519689	-0,000711	0,936241	-5,81862	-0,29382	-0,06582	0,09883	0,076756	-1,24953	-1,99935	-2,08763	-0,34886	0,188838	-0,5402	-0,33791	-0,52829	-0,07939	-0,31794	0,440203	0,029426	-0,476264929	-0,750305735	0,6014
Abcc6-Mm00497685_m1	N/A	N/A	N/A	-6,032108	-8,89777	N/A	-4,16012	-5,52394	-7,6662	-6,477	N/A	N/A	N/A	N/A	N/A	N/A	-7,55459	N/A	N/A	N/A	N/A	-7,00844	-6,263549056	-6,665021417	0,3954
Cftr-Mm00445197_m1	-8,050866	-6,112087	-0,437775	-2,764482	-3,89748	-2,46455	-3,9583	-3,51154	-0,99484	-4,95659	-1,69607	-5,62052	-3,99257	-1,28742	-3,98302	-3,21959	-4,11583	-1,2471	-6,05575	-2,09108	-1,11491	-4,12852	-3,046660325	-3,440949326	0,6184
Abcc8-Mm00803450_m1	N/A	-7,641426	-5,030312	-5,83309	-2,99642	N/A	-5,60656	-1,84048	N/A	N/A	-0,75536	N/A	N/A	-8,3485	N/A	-3,48365	N/A	N/A	-7,96012	-3,12544	-4,2526	N/A	-4,598556864	-4,739496042	0,9264
Abcc9-Mm00441638_m1	1,028408	-0,793571	-0,104583	1,345874	0,047303	0,630514	0,462192	0,045672	-0,21071	0,536809	0,115088	0,278203	-0,59115	-0,50586	0,954564	1,35695	0,804606	0,087233	-0,09411	-0,24912	1,42572	1,138021	0,070617786	0,350366129	0,2021
Abcc10-Mm00467403_m1	0,093226	0,257919	0,450988	0,155085	-0,30151	1,272454	-0,19606	0,809875	1,182174	0,628149	0,239291	0,911297	0,026281	-0,25601	-0,33477	0,901017	1,269252	1,633045	1,408716	-0,07459	0,171205	-0,62605	0,451125421	0,437317871	0,7246
Abcc12-Mm00556685_m1	-6,870887	-2,460981	-5,198594	-3,340783	-3,60458	-6,561	-4,00757	-3,96917	-3,17666	-1,62244	-4,81107	-6,61588	-6,31171	-6,44289	N/A	-3,07946	-4,90527	-1,48457	-3,95693	-4,51158	-3,61297	-6,45883	-4,961056214	-4,428753905	0,5973
Abcd1-Mm00431749_m1	0,698244	1,234249	1,479112	1,431522	0,662357	2,130523	0,867214	0,737502	1,407553	1,26344	-0,66033	0,336366	2,045409	1,393771	1,318765	2,047155	1,60023	1,294815	1,620273	0,169628	2,528489	1,691227	0,959968786	1,24079622	0,1552
Abcd2-Mm00496455_m1	-3,585309	-2,204526	-3,300257	-3,559032	-1,01866	-2,85697	-3,94609	-4,01009	-3,92146	-1,52411	-4,44475	-5,84317	-6,97364	-5,35809	0,084084	-5,07073	-2,53492	-4,10551	-3,31088	-3,74777	-2,29903	-1,10888	-4,125216246	-3,392717114	0,0741
Abcd3-Mm00436150_m1	2,439898	1,49592	2,599972	2,170271	1,205702	2,420024	1,62925	2,164777	2,888793	2,350415	2,33958	1,868507	2,528189	1,502466	1,920972	2,388223	2,938608	2,701625	1,101662	1,689685	2,951278	2,089896	2,049750992	2,153896053	0,4735
Abcd4-Mm00436180_m1	-0,65024	-0,186845	-0,609382	-0,791371	-0,47987	0,033594	0,146302	-0,0275	-1,03577	0,296555	0,172215	-0,00217	-0,08275	-0,31938	1,575203	-1,04069	-0,08894	-0,22427	-0,4958	-1,23248	-0,66678	0,937339	-0,465806135	-0,216955856	0,2586
Abce1-Mm00649858_m1	1,606159	2,25588	2,656546	2,848788	1,686817	3,244722	1,927433	1,594572	2,855642	2,278204	2,702052	2,311212	2,504334	2,805746	2,828006	2,942538	3,165742	2,095105	1,908817	2,714452	1,635735	2,919427	2,801710786	2,431269417	0,2387
Abcf2-Mm00457400_g1	7,062225	1,690108	1,613456	1,386915	0,614204	2,891243	0,952449	1,060973	1,063814	2,06204	1,71226	1,018639	2,674861	2,302234	1,864751	1,677823	1,587181	1,719342	1,07382	1,249731	1,163063	0,978234	1,503566437	1,791789229	0,7799
Abcf3-Mm00658695_m1	-0,257028	-0,588971	-0,032476	0,077839	-0,2508	0,796292	-0,10639	0,112001	0,540851	-0,01234	-0,38855	-0,25493	-0,08408	-0,04406	0,14625	-0,28379	0,06872	-0,24962	-0,00488	0,041545	0,242897	-0,09952	0,074743452	-0,028683614	0,2586
Abcg1-Mm00437390_m1	2,861426	1,919831	1,838475	0,490395	0,585738	3,72684	1,289361	0,772366	1,646935	1,477232	0,987413	1,93557	2,632652	1,52324	2,105253	2,119464	2,493033	1,617548	1,175626	1,068582	1,820712	1,827289	1,659281452	1,723408174	0,8364
Abcg2-Mm00496364_m1	2,449467	0,230992	-1,634547	-1,154165	0,024619	1,199518	-1,89896	-0,0128	-1,09297	-0,88169	-2,04854	-1,61388	-1,08883	-0,78683	-0,10911	-1,19877	-0,6981	0,491935	-1,4631	-2,17256	-0,44	-0,38155	-0,938195817	-0,649085038	0,5197
Abcg3-Mm00446072_m1	-5,445654	-0,625836	-2,108702	-2,42981	-4,47694	-4,70401	-1,99899	-3,06405	-4,30746	-3,96205	-5,58365	-6,01946	-5,13902	-4,80789	-2,39359	-4,32503	-4,25164	-4,50858	-2,24732	-4,49573	-3,95297	-1,37363	-4,002352611	-3,737363659	0,7246
Abcg4-Mm00507250_m1	-5,785092	-6,045265	-6,021838	-4,468993	-5,45114	-4,30536	-5,46904	-6,58125	-5,64394	-4,16141	-6,88378	-7,02292	-4,93451	-4,92771	-6,46795	-3,2833	-5,6604	-3,72929	-6,05205	-5,91087	-3,79591	-7,19349	-5,755031286	-5,445249871	0,568
Abcg5-Mm00446249_m1	-9,078412	-8,496801	N/A	-4,451976	N/A	-4,51353	-6,1152	-5,4708	N/A	-3,20197	-6,56959	N/A	-8,52	N/A	N/A	-3,78184	N/A	-1,20477	-7,2339	N/A	N/A	N/A	-5,941825611	-5,719898722	0,8262
Abcb7-Mm01235258_m1	0,15496	0,835697	1,501006	1,083352	0,091078	2,074657	1,977048	0,420754	1,145929	0,930783	0,32459	0,408948	-0,20881	0,837835	-0,13268	1,022148	1,417078	1,020851	0,880935	0,495911	1,89712	-0,17653	1,00412404	0,818303098	0,4887
Abcc4-Mm01226380_m1	-4,004455	-0,184514	-0,052782	-0,689475	-2,52584	-0,59928	1,271524	-1,19257	0,191883	-1,34967	0,151259	-0,4329	1,282021	-1,30379	-0,75804	-2,24227	-1,01615	-2,23586	2,308105	-0,51063	-2,23258	-1,59757	-0,53575231	-0,805617886	0,3496
Abcf1-Mm01275245_m1	1,910451	1,937094	2,823488	2,671019	1,491351	3,589945	2,638529	2,174454	2,573885	2,381491	3,179615	2,164712	2,788697	2,911408	1,668125	2,695298	3,518884	3,011513	1,649735	2,133829	2,108193	1,990599	2,579229325	2,455105129	0,4887

Supplementary Table 3.

	description	primary Accession	refseq	Expected score (dExp)	Observed score(d)	Numerator(r)	Denominator (s+s0)	Fold change (Unlogged)
1	guanine nucleotide binding protein (G protein), gamma 10	mMR029692	NM_025277	0.7424547	4.3159986	0.6756037	0.15653473	1.6132612
2	glycoprotein 49 A	mMR031223	NM_008147	0.9148826	4.0678153	1.1747769	0.288798	2.516413
3	lysozyme	mMR030807	NM_017372	0.86525834	3.9086351	1.2845502	0.3286442	2.7509046
4	integrin beta 2	mMC006392	NM_008404	-0.81892484	3.9084783	1.1454594	0.29307044	2.0792363
5	P lysozyme structural	mMR027404	NM_013590	0.53708607	3.8952055	1.1643862	0.29892805	2.4603899
6	triggering receptor expressed on myeloid cells 2	mMC000876	NM_031254	-1.7120212	3.8331485	0.83543503	0.21795009	1.8520628
7	leukocyte immunoglobulin-like receptor, subfamily B, member 4	mMR030747	NM_013532	0.8571193	3.8218963	1.05341	0.27562496	2.2524378
8	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	mMC020918	NM_013612	0.09594909	3.7005484	0.6877517	0.18585129	1.6330663
9	lysozyme	mMR028073	NM_017372	0.5997738	3.6986074	1.1160176	0.30173993	2.4080336
10	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	mMC005807	NM_011075	-0.8773627	3.6660628	1.5410365	0.4203519	4.7408605

Supplementary Table 4. Top 50 outlier genes of poor docetaxel responding tumors using the Illumina gene expression data.

	probe	refseq	symbol
1	1813125	NM_008546.2	Mus musculus microfibrillar-associated protein 2 (Mfap2), mRNA.
2	1813124	NM_008546.2	Mus musculus microfibrillar-associated protein 2 (Mfap2), mRNA.
3	1805820	NM_054049.2	Mus musculus odd-skipped related 2 (Drosophila) (Osr2), mRNA.
4	1788439	NM_007802.3|NM_007802.2	Mus musculus cathepsin K (Ctsk), mRNA.
5	1781201	XM_001474270.1	PREDICTED: Mus musculus similar to reproductive homeobox on X chromosome 2, transcript variant 1 (LOC100040016), mRNA.
6	1800067	NM_010052.4	Mus musculus delta-like 1 homolog (Drosophila) (Dlk1), mRNA.
7	1767873	NM_145592.2|NM_145592.1	Mus musculus dickkopf homolog 4 (Xenopus laevis) (Dkk4), mRNA.
8	1767194	NM_153529.1	Mus musculus neuritin 1 (Nrn1), mRNA.
9	1818847	NM_023456.2	Mus musculus neuropeptide Y (Npy), mRNA.
10	1788103	NM_011058.2|NM_011058.1	Mus musculus platelet derived growth factor receptor, alpha polypeptide (Pdgfra), transcript variant 1, mRNA.
11	1792994	NM_183171.1	Mus musculus fasciculation and elongation protein zeta 1 (zygin I) (Fez1), mRNA.
12	1820425	NM_008002.3	Mus musculus fibroblast growth factor 10 (Fgf10), mRNA.
13	1820424	NM_008002.3	Mus musculus fibroblast growth factor 10 (Fgf10), mRNA.
14	1770441	NM_011333.3|NM_011333.1	Mus musculus chemokine (C-C motif) ligand 2 (Ccl2), mRNA.
15	1813126	NM_008546.2	Mus musculus microfibrillar-associated protein 2 (Mfap2), mRNA.
16	1811789	NM_183171.1	Mus musculus fasciculation and elongation protein zeta 1 (zygin I) (Fez1), mRNA.
17	1790432	NM_029861.2	Mus musculus RIKEN cDNA 1500041B16 gene (1500041B16Rik), mRNA.
18	1763846	NM_001043335.1| XM_127139.5	Mus musculus echinoderm microtubule associated protein like 1 (Eml1), transcript variant 1, mRNA.
19	1800066	NM_010052.4	Mus musculus delta-like 1 homolog (Drosophila) (Dlk1), mRNA.
20	1808766	NM_010465.2|NM_010465.1	Mus musculus homeo box C6 (Hoxc6), mRNA.
21	1815089	NM_054049.1	Mus musculus odd-skipped related 2 (Drosophila) (Osr2), mRNA.
22	1811480	NM_007855.1	
23	1815503	NM_013654.2	Mus musculus chemokine (C-C motif) ligand 7 (Ccl7), mRNA.
24	1808410	NM_008516.2|NM_008516.1	Mus musculus leucine rich repeat protein 1, neuronal (Lrrn1), mRNA.
25	1781273	NM_028122.3	Mus musculus solute carrier family 14 (urea transporter), member 1 (Slc14a1), mRNA.

Supplementary Table 4. Top 50 outlier genes of poor docetaxel responding tumors using the Illumina gene expression data.

	probe	refseq	symbol
26	1822062	NM_133237.2	Mus musculus adenomatosis polyposis coli down-regulated 1 (Apcdd1), mRNA.
27	1784269	NM_028386.1	Mus musculus aspartate beta-hydroxylase domain containing 2 (Asphd2), mRNA.
28	1781939		
29	1806476	NM_010356.3|NM_010356.2	Mus musculus glutathione S-transferase, alpha 3 (Gsta3), transcript variant 2, mRNA.
30	1793331	NM_008409.2|NM_008409.1	Mus musculus integral membrane protein 2A (Itm2a), mRNA.
31	1818098	NM_001081669.1	Mus musculus predicted gene, EG434760 (EG434760), mRNA.
32	1773194	NM_008344.2|NM_008344.1	Mus musculus insulin-like growth factor binding protein 6 (Igfbp6), mRNA.
33	1778490	NM_009382.3|NM_009382.2	Mus musculus thymus cell antigen 1, theta (Thy1), mRNA.
34	1813583	NM_010577.2	Mus musculus integrin alpha 5 (fibronectin receptor alpha) (Itga5), mRNA.
35	1800364	NM_015734.1	Mus musculus procollagen, type V, alpha 1 (Col5a1), mRNA.
36	1821236	NM_011075.1	Mus musculus ATP-binding cassette, sub-family B (MDR/TAP), member 1B (Abcb1b), mRNA.
37	1809451	NM_007833.4|NM_007833.1	Mus musculus decorin (Dcn), mRNA.
38	1812062	NM_007542.3	Mus musculus biglycan (Bgn), mRNA.
39	1799173	NM_010729.2	Mus musculus lysyl oxidase-like 1 (Loxl1), mRNA.
40	1771878	XM_001476984.1| NM_024217.2	PREDICTED: Mus musculus similar to CKLF-like MARVEL transmembrane domain containing 3 (LOC100046883), mRNA.
41	1774745		
42	1803582	NM_010430.2|NM_010430.1	Mus musculus hypermethylated in cancer 1 (Hic1), transcript variant 1, mRNA.
43	1790878	NM_011340.3|NM_011340.2	Mus musculus serine (or cysteine) peptidase inhibitor, clade F, member 1 (Serpinf1), mRNA.
44	1787082	NM_009317.3|NM_009317.2	Mus musculus T-cell acute lymphocytic leukemia 2 (Tal2), mRNA.
45	1804003	NM_024263.3	Mus musculus matrix-remodelling associated 8 (Mxra8), mRNA.
46	1773254	NM_175686.3|NM_011127.1	Mus musculus paired related homeobox 1 (Prrx1), transcript variant 2, mRNA.
47	1765670	XM_001475459.1| NM_010151.1	PREDICTED: Mus musculus similar to COUP-TFI (LOC100046044), mRNA.
48	1811383	NM_015814.2	Mus musculus dickkopf homolog 3 (Xenopus laevis) (Dkk3), mRNA.
49	1788086	NM_026835.2|NM_026835.1	Mus musculus membrane-spanning 4-domains, subfamily A, member 6D (Ms4a6d), mRNA.
50	1776647	NM_212485.2	Mus musculus keratin 73 (Krt73), mRNA.

Supplementary Table 5A. Patient characteristics distributed by *XIST* expression.

	***XIST* expression (exon 2-3)**					***XIST* expression (exon 4-5)**						
	Low		**High**		**p**	**Low**		**High**		**p**	**Total**	
Variable	**n**	**%**	**n**	**%**	**values***	**n**	**%**	**n**	**%**	**values***	**n**	**%**
Total	**39**	**65.0**	**21**	**35.0**		**42**	**70.0**	**18**	**30.0**		**60**	**100.0**
Treatment												
Conventional Chemotherapy	19	48.7	12	57.1	0.60	21	50.0	10	55.6	0.78	31	51.7
IPB chemotherapy	20	51.3	9	42.9		21	50.0	8	44.4		29	48.3
Age in categories												
≤ 40 years	19	48.7	7	33.3	0.29	19	45.2	7	38.9	0.78	26	43.3
> 40 years	20	51.3	14	66.7		23	54.8	11	61.1		34	56.7
Type of surgery												
Breast conserving therapy	10	25.6	6	28.6	1.00	11	26.2	5	27.8	1.00	16	26.7
Mastectomy	29	74.4	15	71.4		31	73.8	13	72.2		44	73.3
Tumour classification												
T1 or T2	33	84.6	18	85.7	1.00	36	85.7	15	83.3	1.00	51	85.0
T3	6	15.4	3	14.3		6	14.3	3	16.7		9	15.0
No. of positive lymph nodes												
4-9	26	66.7	11	52.4	0.40	26	61.9	11	61.1	1.00	37	61.7
≥ 10	13	33.3	10	47.6		16	38.1	7	38.9		23	38.3
Histologic grade												
I + II	6	15.4	9	42.9	0.02	9	21.4	6	33.3	0.32	15	25.0
III	31	79.5	10	47.6		31	73.8	10	55.6		41	68.3
Not determined	2	5.1	2	9.5		2	4.8	2	11.1		4	6.7
Hormone receptor status												
ER and PR negative (<10%)	33	84.6	13	61.9	0.02	34	81.0	12	66.7	0.16	46	76.7
ER or PR positive (≥10%)	4	10.3	8	38.1		6	14.3	6	33.3		12	20.0
Unknown	2	5.1	0	0.0		2	4.8	0	0.0		2	3.3
P53 status												
Negative (<10%)	21	53.8	11	52.4	1.00	23	54.8	9	50.0	0.78	32	53.3
Positive (≥10%)	18	46.2	10	47.6		19	45.2	9	50.0		28	46.7

* p values were calculated using the Fisher's exact test; patients with unknown values were omitted from analyses. *Abbreviations:* IPB-chemotherapy, intensive, platinum-based chemotherapy; ER, estrogen receptor; PR, progesterone receptor.

Supplementary Table 5B. Multivariate Cox proportional-hazard analysis of the risk of recurrence (RFS) and *XIST* expression.

Variable	No. Events / No. patients	Hazard Ratio	95% CI	p values
***XIST* expression (exon 2-3)**				
Low	18 / 39	1.00		
High	15 / 21	1.56	0.60 – 4.04	0.36
***XIST* (exon 2-3) Low**				
Conventional chemotherapy	13 / 19	1.00		
IPB chemotherapy	5 / 20	0.31*	0.11 – 0.88	0.03
***XIST* (exon 2-3) High**				
Conventional chemotherapy	9 / 12	1.00		
IPB chemotherapy	6 / 9	0.63*	0.20 – 1.91	0.41
***XIST* expression (exon 4-5)**				
Low	21 / 42	1.00		
High	12 / 18	1.07	0.41 – 2.82	0.89
***XIST* (exon 4-5) Low**				
Conventional chemotherapy	15 / 21	1.00		
IPB chemotherapy	6 / 21	0.30#	0.11 – 0.82	0.02
***XIST* (exon 4-5) High**				
Conventional chemotherapy	7 / 10	1.00		
IPB chemotherapy	5 / 8	0.81#	0.23 – 2.89	0.74

All analyses shown were adjusted for: number of positive lymph nodes (4-9 vs. >10); pathological T-stage (1 or 2 vs. 3); histologic grade (I or II vs. III). Homogeneity of both hazard ratios was not rejected based on an interaction term with * p value = 0.36 for *XIST* expression exon 2-3 and # p value = 0.24 for *XIST* expression exon 4-5; Number of events is not equal for all variables, since some patients have missing data; maximum missing variables (i.e. events) is 1/33. *Abbreviations:* CI, confidence interval.

Supplementary Table 6. Sequence of oligonucleotides used for the RT-MLPA reactions to quantify human XIST gene expression.

length	number	gene	mapview	chr. pos.	LPO
123	11317-L07566	LDHA	11-018,374941	11p15.1	TCCCGACGTGCATTC-CCGATT
148	S0224-SP0028-L09843	GAPDH	12-006,517038	12p13	CGTGGCCAAGGTCATCCAT-GACAACTTTGGTA
193	00954-L00185	B2M	15-042,795157	15q21.1	GTGACTTTGT-CACAGCCCAAGATAGTTAAG
246	08097-L09379	ARHGDIA	17-077,420356	17q25.3	GGATAAAAATCTCTTTCCG-GGTTAACCGA
344	08098-L07879	FAU	11-064,645661	11q13.1	CAGGAAACGGTCGCCCA-GATCAAGGCT
373	14709-L16365	XIST	X-072,969881	Xq13.2	GTCATGTCTCCTTAGGCTC-CTCTTGGACAT
400	01660-L01235	GAPDH	12-006,516114	12p13	GACCCCTTCATTGACCT-CAACTACATGGTT
445	14710-L16366	XIST	X-072,965736	Xq13.2	CTCAAAAACAACCAC-CACACGTCAAGCTCT
454	14679-SP0228-L16331	OAZ1	13-002,222792	13p13.3	GAGGGGAGCAAG-GACAGCTTTGCAGTT
472	14680-L00676	BIRC2	11-101,744461	11q22	GAAAAACAAGCTGAA-GAAATGGCATCAGAT

	SP	RPO	start	end	RT primer sequence
		CCTTTTGGTTC-CAAGTCCAATAT-GGCAA CGC	18374941	18374975	TATAAATCAGCT-GATCCTTTAG
	TCGTGGAAG-GACTCATGACCAC AGTCCATGCCAT-CACTGCCACC	CAGAAGACTGTGGAT-GGCCCCTCCAA TGAG	6517038	6517061	GCAGGGATGATGT-TCTG
		TGGGATCGAGACATG-TAAGCAGCATCA TGGA	42795157	42795845	CAAATGCG-GCATCTTCA
		GAGATAGTGTCCG-GCATGAAGTACATCC AGCATACGT	77420356	77420418	ACCATGTAGT-CAGTCTTGTCA
		CATGTAGCCTCACT-GGAGGGCATTGCCC	64645661	64645715	CGCCTGCCAGGAG
		TCTGAGCATGTGA-GACCTGAGGACTGCA AACAGCTATAAGAG-GCTCCAA	72969881	72974024	GATATGATTAATTT-GGAGCC
		TACATGTTCCAATAT-GATTCCACCCATGGCA AATTCCATGGCA	6516114	6516156	CCCGTTCT-CAGCCTT
		TCATTGTTCCTATCT-GCCAAATCATTATACT TCCTACAAGCAGT-GCAGAGAG	72965736	72967651	CTGAAGACT-CAGCTCTCT
	CTCCTGGAGT-TCGCT GAGGAGCAGCT-GCGA	GCCGACCATGTCT-TCATTTGCTTCCACAAG AACCGCG	2222792	2223810	CTCTGTCCTCGCG-GT
		GATTTGTCATTAATTCG-GAAGAACAGAAT GGCTCTCTTTCAAC	101744461	101753481	GAAGCACACATGT-CAATTGT

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Submitted

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LACK OF GENETIC HETEROGENEITY AT HIGH-RESOLUTION aCGH BETWEEN PRIMARY BREAST CANCERS AND THEIR PAIRED LYMPH NODE METASTASES

ABSTRACT

Lymph node (LN) metastases predict for high recurrence rates in breast cancer patients. Eradication of micro-metastatic tumor cells is the primary goal of adjuvant systemic treatment. Decisions regarding systemic treatment depend largely on primary tumor characteristics rather than on characteristics of their LN metastases. However, it remains unclear to what extent LN metastases, having already metastasized locally, resemble their primary breast tumors and as such will be eradicated by the systemic therapy chosen. In this study we investigated the genetic differences between primary breast cancers and their paired LN metastases using array comparative genomic hybridization analyses on a high resolution 720K Nimblegen platform. Thus far, no metastasis-specific genomic aberrations have been identified. We hypothesized that this is due to low-resolution platforms and lack of stratification on breast cancer subtypes (specifically, triple-negative (TN) versus luminal). Furthermore, we speculated that as TN tumors are known to be more genetically unstable, their LN metastases would show an increase in random copy number aberrations (CNAs). Therefore, we studied 10 primary TN breast tumor–LN pairs and 10 luminal pairs and found that all LN metastases clustered nearest to their matched tumor except for two. These two were explained by poor hybridization quality and, interestingly, the presence of two histological components in one tumor. We found no significantly altered CNAs between pairs in the whole group, nor when subdivided over subtypes; neither did we find a CNA increase in LN metastases compared to primary tumors within the TN subgroup, suggesting most CNAs are functional and not random. Our findings suggest a strong clonal relationship between primary breast tumors and its LN metastases and support the use of the primary tumor characteristics to guide adjuvant systemic chemotherapy in breast cancer patients, since primary tumors and their subsequent LN metastases seem remarkably similar, at least prior to treatment.

INTRODUCTION

Breast cancer is a multistep process of genetic alterations in which normal breast cells are transformed into malignant tumor cells and finally into tumor cells with metastatic capacity [1]. Understanding lymph node (LN) metastasis formation is essential as their presence predicts for a poor prognosis and occurs irrespective of tumor size, grade or hormone receptor status. It has been speculated that a specific trait should be present in order for breast cancer cells to be able to metastasize resulting in a high risk of recurrence or distant metastases [2]. However, it remains unclear to what extent LN metastases differ from their primary breast tumors and whether this trait is actually present. Answering this question is essential, since decisions regarding systemic adjuvant therapy are increasingly based on predictive markers tested in primary tumors but whether LN metastases resemble their primary tumor and as such will have a similar response has not been clarified.

A fair number of studies have investigated the genetic differences between primary breast tumors and lymph node (LN) or distant metastases by visualizing chromosomal losses and amplifications using either comparative genomic hybridization (CGH) or array-based CGH (aCGH) [3-9]. These studies either identified different regions of significant difference[4,7,9] or found no significant differences [3,5,6,8]. However, most of these studies used low resolution platforms detecting only large differences in copy number aberrations (CNAs). Smaller or more subtle changes between primary breast tumors and their metastases could have been missed by these studies. Furthermore, previous investigations did not differentiate between subtypes of breast cancer, while it has become clear that breast cancer is a heterogeneous disease with subtypes influencing prognosis and genomic profile [10,11]. These subtypes roughly follow the distribution of hormone receptor and HER2 status, with the luminal subtype expressing the estrogen-receptor (ER) and the basal-like subtype lacking ER, progesterone-receptor (PgR) and HER2 expression (also known as triple-negative (TN) breast cancers) on immunohistochemistry (IHC) [10,11]. Using aCGH, the differences between these subtypes mainly regard the extent of CNAs, i.e. amount of genomic instability. Basal-like, TN breast tumors have been shown to have the highest number of CNAs, i.e. the highest level of genomic instability [12-15]. Interestingly, this is one of the main features of *BRCA1*-mutated breast cancer. BRCA1 is involved in DNA-repair of double-strand breaks (DSBs) by homologous recombination (HR). Failure of HR will result in error-prone repair of DSBs, resulting in gross chromosomal rearrangements and consequently genomic instability. These TN tumors resemble breast cancers of *BRCA1*-mutation carriers not only on their level of genomic instability but also on their gene expression patterns and their lack of expression of hormone receptors [16]. This suggests that a subset of sporadic TN tumors might share this deficiency in HR DNA-repair.

We hypothesized that using a high resolution aCGH platform, and stratifying on different subtypes of breast cancer, we would be able to find specific genetic differences between primary tumors and their LN metastases if they occur. Furthermore, we speculated that TN LN metastases would have more genetic

differences than their primary tumors when compared to luminal tumors due to their potential HR-deficient background. To explore these hypotheses, we analyzed 10 TN breast tumors, 10 ER-positive tumors and their paired LN metastases on a 720K Nimblegen aCGH platform. We found that regardless of subtype, metastases closely resembled their primary tumors, without specific recurrent genetic differences being present, suggesting that the potential to form metastasis does not require new relevant aberrations and might be present from the start. Furthermore, these data suggest that the CNAs present are likely to be functional and not random.

METHODS

Patients

Patients were part of a multicentre trial performed in the Netherlands (1993 – 1999), in which breast cancer patients with at least four tumor-positive axillary LN but no distant metastases participated [17]. For this study we randomly selected ten patients with ER-positive, HER2-negative tumors, i.e. the luminal group, and ten patients with ER-, PR- and HER2-negative tumors, i.e. the TN group. Selection was based upon availability of formalin-fixed paraffin-embedded (FFPE) tissue containing more than 60% tumor cells in both the primary tumor and their paired LN.

Histopathology

Hematoxylin & eosin slides were scored for tumor percentages by a breast cancer pathologist (JW). ER, PR, P53, and HER2 were determined by immunohistochemistry as described previously [17,18].

DNA extraction and array comparative genomic hybridization

Genomic DNA was extracted from FFPE primary tumors and their LNs as previously published [19]. DNA from Promega (Human Genomic DNA: Female, G1521) was used as reference DNA. Tumor and reference DNA was labeled as previously described [20], except for the amount of input which was increased from 500ng DNA previously, to 1ug in concordance with the larger hybridization area of the 720K Nimblegen arrays. Labeled DNA was hybridized on a 720K Nimblegen CGH array (human CGH 3x720k whole-genome tiling v3.1 arrays, GEO platform GPL 14965), containing 719690 unique in situ synthesized 60-mer oligonucleotides, according to manufacturer's protocol (Roche Nimblegen, Madison, USA). Slides of the arrays were scanned using a G2505C microarray scanner (Agilent Technologies). Images were analyzed using DNAcopy, part of Nimblescan version 2.5.26 feature extraction software (Roche Nimblegen, Madison, USA). Oligonucleotides were mapped according to the human genome build NCBI36.

Bioinformatics analyses

Background corrected, non-segmented data output by the NimbleScan software was analyzed using the R statistical programming language. All analyses were performed in this language unless otherwise noted.

Heatmap

We computed a smoothed profile for each sample using the comparative module in the ***KCsmart*** R package [21], as implemented in the Bioconductor toolbox [22]. We set the kernel size for smoothing at σ = 1 Mb. We calculated the correlation distance (1-correlation) between all smoothed tumor profiles and used hierarchical clustering (average linkage) to construct the heatmap.

Comparative analysis of CNAs

Using the comparative module in the ***KCsmart*** package, designed to identify significantly differential recurrent CNAs between two groups of samples, we analyzed all samples for significant copy number differences between the primary tumor and the LN metastasis [22]. We also analyzed the TN and luminal groups separately. We used a kernel size of σ = 1 Mb, significance cut-off at $p < 0.05$ and 1000 permutations.

Pairwise analyses of primary tumor and lymph node

As any group-wise analysis might miss low-frequency or specific pair-wise changes, we analyzed each tumor-LN pair separately. To allow for direct comparison of tumor and LN samples we applied quantile normalization on the dataset. As the distributions of the data did not differ dramatically this makes the samples directly comparable by profile subtraction without losing information. After normalization we subtracted the tumor profile from the LN profile, creating a so-called 'delta-profile'. These delta profiles were then segmented by the *DNAcopy* package as implemented in the *Bioconductor* toolbox (version 2.8). Segmentation parameters were standard, except we used the option to undo a breakpoint call based on two standard deviations. Segments that were not at least two standard deviations apart were merged. To find recurrent pair-wise differential CNAs we searched for overlapping segments among all delta profiles As a measure of genomic instability-induced change between the primary tumor and the LN metastasis we counted the number of segments in the delta profiles that showed an absolute difference between the primary tumor and the LN above .2 on a log2 scale.

Concordance of an aCGH-based predictive marker between primary tumor and paired LN

Previously, we developed an aCGH classifier that determines the likelihood of patients being a *BRCA1*-mutation carrier by comparing CNAs in tumors of *BRCA1*-mutation carriers and tumors of sporadic breast cancer patients on an aCGH BAC platform [23,24]. We found that this BRCA1-classifier was a predictive marker for benefit to high-dose platinum-based chemotherapy [25]. This classifier was translated to the nimblegen platform by training a new classifier on the same regions as the BAC aCGH BRCA1-classifier and validated this on an independent dataset. We tested whether the primary tumors showed a concordant score (BRCA1-like^{CGH} or non-BRCA1-like^{CGH}) with their paired LN metastasis.

RESULTS

DNA from tumors and their paired LN metastases of 20 patients were successfully hybridized, resulting in 40 aCGH profiles (supplemental file 1). Patient characteristics are represented in supplementary table 1. Using hierarchical clustering on the correlation distance between the genomic profiles of the samples, we found that all LN metastases clustered nearest to their matched tumor, except for two (Figure 1).

The aCGH profiles of these two pairs were evaluated to try to explain why the LN metastases did not resemble their primary tumor. For patient 456 we found that while visually the profiles looked similar, the hybridization was of such poor quality that the correlation distance was quite high (Figure 2, A and B). In contrast, the LN metastasis of patient 592 appeared to have originated from a different tumor (Figure 2, C and D). By reviewing the HE slide of the tumor, we determined that it held two components: an expansively growing, poorly differentiated part and a diffusely infiltrating with lobular features part (Figure 2E). DNA from both components was isolated and hybridized separately (Figure 2E). Using the same clustering method, now including the profiles of these two separately isolated components, we found that the 592 expansively growing component clustered most closely with the 592 LN metastasis, indicating that this component was the origin of the metastasis (Supplementary Figure 1).

We excluded both non-resembling pairs (patient 456 and 592) from further analyses, since they would introduce artificial differences (either due to noise or due to differences between tumors itself).

Using the remaining samples we searched for any recurrent CNAs differing between the primary tumor and its LN metastasis. The smoothed KCsmart profiles were highly similar and no significant differences were detected when all samples were analyzed simultaneously (Figure 3A). However, subtype-specific differences might have been diffused due to pooling of data instead of taking into account the specific breast cancer subtypes. We therefore analyzed the pairs within the luminal and TN subtypes separately; but again found no significantly different regions between the pairs within either subtype (Figure 3B and 3C).

We next tested our hypothesis whether TN LN metastases showed more genetic differences than their primary tumors when compared to luminal tumors. To do this we constructed 'delta-profiles', normalized pair-wise subtractions of the tumor profile from its paired LN profile. By counting the number of segments of the delta profiles per tumor (delta-segments) we found no significant difference between the TN and luminal subtype (Figure 4).

We also checked whether we could identify any genomic region that contained a delta segment in different patients, indicating a recurrent change between tumor and LN. Although, we found a number of overlapping delta segments between tumor and LN metastasis (Supplementary Table 2), none of these showed a consistent direction of change between the overlapping CNAs, indicative of random change instead of driving alteration (Supplemental File 2).

Lastly, we tested the concordance between the primary tumor and its paired LN metastasis of an aCGH-based predictive marker and found that all tumor – LN pairs scored similarly, except for one which was the biphasic tumor 592

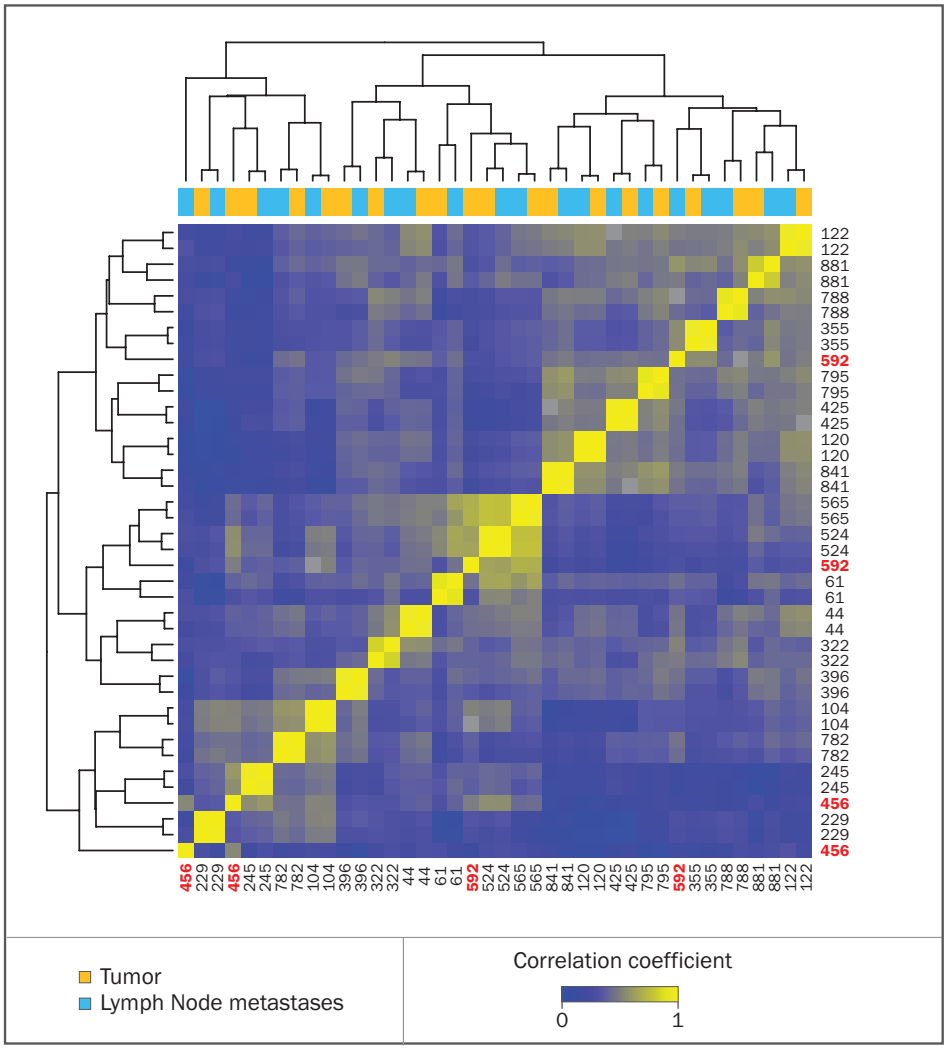

Figure 1. Heatmap of a hierarchical clustering based on copy number profile. Samples are hierarchically clustered with correlation as distance on their smoothed copy number profiles. The intensity of correlation is shown from blue to yellow. The coloured bar above the heatmaps denotes the origin of the sample, either a primary tumor or a lymph node metastasis. The numbers on the y-axis and x-axis are the patient numbers. Patient numbers in red show primary tumors and lymph node samples that do not cluster together.

(Supplementary Table 3). These findings support testing this predictive aCGH-based marker not only on the primary tumor but also on lymph node metastases. This might be especially helpful in case small primary tumors or tumors that grow diffusely, which are therefore less likely to yield sufficient quantities of DNA with a high tumor cell percentage.

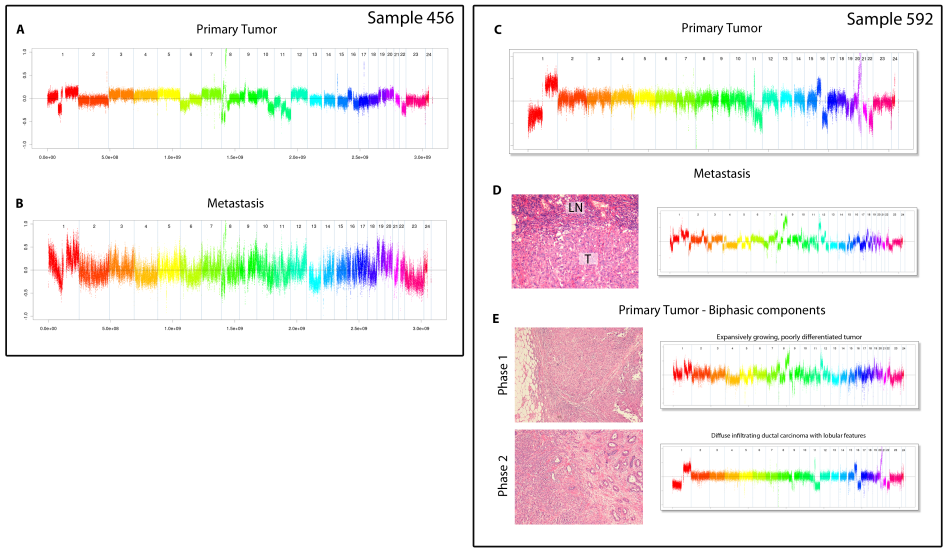

Figure 2. Investigation into discordant samples. Two samples that did not cluster together based on correlation were investigated for their discordance. A and B) Primary tumor and metastasis of patient 456 showed visually similar profiles, but the metastasis was a highly noisy hybridization. C and D) Primary tumor and metastasis profiles for patient 592 showed two highy different profiles. E) Two different histological phases were detected in the primary tumor, of which DNA was isolated separately and hybridized again. It is clear that the expansively growing component gave rise to the metastasis.

DISCUSSION

Eradication of disseminated tumor cells is the primary goal of adjuvant systemic treatment in breast cancers patients. Decisions regarding systemic treatment in all stages of breast cancer (early to metastatic) depend largely on tumor characteristics, which are based on the primary tumor rather than disseminated tumor cells. In this study we investigated the genetic differences between primary breast cancers and their paired LN metastases by using a high resolution aCGH platform. We hypothesized that besides lower resolution, the lack of stratification on subtypes (specifically, TN versus luminal) of breast cancer might explain not having identified metastasis-specific DNA alterations in the past. Furthermore, we speculated that the genetic instability of TN tumors might result in an increase in CNAs in metastases, simply due to random occurrence in this subgroup. We therefore studied 10 primary breast tumor – LN pairs of the TN subtype and 10 of the luminal subtype and found that 1) most LNs closely resembled their primary tumor with only small focal differences; 2) lack of resemblance was explained by poor quality hybridization or the presence of two histological components within one tumor; 3) no significantly regional differences occurred between primary tumors and LNs overall or within the specific subtypes; 4) the number of CNAs in the LN metastasis compared to the tumor did not differ between TN and luminal tumors; 5) no consistent regions of DNA alteration were found when analyzing the

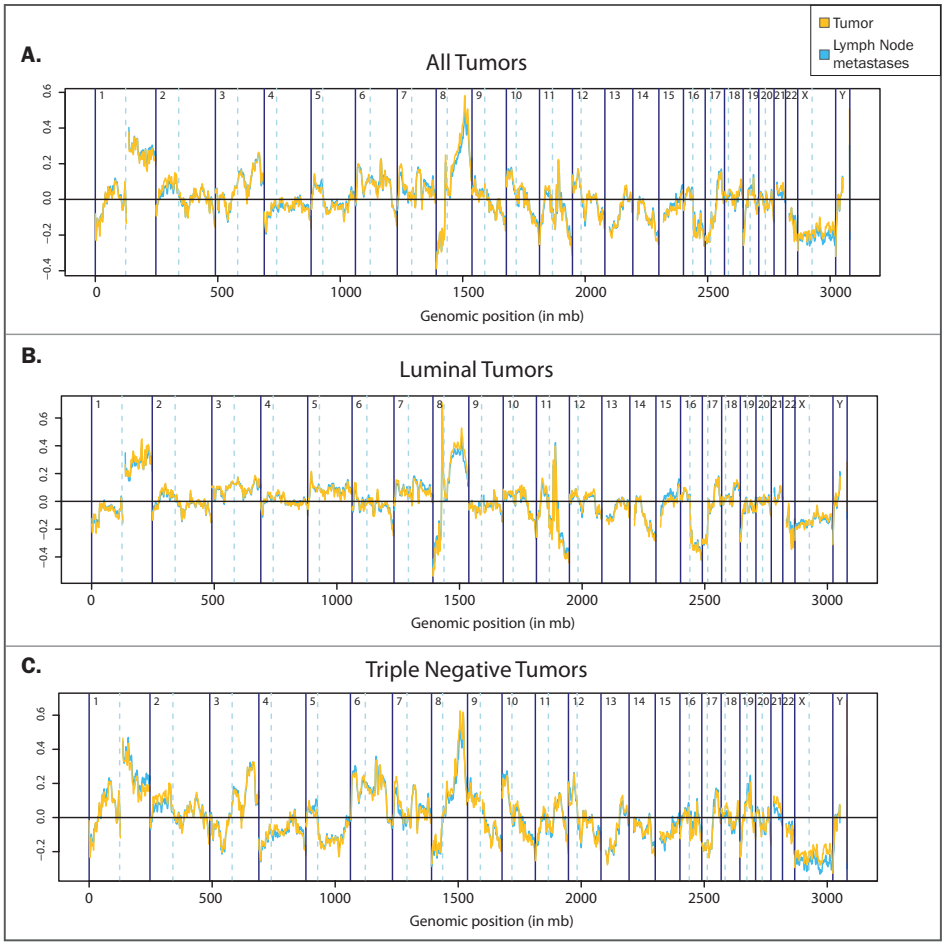

Figure 3. Group-wise comparison of primary tumor copy number profiles with lymph node metastasis copy number profiles. Overall profiles were generated using the comparative module of the *KCsmart* package on either (A) the whole group, (B) just the luminal tumors and (C) the triple negative tumors.

tumors in a pair-wise segmentation-based approach; and 6) a predictive marker based on aCGH can be used on both the primary tumor and on LN metastasis as both scored highly similar on this test.

This is the first study in which an aCGH platform was used which is capable of identifying true differences between primary breast tumors and LN pairs, since with this resolution even small differences can be detected. Furthermore, this is the first studying taking into account breast cancer subtypes. Although, our study did not include all subtypes (for example: we did not analyze the HER2-subtype), we speculated that by stratifying on the two most extreme subtypes with regard to number of CNAs [15], we would be most likely to find potential differences.

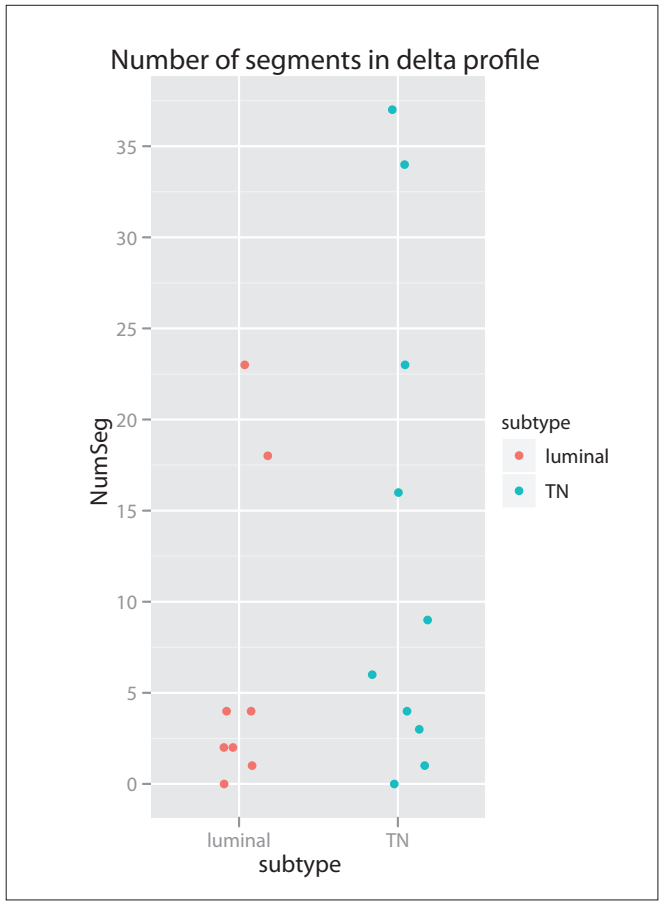

Figure 4. Quantification of copy number differences between the tumor and lymph node metastasis. For each sample pair (Tumor-Lymph Node) we constructed a delta profile, which contains only the copy number differences between the two. For each delta profile we counted the unique segments that showed a difference above .2 log2 or below -.2 log2 and we only counted the segments that contained 10 or more probes. We split the tumors into the Triple Negative (TN) and luminal subgroups.

Our findings of a high resemblance between primary breast tumors and their LN metastases on CGH for most cases but not in all, were in accordance with most previous studies comparing breast cancer with their LN on CGH [3,6,8]. This suggests that metastasis formation does not require new relevant aberrations to endure the selection pressure of metastasis. Comparable results were seen with regard to similarity between primary breast cancers and their metastases on gene expression arrays[10,26] and on immunohistochemistry [27]. In our study only 5% (1/20) of the LN metastasis truly did not resemble its primary tumor, suggesting a high level of clonality between primary breast cancers and their LN metastases.

Recently, two studies have investigated the clonality within breast cancer by profiling genomes of cells sorted on ploidy of different subsections within the same tumor[28] and by quantifying copy number using next generation sequencing of single breast cancer cells [29]. It was shown that half of the breast tumors consisted of a homogenous clonal population, while the other half had only three clonal subpopulations. Even within these subpopulation the majority of chromosome breakpoints were shared and only small differences based on focal amplifications or deletions were seen [28,29]. This corresponds to our findings of small focal differences between primary breast tumors and their LNs in 16 pairs (supplementary table 2). The high level of similarity of even highly complex, aberrant patterns (such as in TN) suggests that LN metastases is not an early event in tumorigenesis, since this complex pattern would need time to evolve. On the other hand, the lack of more than three subclones in the clonality studies [28,29] suggests that either gradual accumulation of many changes are required in single cells before these are able to form a mass, in which case metastases could arise simultaneously with the tumor mass formation and still be a relatively early event. Alternatively, it could be that a tumor is formed after sudden accumulation of changes based on for example mitotic missegration as proposed by Navin et al [28]. However, this last scenario seems less likely since the chances of acquiring all the advantages needed for proliferation and survival in a single event seem small. Using aCGH even with high resolution will not clarify these processes as it is only possible to assess a relatively large subset of cells. Furthermore, in our study copy number neutral changes, such as point mutations or balanced translocations and inversions, were not explored and therefore primary tumors might still differ more from their LN metastases than we now found.

From previous studies it is known that basal-like and TN breast tumors not only resemble *BRCA1*-mutated breast cancers closely with regard to their genomic instability upon aCGH [12-15], but a subgroup of TN breast cancers also share the characteristic genomic pattern of specific CNAs associated with *BRCA1*-mutated breast cancer [25,30]. This suggests that this subgroup of sporadic TN tumors might share the HR-deficient background with *BRCA1*-mutated breast cancers. This hypothesis was strengthened by the fact that some of these on aCGH BRCA1-like tumors showed *BRCA1*-promoter methylation [25,30]. One could envision that due to HR-deficiency, repair of DSBs depends on error-prone DNA repair pathways resulting in random CNAs and genomic instability. However, we found that even within TN breast tumors no increase of CNAs was seen in their LN metastases, suggesting that regardless of the complexity of CNAs, the CNAs present are likely to be functional and not random. This concept is supported by the CGH findings in a mouse model of BRCA1-associated breast cancer, showing a highly similar pattern of CNAs in different BRCA1-deficient mouse mammary tumors, which was highly reproducible over different studies [31].

The finding of one truly discordant pair in our study is very interesting with regard to systemic treatment response. This result could explain the mixed response (mostly a responding primary tumor with no response of the LN metastasis) sometimes seen within breast cancer patients upon systemic treatment. Furthermore, this suggests that upon observing a mixed response it

could be useful to histologically evaluate the primary tumor again for evidence of multiple components within the tumor and if applicable adjust treatment. However, the general findings of our study imply that differences in response of the LN metastases and the primary breast tumor are not due to genomic differences. Of course these could be due to differences in methylation pattern or protein modification, however also on immunohistochemistry no substantial differences were found [27]. Therefore, it could also be that the intrinsic mechanisms of resistance/ sensitivity are equal in the LN metastasis to that in the primary breast tumor, at least with regard to non-single gene targeted therapies (most chemotherapeutics). This implicates that the choice of first therapy might be even more essential for eradication as both tumor and LN can still respond similarly, but might develop separate mechanisms of resistance after this therapy. Therefore they might react differently to second-line therapy, complicating treatment choice. Of course this remains mere speculation but could make the search for predictive biomarkers even more important as the first-line treatment is more likely to target both primary tumor and LN metastasis, and after which response of both might diverge.

The high level of similarity of genomic profiles further implicates that biomarkers based on genome-wide aberrations can also be tested on DNA from the LN metastases instead of the primary tumor, which can be helpful in case of limited tumor material. This is supported by our findings of the high level of concordance with the aCGH BRCA1-classifier.

In summary, in this study we compared the genomic profiles of primary breast cancers and their paired LN metastases using aCGH. To our knowledge this is the first study investigating these potential differences using a high resolution platform and by stratification on breast cancer subtypes. We found a high level of similarity between the pairs, suggesting a strong clonal relationship between the two, which makes the hypothesis of a small subpopulation of the tumor acquiring metastatic capacity unlikely. Furthermore, the findings in our study emphasize the value of being able to predict response to first-line systemic chemotherapy in breast cancer patients, since primary tumors and their subsequent LN metastases seem remarkably similar, at least prior to treatment.

COMPETING INTERESTS

All authors of this paper declare to have no financial and/or personal relationships with other people and/or organizations that could influence or bias their work.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Patient characteristics.

Variable	No. of patients with luminal tumors	No. of patients with TN tumors	p-value
Age			
< 40 years	3	3	
40 - 49 years	7	4	0.51*
≥ 50 years	0	3	
Pathological tumor classification			
T1	1	2	
T2	7	6	1.00*
T3	2	2	
No. of positive lymph nodes			
4-9	9	6	0.30
≥ 10	1	4	
Histologic grade			
I	2	0	
II	5	2	0.04*
III	3	7	
Not determined	0	1	
Estrogen receptor status			
Negative (<10%)	0	10	nr
Positive (≥10%)	10	0	
Progesterone receptor status			
Negative (<10%)	2	10	nr
Positive (≥10%)	8	0	
P53 status			
< 10%	6	2	
10 – 50%	3	1	0.06*
> 50%	1	5	
Unknown	0	2	

p values: patients with unknown values were omitted. p values were calculated using the Fisher's exact test, except for * Chi square test for trend. *Abbreviations:* no. number; nr, not relevant selection on these variables

Supplementary Table 2: Overview of all overlapping delta segments.

Patient nr	chrom	chrom start	chrom end	num. mark	seg. mean	subtype	Tumor state	LN state	combined state
355	1	39679781	47438957	2019	0.2541	TN	none	gain	none->gain
881	1	45854854	46069799	49	0.3261	TN	none	none	none->none
355	1	47615749	53435570	1623	0.2267	TN	none	gain	none->gain
565	1	49669820	50287337	152	-0.5004	luminal	none	loss	none->loss
565	1	50583807	50722208	40	-0.4561	luminal	none	loss	none->loss
565	1	51123165	51458679	69	-0.4916	luminal	none	loss	none->loss
881	1	158175215	187127014	7617	-0.2519	TN	gain	gain	gain->gain
396	1	160415206	160521574	44	-0.3742	TN	gain	gain	gain->gain
322	1	171421653	172717046	281	-0.3455	TN	gain	gain	gain->gain
122	1	172061131	172105207	11	0.2882	luminal	gain	gain	gain->gain
881	2	19799	75357291	19716	0.2314	TN	none	none	none->none
841	2	9997778	10039866	10	0.246	TN	gain	gain	gain->gain
355	2	19519744	20741756	328	0.3051	TN	loss	none	loss->none
524	3	65164956	65194349	11	0.4146	luminal	loss	loss	loss->loss
355	3	65164956	65188166	10	-0.2444	TN	loss	loss	loss->loss
881	4	132802986	133122935	23	-0.3888	TN	gain	none	gain->none
122	4	132805248	133077170	18	-0.3762	luminal	gain	none	gain->none
61	5	150120655	180814162	9355	-0.2229	TN	gain	none	gain->none
355	5	177296269	180344711	718	0.2825	TN	loss	none	loss->none
881	5	180275126	180362377	11	-0.3516	TN	loss	loss	loss->loss
322	5	180293675	180718374	66	-0.4276	TN	gain	gain	gain->gain
355	5	180362377	180814162	62	0.2717	TN	loss	none	loss->none
881	7	35249507	57575557	6083	0.2795	TN	loss	none	loss->none
782	7	38281394	38326725	14	0.3444	luminal	none	gain	none->gain
355	7	118942521	158816034	10700	-0.2083	TN	gain	none	gain->none
44	7	158306644	158816034	117	-0.2824	luminal	none	loss	none->loss
881	8	7219480	137748739	32716	0.2968	TN	gain	gain	gain->gain
122	8	8442371	9047328	159	0.2402	luminal	gain	gain	gain->gain
122	8	67605281	67856039	63	0.3198	luminal	gain	gain	gain->gain
355	8	117303752	117333429	15	0.2793	TN	gain	gain	gain->gain
322	8	132681101	146264232	3519	-0.2726	TN	gain	none	gain->none
881	8	137984221	146264232	1876	0.3458	TN	gain	gain	gain->gain
355	10	128680	2982309	837	-0.2681	TN	none	loss	none->loss
425	10	128680	38553648	10018	-0.2595	TN	gain	none	gain->none
355	10	84587705	89380669	1143	-0.2498	TN	none	none	none->none
122	10	89038683	89248990	41	-0.2747	luminal	none	none	none->none
322	11	66612	58026741	12984	-0.2275	TN	none	loss	none->loss
565	11	3217293	3326093	22	-0.3249	luminal	gain	gain	gain->gain
841	11	12649765	12671448	11	0.25	TN	loss	none	loss->none
841	11	57285610	57325807	12	0.278	TN	none	gain	none->gain
322	11	58027476	82380367	5835	0.3028	TN	none	gain	none->gain
104	11	60728061	60774450	12	-0.2091	luminal	loss	loss	loss->loss

Supplementary Table 2: Overview of all overlapping delta segments.

Patient nr	chrom	chrom start	chrom end	num. mark	seg. mean	subtype	Tumor state	LN state	combined state
841	11	64943448	65048463	24	0.2366	TN	none	gain	none->gain
355	11	76894678	134444816	15428	-0.2282	TN	gain	none	gain->none
122	11	125795630	125853430	17	0.3797	luminal	loss	none	loss->none
881	12	3853197	5978832	659	-0.6829	TN	gain	loss	gain->loss
355	12	4399991	4431887	12	-0.5666	TN	gain	gain	gain->gain
355	12	4446083	8200963	942	-0.2739	TN	gain	gain	gain->gain
881	12	5990965	8200963	459	-0.3743	TN	none	loss	none->loss
881	12	8495584	10031215	251	-0.4175	TN	none	loss	none->loss
355	12	8495584	14822550	1361	-0.2617	TN	gain	gain	gain->gain
881	12	13172917	13359121	64	-0.3948	TN	loss	loss	loss->loss
122	14	18144040	19309732	55	-0.2613	luminal	none	none	none->none
425	14	18144040	19270761	51	0.2501	TN	loss	loss	loss->loss
122	14	66868322	68623996	533	0.2931	luminal	loss	none	loss->none
788	14	67621362	68162212	170	-0.4298	TN	loss	loss	loss->loss
788	14	89712531	106342076	4523	-0.2268	TN	loss	loss	loss->loss
104	14	105920708	106015985	15	-0.2215	luminal	loss	loss	loss->loss
245	15	18420959	100296299	20478	-0.2134	luminal	gain	none	gain->none
104	15	85629520	85679279	12	-0.283	luminal	loss	loss	loss->loss
122	15	91141932	91271477	27	0.3847	luminal	gain	gain	gain->gain
322	15	96644380	97874183	339	-0.3225	TN	gain	none	gain->none
322	15	99302092	100296299	253	-0.3093	TN	gain	none	gain->none
322	17	54833495	55604525	161	-0.5894	TN	gain	gain	gain->gain
841	17	55191837	55290343	12	0.2712	TN	none	none	none->none
355	17	55218888	55333850	16	0.2932	TN	gain	gain	gain->gain
795	20	15928	32627779	7504	0.2222	TN	none	none	none->none
322	20	13176553	13594409	109	-0.5331	TN	gain	gain	gain->gain
322	20	15945815	16107094	46	-0.5627	TN	gain	gain	gain->gain
524	22	14434579	49568253	8898	0.2147	luminal	loss	loss	loss->loss
881	22	14434579	15535092	37	-0.2014	TN	gain	none	gain->none
565	22	25766514	26701689	344	-0.446	luminal	none	loss	none->loss
565	22	27009001	27058008	14	-0.5082	luminal	none	loss	none->loss
565	22	27995406	28319989	99	-0.4624	luminal	none	loss	none->loss
565	22	28551441	28603176	24	-0.5078	luminal	none	loss	none->loss
565	22	28651960	28740011	22	-0.4262	luminal	none	loss	none->loss
565	22	29117499	29306821	48	-0.4308	luminal	none	loss	none->loss
565	22	29853956	35510186	1809	-0.4701	luminal	none	loss	none->loss
565	22	35723318	35809021	23	-0.4355	luminal	none	loss	none->loss
565	22	35868455	35972684	27	-0.4073	luminal	none	loss	none->loss
565	22	36419455	37109004	162	-0.4493	luminal	none	loss	none->loss
782	22	36924170	37039453	30	0.254	luminal	none	none	none->none
565	22	37128927	41456123	1144	-0.4598	luminal	none	loss	none->loss
565	22	41573513	41897062	76	-0.4411	luminal	none	loss	none->loss

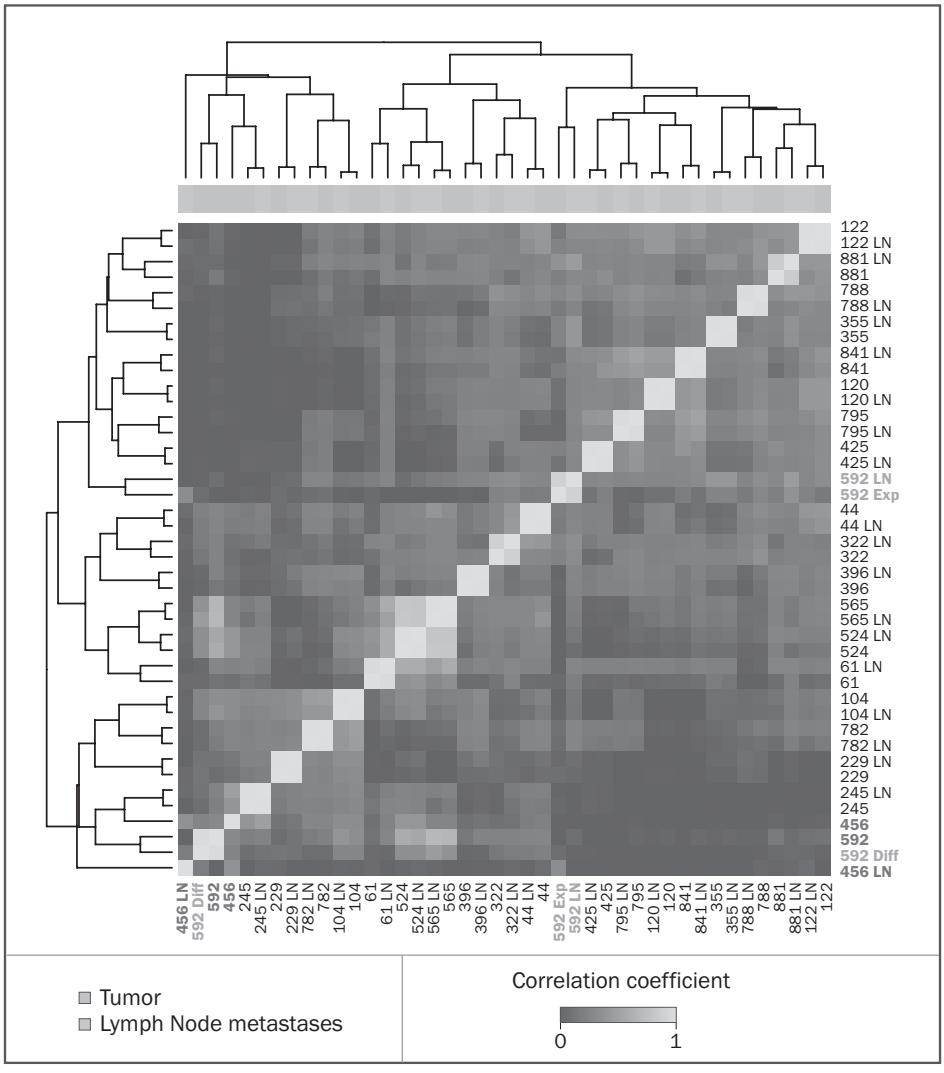
Supplementary Table 2: Overview of all overlapping delta segments.

Patient nr	chrom	chrom start	chrom end	num. mark	seg. mean	subtype	Tumor state	LN state	combined state
565	22	42023735	42071537	16	-0.4206	luminal	none	loss	none->loss
782	22	42988464	43251496	55	0.3099	luminal	none	none	none->none
795	23	146809526	154895334	2316	0.3669	TN	loss	none	loss->none
396	23	153075386	153153323	24	-0.3291	TN	gain	none	gain->none
44	24	57458252	57758621	86	0.2915	luminal	none	gain	none->gain
795	24	57490740	57758621	81	0.3164	TN	loss	none	loss->none

Abbreviations: nr, number; chrom, chromosome; seg, segment. Samples below dashed lines also share a regions with tumors above this line.

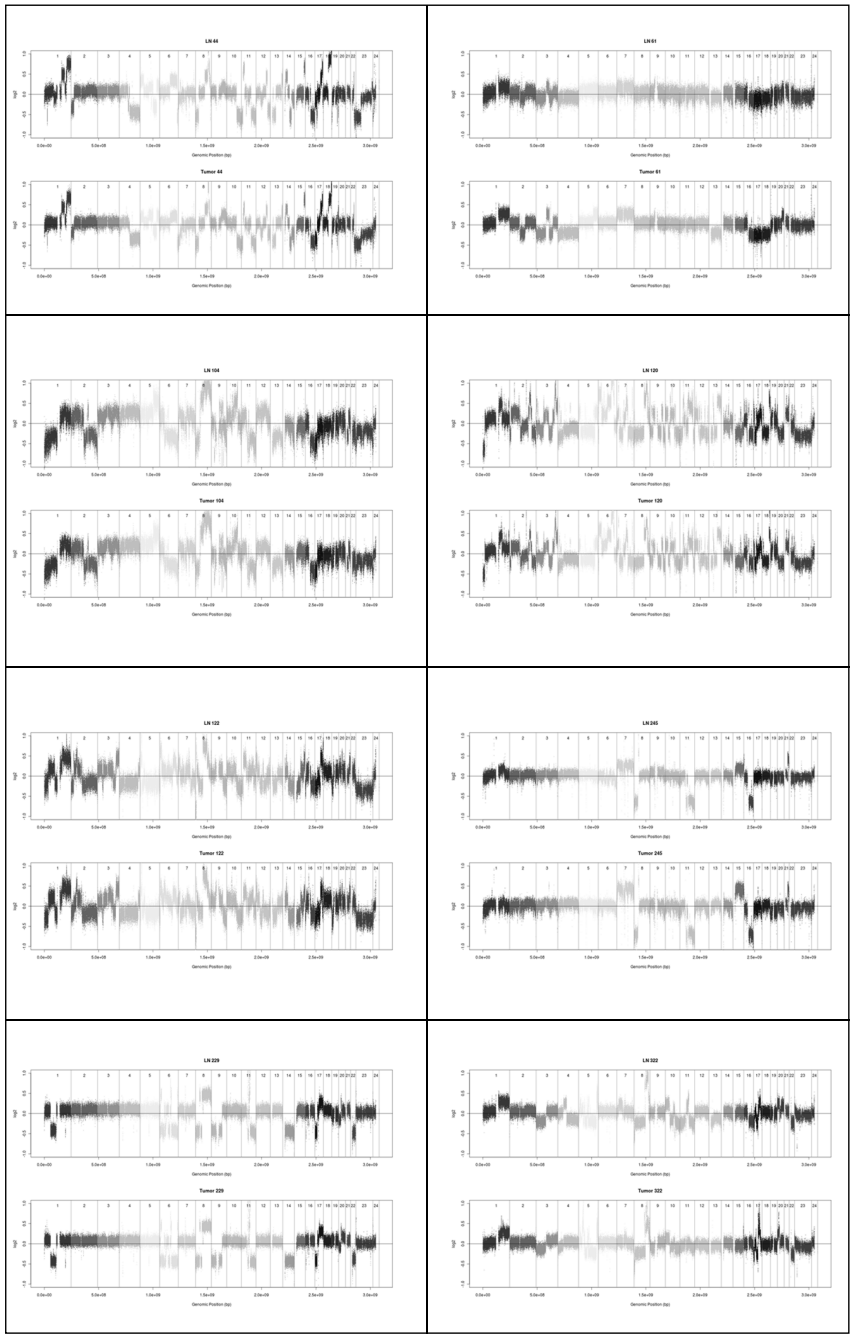
Supplementary Table 3. Concordance between primary tumor and their LN metastasis regarding the aCGH BRCA1-classifier.

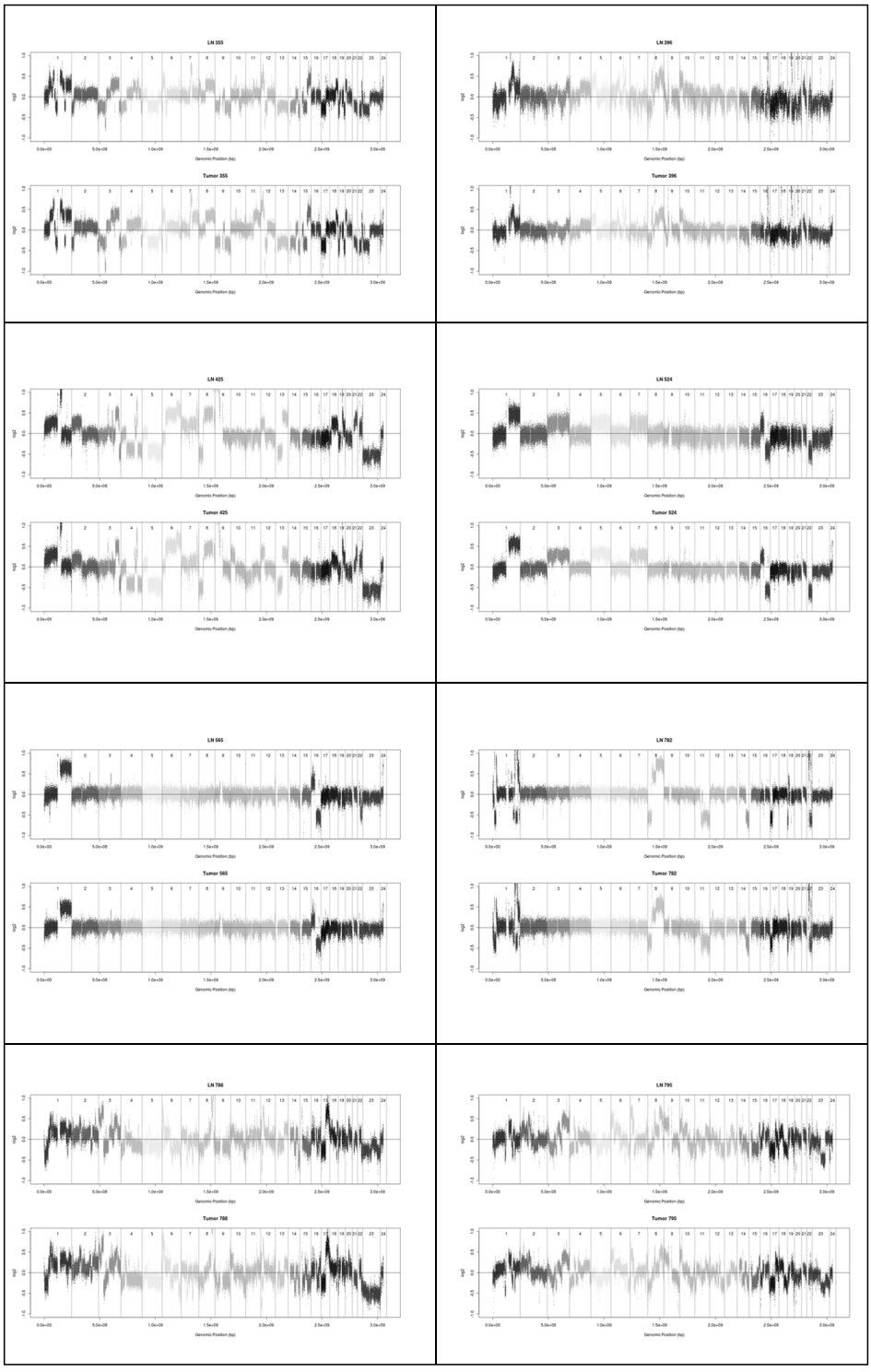
		Lymph node metastasis	
		BRCA1-like^{CGH}	**non-BRCA1-like^{CGH}**
Primary Tumor	BRCA1-like^{CGH}	8	1
	non-BRCA1-like^{CGH}	0	11

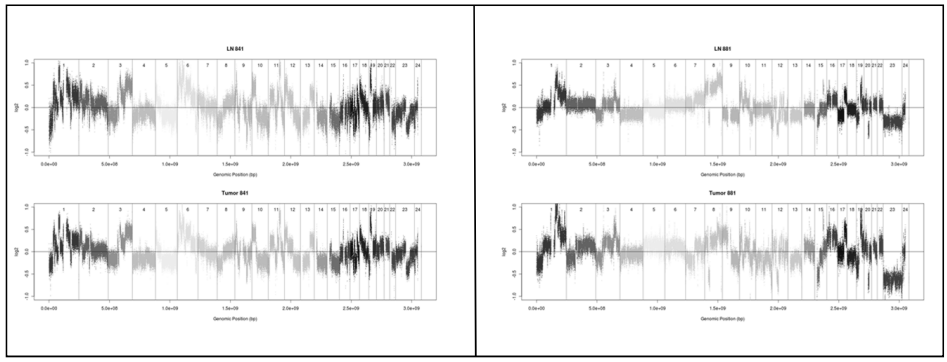


Supplemental Figure 1. Heatmap of a hierarchical clustering based on copy number profile. Samples are hierarchically clustered with correlation as distance on their smoothed copy number profiles. The intensity of correlation is shown from blue to yellow. The colored bar above the heatmaps denotes the origin of the sample, either a primary tumor or a lymph node metastasis. The numbers on the y-axis are the patient numbers. Patient numbers in red show primary tumors and lymph node samples that do not cluster together. Patient numbers in green show samples that were reassessed and visualized in Figure 2.

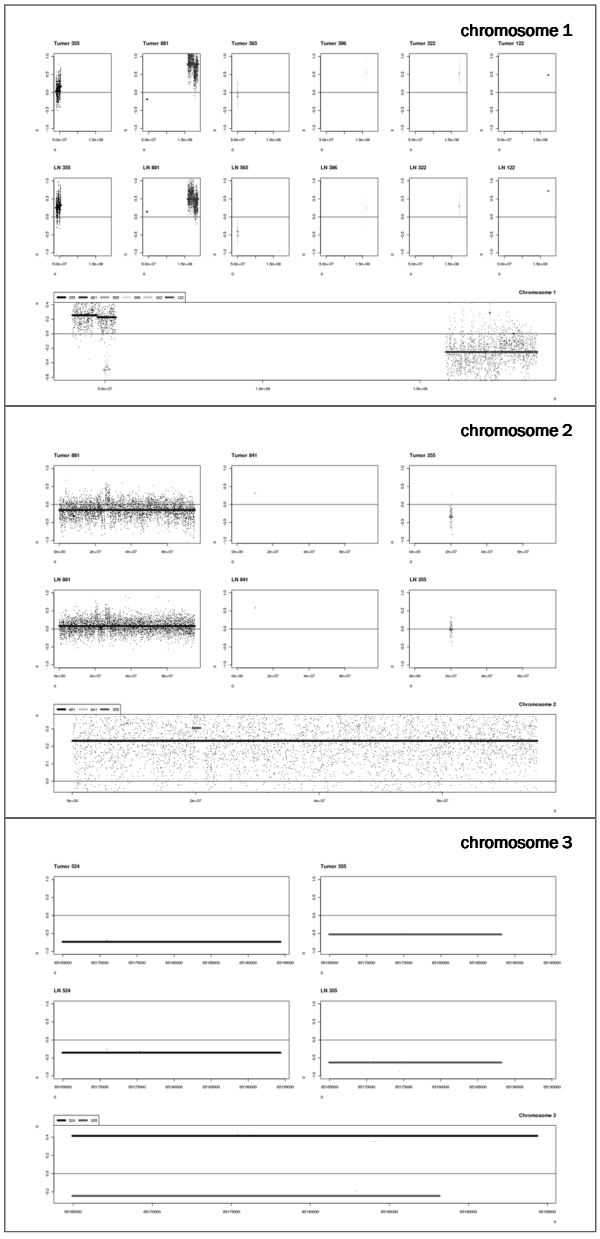
Supplemental File 1. Visualizations of the copy number profile of all primary tumor – lymph node metastasis pairs that were included in all analyses. Each figure shows the profiles for a single patient.

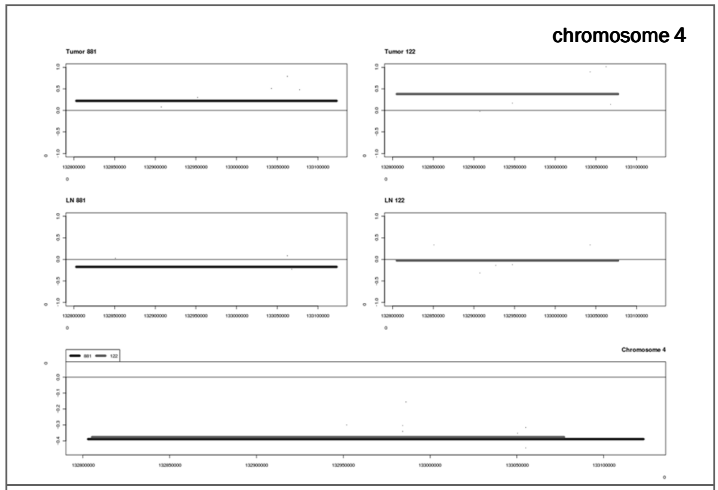



LN 355
Tumor 355
LN 396
Tumor 396
LN 425
Tumor 425
LN 524
Tumor 524
LN 565
Tumor 565
LN 782
Tumor 782
LN 788
Tumor 788
LN 795
Tumor 795
log2
Genomic Position (bp)


LN 841
Tumor 841
LN 881
Tumor 881
log2
Genomic Position (bp)

Supplemental File 2. Visualizations of the delta segments that overlapped and exceeded an absolute log2 value of 0.1. Each figure shows all delta segments found on a single chromosome. The upper row shows the raw data for a segment in the tumor sample. The middle row shows the raw data for a segment in the lymph node sample. The lower panel shows all delta segments and the values of the probes in the delta profile. Each patient is coloured differently.

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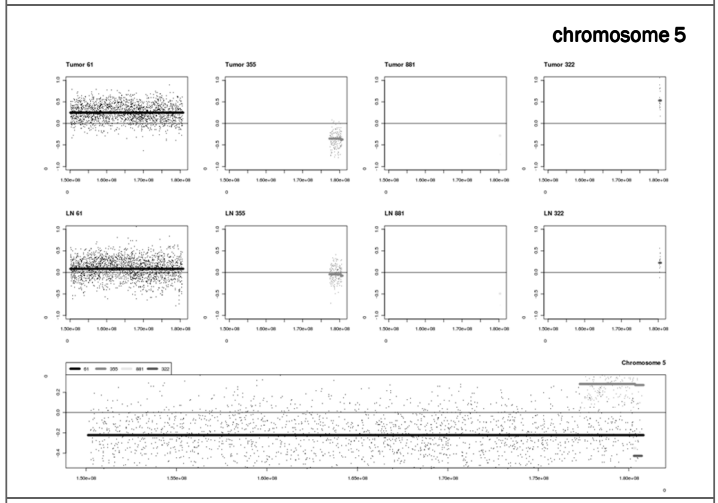
Tumor 881
Tumor 122
LN 881
LN 122
Chromosome 4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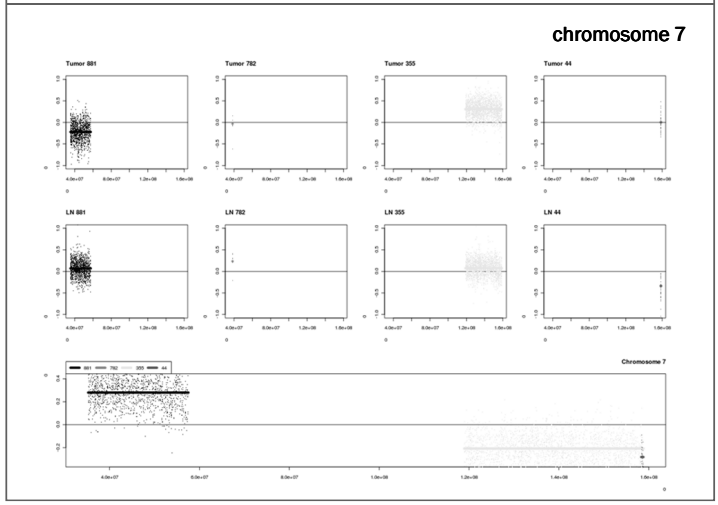
Tumor 355
Tumor 881
Tumor 322
LN 61
LN 355
LN 881
LN 322
Chromosome 5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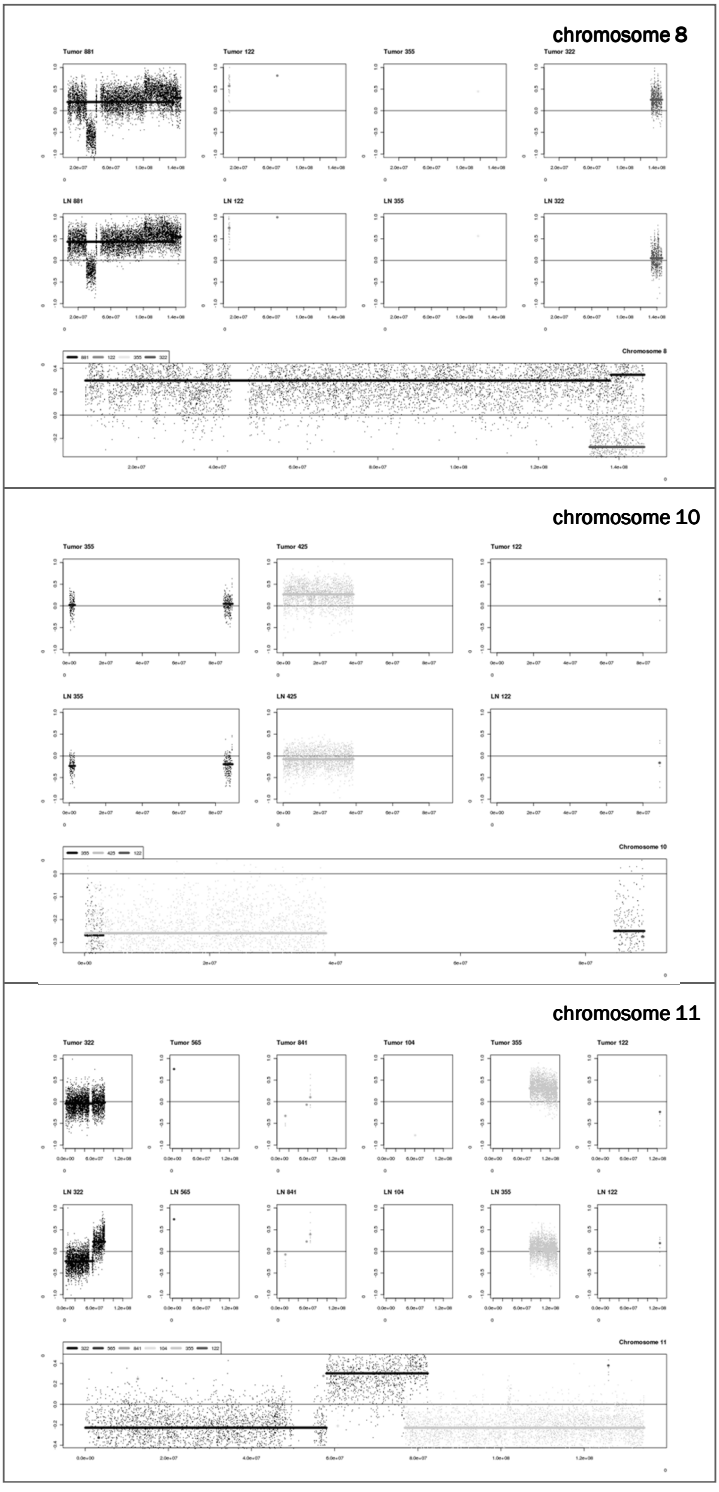
Tumor 782
Tumor 355
Tumor 44
LN 881
LN 782
LN 355
LN 44
Chromosome 7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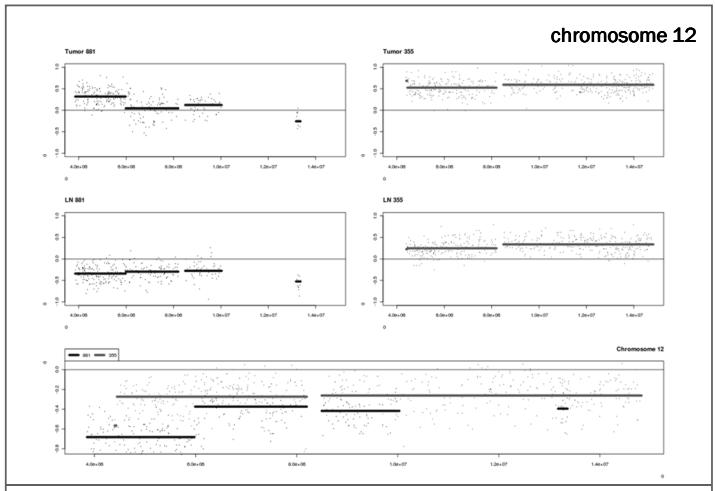
Tumor 122
Tumor 355
Tumor 322
LN 881
LN 122
LN 355
LN 322
Chromosome 8
chromosome 10
Tumor 355
Tumor 425
Tumor 122
LN 355
LN 425
LN 122
Chromosome 10
chromosome 11
Tumor 322
Tumor 565
Tumor 841
Tumor 104
Tumor 355
Tumor 122
LN 322
LN 565
LN 841
LN 104
LN 355
LN 122
Chromosome 11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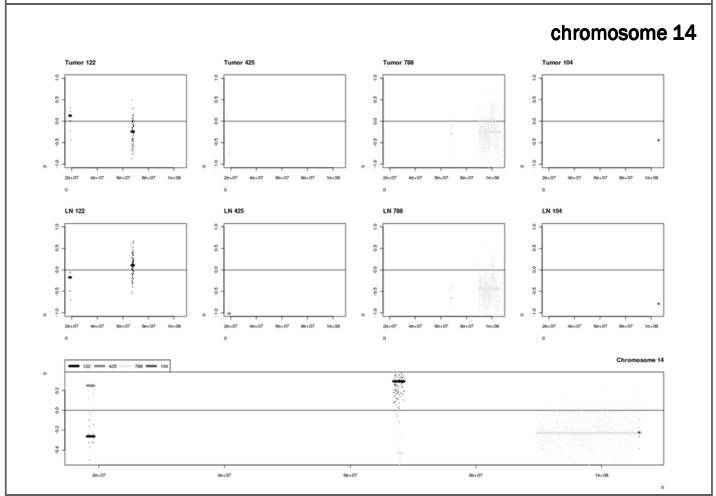
Tumor 355
LN 881
LN 355
Chromosome 12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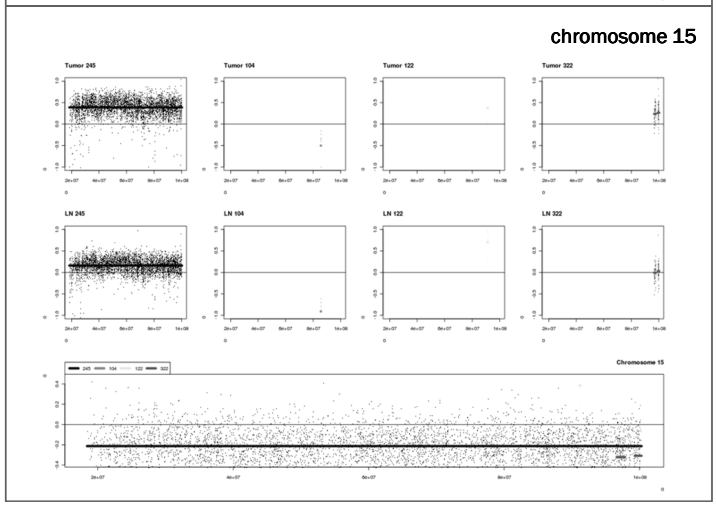
Tumor 425
Tumor 788
Tumor 104
LN 122
LN 425
LN 788
LN 104
Chromosome 14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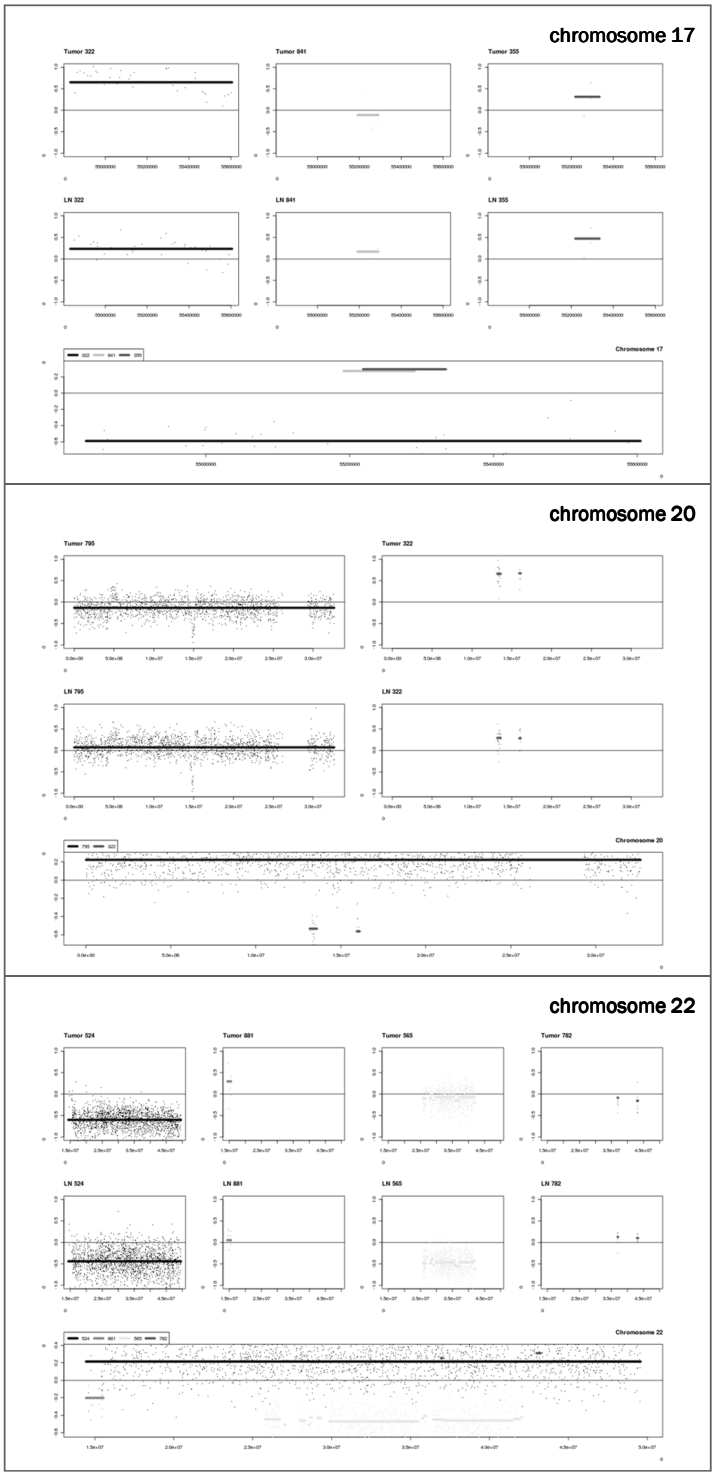
Tumor 104
Tumor 122
Tumor 322
LN 245
LN 104
LN 122
LN 322
Chromosome 15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Tumor 322
Tumor 841
Tumor 355
LN 322
LN 841
LN 355
Chromosome 17
chromosome 20
Tumor 795
Tumor 322
LN 795
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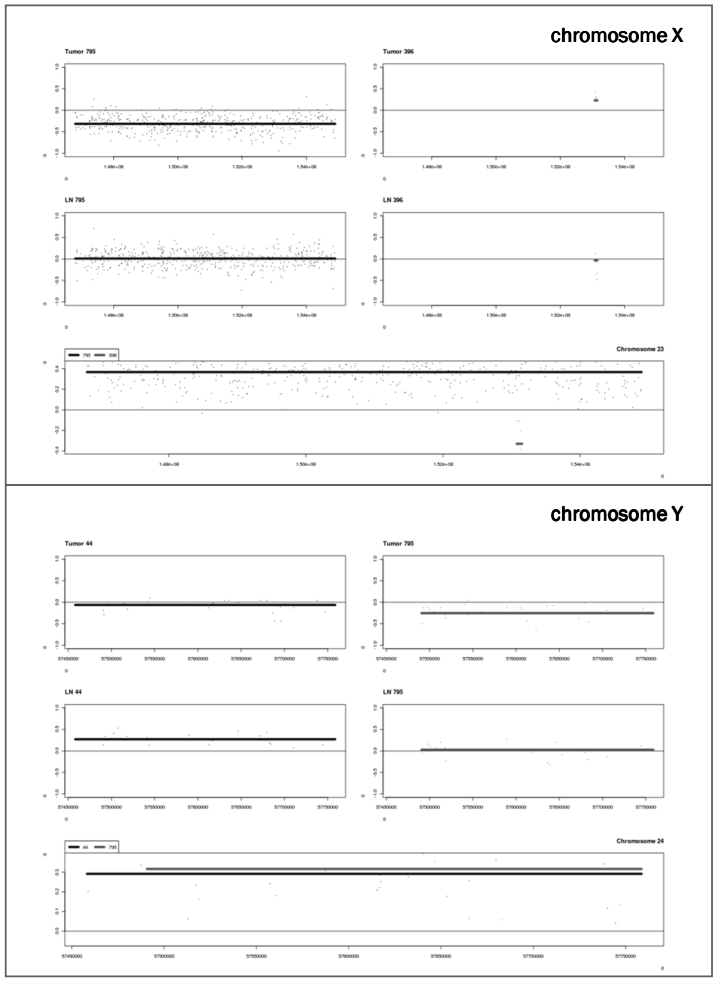
Chromosome 20
chromosome 22
Tumor 524
Tumor 881
Tumor 565
Tumor 782
LN 524
LN 881
LN 565
LN 782
Chromosome 22


chromosome X
Tumor 795
Tumor 396
LN 795
LN 396
Chromosome 23
chromosome Y
Tumor 44
Tumor 795
LN 44
LN 795
Chromosome 24

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7

SOLUBLE EPIDERMAL GROWTH FACTOR RECEPTOR (SEGFR) AND CARCINOEMBRYONIC ANTIGEN (CEA) CONCENTRATION IN PATIENTS WITH NON-SMALL CELL LUNG CANCER : CORRELATION WITH SURVIVAL AFTER ERLOTINIB AND GEFITINIB TREATMENT

ABSTRACT

Background: In patients with non-small cell lung cancer (NSCLC) a higher response rate can be achieved with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI's) when selection for therapy is guided by mutation analysis or gene amplification. However, both tests are complex and require tumour tissue. Simple methods to identify responders prior to EGFR-TKI treatment are urgently needed. This study aimed to define the relation between serum sEGFR levels, carcinoembryonic antigen (CEA) and survival in NSCLC patients treated with EGFR-TKI's.

Methods: Patients with stage III/IV NSCLC treated with gefitinib or erlotinib between July 2002 and December 2005 were reviewed. Levels of serum sEGFR were determined by a sandwich quantitative enzyme-linked immunosorbent assay. A chemiluminescence immunoassay was used for CEA. The relation between sEGFR and survival was investigated.

Results: One-hundred-and-two NSCLC patients, mainly stage IV (80%), were identified. Mean sEGFR at baseline was 55.9 µg/l (range 35.3 – 74.5µg/l). The median CEA level was 11.1 µg/l (range <1.0 – 2938.0 µg/l). Median overall survival was 5.2 months (range 1 - 52 months). Decreasing log CEA values (HR 1.51, 95% CI 1.11-2.04, multivariate analysis) and increasing sEGFR values (HR 0.96, 95% CI 0.93-0.99, multivariate analysis) were both independently associated with prolonged survival. Higher levels of pre-treatment sEGFR were associated with lower risk of progressive disease within 3 months (p=0.04).

Conclusions: Both baseline sEGFR and CEA levels in NSCLC patients receiving EGFR-TKIs showed a significant correlation with survival. To distinguish whether these factors have a predictive or a prognostic value, validation is warranted in an independent patient series containing a control arm without EGFR-TKI treatment.

BACKGROUND

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally activated in different types of epithelial malignancies. A constitutively activated EGFR can lead to malignant transformation of the cell. It was shown twenty years ago that blocking of the EGFR could inhibit cell proliferation in these transformed cells [1]. Since these first observations various drugs have been developed that target either the extracellular domain or the intracellular tyrosine kinase domain of the EGFR. Especially drugs of the latter category, small molecule adenosine triphosphate-competitive inhibitors of the receptor's tyrosine kinase (EGFR-TKIs) such as erlotinib and gefitinib, have proven their efficacy in the treatment of non-small cell lung cancer (NSCLC) [2-4]. However, response rates of erlotinib and gefitinib in unselected patient populations are low, and selection of patients is warranted to increase response rates to a more satisfying level. A response rate of 30% can be achieved when selection of patients is based on their phenotype (female gender, non-smoking status, Asian origin, adeno- or bronchioloalveolar carcinoma) [2,3,5-7]. This can be increased up to 70% when selection is based on EGFR mutation or FISH analysis [8-16]. However, for these assays availability of tumour tissue is a prerequisite, while this is frequently not at hand in advanced NSCLC. More simple and accessible predictors of response are warranted.

Recently, soluble EGFR (sEGFR) was recognized as a potential screening tool for epithelial cancer [17,18]. sEGFR is a proteolytically cleaved form of the extracellular domain of the EGFR, and can be measured directly in the serum [19,20]. The plausibility of sEGFR being a surrogate marker for response to treatment with an EGFR-TKI is based on the hypothesis that the level of sEGFR reflects the absolute number of activated receptors, susceptible to inhibition [21]. A decrease in sEGFR during treatment with gefitinib has been recognized to correlate with disease control in patients with non-small cell lung cancer (NSCLC) [21]. However, the role of baseline sEGFR as a predictive marker for response and survival in clinical practice is still uncertain.

The conventional tumour marker carcinoembryogenic antigen (CEA) is a member of the immunoglobulin supergene family, a cell surface adhesion protein, and it is thought to play a role in cell-to-cell adhesion(22). Since there is evidence that elevated pre-treatment levels of CEA are also predictive for response and outcome after EGFR-TKI treatment [22], independent of histological subtype, we decided to study both baseline sEGFR and CEA levels in relation to survival after EGFR-TKI treatment.

PATIENTS AND METHODS

Patients selection and study design

Between July 2002 and December 2005, patients with advanced non-small cell lung cancer, not responding to conventional chemotherapy or unable to receive chemotherapy due to poor medical condition, were offered treatment with gefitinib (Iressa®) or erlotinib (Tarceva®) as part of the Expanded Access

Programme, on a compassionate use basis. Consecutive patients who were treated for more than 14 days were identified and enrolled in this study if pre-treatment serum was available for sEGFR analysis. The final sample size was determined according to the number of available patient serum samples. Hospital records were retrospectively reviewed for age, gender, race, smoking status, histological subtype, stage, side effects and toxicity of the EGFR-TKI treatment and best overall response to EGFR-TKI. Informed consent was obtained from all patients. For design and report of this study the REMARK guidelines were followed [23].

Patients receiving gefitinib were treated with a daily dose of 250 mg. In case of unacceptable or severe (grade 3-4) toxicity the treatment with gefitinib was interrupted. Erlotinib was administered in daily doses of 150 mg. Dose changes of 50 mg were possible in case of unacceptable toxicity. Adverse events were assessed according to the National Cancer Institute - Common Toxicity Criteria version 2. Treatment of gefitinib or erlotinib was continued until disease progression or the occurrence of a serious adverse event.

Assessment of sEGFR and CEA levels

Blood samples had to be collected within 2 months before start of treatment with EGFR-TKIs. Serum was stored at -30˚C. Concentration levels of the EGFR-extracellular binding domain were determined by a sandwich quantitative enzyme-linked immunosorbent assay (EGFR Microtiter ELISA; Oncogene Science, Cambridge, MA) according to the manufacturer's instructions. The normal range is 45-78 µg/l as described previously [18]. CEA was measured on the E170 analyzer, which is based on chemiluminescent immunometric technology (Roche Diagnostics, Mannheim, Germany) [24].

Response assessment and statistical analysis

Correlation among sEGFR, CEA and age were studied using Pearson correlation analysis. Associations between sEGFR, CEA, gender, stage (III, IV), smoking status (smoker, non-smoker) and histology (adenocarcinoma, squamous cell carcinoma and large cell undifferentiated) were investigated by means of the student t-test or generalized linear regression.

Overall survival was calculated using the Kaplan-Meier method, from the first day of treatment with the EGFR-TKI to the date of death. Differences in survival between subgroups of patients were determined using the log rank test. Univariate analysis (Cox proportional hazard regression analysis) was used to detect associations between sEGFR and CEA levels and survival. Furthermore, age, gender, smoking status, tumour stage, histology, and treatment drug were investigated. The assumptions of linearity and proportional hazards for sEGFR and CEA were checked by means of Martingale residuals and scaled Schoenfeld residuals [25,26]. Continuous variables (age, sEGFR and logCEA) were tested for possible non-linear associations (violence of the proportional hazards assumptions). To present Kaplan-Meier plots for sEGFR and logCEA, a cut-off was used to divide these factors into two separate groups (i.e. high vs low). A spline function through the Martingale residuals of sEGFR and logCEA was used to determine possible cut-off values; i.e., the concentration of sEGFR or CEA where

the line crossed through zero of the Martingale residuals, was used as the cut-off. Variables achieving a probability value of less than 0.10 in the univariate analysis as well as preoperative factors considered relevant in the available literature[27-29] were introduced in a multivariate stepwise proportional hazard analysis to identify variables significantly associated with survival. P-values < 0.05 were considered statistically significant.

Response evaluation was performed using computed tomography (CT) according to Response Evaluation Criteria In Solid Tumors (RECIST) [30]. Response measurement at fixed intervals was not available for every patient. The occurrence of early progressive disease (within 3 months) was investigated to analyze the relation between (non-) response and sEGFR and/or CEA levels. Associations between high or low sEGFR and/or CEA levels, and early occurrence of PD were tested using non-parametric tests. For this purpose sEFGR and log CEA were dichotomized by the cut-off value described above.

RESULTS

Over a 3.5 years period, 145 patients with advanced non-small cell lung cancer were treated with gefitinib or erlotinib. Of these, 102 patients with available serum samples were eligible, 54 men and 48 women, with a mean age of 59 years (95% CI 57 – 61 years). Patient characteristics are shown in Table 1. The median follow up was 161 days (range 17-1581 days). EGFR mutation status was assessed in 13 patients, of whom 6 patients had mutations: 3 patients had a mutation in exon 19, one patient in exon 20, and 2 patients in exon 21. Sixty-seven patients were treated with gefitinib and 35 patients were treated with erlotinib. The median duration of treatment with gefitinib was 69 days (range 14-1259 days), and with erlotinib 78 days (range 15-814 days).

Baseline sEGFR levels were available for all 102 patients and showed a Gaussian distribution. The mean sEGFR level at baseline was 55.9 µg/l (SD 8.9). Given the normal range provided by the manufacturer of the test (48-72 µg/l), 23% of patients had decreased sEGFR levels. Patients with a squamous cell tumour had significant lower values of sEGFR compared to

Table 1. Patient and tumour characteristics (n=102).

Variable	No.
Age mean (SD)	59 yrs (12.2)
Gender	
Male	54
Female	48
Smoking status (n=99)	
Non	21
Former/ Current	78
Tumour stage (n=101)	
III	20
IV	81
Histology (n=99)	
Adenocarcinoma	66
Non-small cell, undifferentiated	20
Squamous cell carcinoma	13
Drug	
gefitinib	67
erlotinib	35
sEGFR µg/l mean (SD)	55.9 (8.9)
< 55 µg/l	43
≥ 55 µg/l	59
CEA µg/l (n=100) mean (SD)	1.17 (0.75)
< 12.6	57
≥ 12.6	43

patients with tumours of the undifferentiated cell type (p=0.0267); sEGFR levels of patients with adenocarcinoma were found in between. Age was the only patient characteristic that significantly inversely correlated with sEGFR (correlation -0.31, p=0.0014). No significant associations were detected for sEGFR levels with gender, smoking status or tumour stage.

Baseline CEA values were available for 100 patients. CEA values did not follow a normal distribution. The median serum CEA value overall was 11.1 µg/l (range <1.0 – 2938.0 µg/l). Using the internationally accepted upper limit of normal of 6.5 µg/l for smokers and 5.0 µg/l for non-smokers, 67 patients (67 %) had elevated CEA levels. Because of the skewed distribution of CEA, further analyses were performed using the logarithm of CEA (log CEA, mean 1.17, SD 0.75). Log CEA levels were significantly lower for stage III patients (p=0.01197), and for squamous cell compared to undifferentiated large cell type (p=0.0359). Values of patients with adenocarcinoma were very close to values of patients with tumours of the undifferentiated large cell type. For age, gender or level of sEGFR no association with log CEA was found.

When continuous variables (age, sEGFR and logCEA) were checked for possible non-linear associations, none were found to be significant. Consequently the continuous variables could be included as linear continuous parameters.

The median overall survival was 5.2 months (range 0.6 – 52.0 months). In an univariate analysis, smoking status and sEGFR were shown to be significant prognostic factors (Table 2, p=0.001 and p=0.018, respectively). Cut-off values for sEGFR and log CEA were found at 55 µg/l and 1.1 (corresponding with CEA= 12.6

Table 2. Univariate analysis in patients with advanced NSCLC before treatment with EGFR-TKIs.

Variable	No. of events	Median survival (95% CI)	Hazard ratio	95% CI	P
Age per year increment	95	5.2 (4.3 – 6.1)	0.999	0.983 – 1.015	0.857
Gender					
Male	51	5.4 (4.3 – 6.4)	1.009	0.673 – 1.514	0.965
Female	44	4.6 (2.4 – 6.8)			
Smoking status (n=99)					
Non	17	13.4 (8.1 – 18.6)	0.383	0.220 – 0.668	0.001
Former/Current	75	4.6 (3.1 – 6.0)			
Tumour stage (n=101)					
III	19	4.7 (3.4 – 6.0)	1.172	0.705 – 1.946	0.541
IV	75	5.4 (4.3 – 6.5)			
Histology (n=99)					
Adenocarcinoma	58	5.5 (4.1 – 6.9)	0.670	0.437 – 1.028	0.066
Non-adenocarcinoma	34	3.6 (1.1 – 6.2)			
Drug					
gefitinib	64	5.1 (4.0 – 6.3)	1.338	0.867 – 2.065	0.188
erlotinib					
sEGFR µg/l, per µg/l increment	31	7.2 (2.6 – 11.7)	0.970	0.946 – 0.995	0.018
Log CEA µg/l (n=100), per µg/l increment			1.174	0.908 – 1.519	0.222

Abbreviations: NSCLC, non-small cell lung cancer; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitors; sEGFR, soluble EGFR; CEA, carcinoembryonic antigen.

μg/l), respectively. Patients with sEGFR levels above 55 μg/l had a significantly longer overall survival (Figure 1A, log-rank p=0.033), while patients with a log CEA level below 1.1 showed a trend towards longer overall survival (Figure 1B, log-rank p=0.06). In a multivariate overall survival model sEGFR and log CEA, in addition to smoking, proved to be independently associated with survival (Table 3).

Early progression of disease or death within 3 months after the start of EGFR-TKI treatment occurred in 67 of 102 patients (66%). High sEGFR (> 55 μg/l) was associated with a lower chance of early PD (Table 4: 57% vs 77%, p = 0.04). No relation was detected between log CEA (or log CEA and sEGFR combined) and PD.

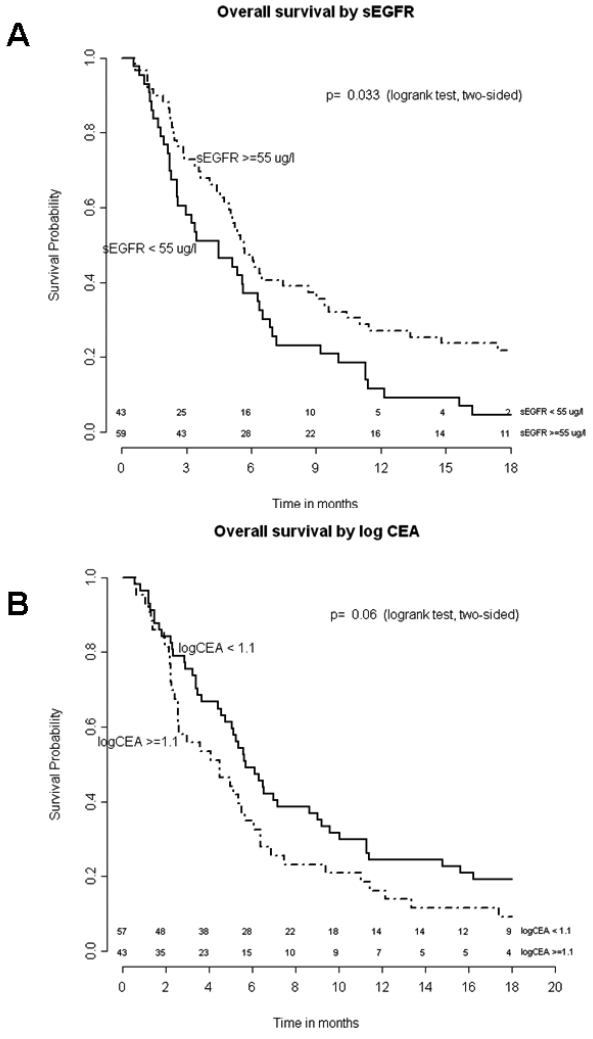

Figure 1. Overall survival by sEGFR (A) and log CEA (B). Numbers of patients at risk are given.

Table 3: Multivariate overall survival model in patients with advanced NSCLC before treatment with EGFR-TKIs: results of the Cox proportional hazard regression analysis.

	Hazard ratio	95% confidence interval	P
Male gender	0.633	0.395 - 1.015	0.058
Age, per year increment	0.990	0.970 - 1.011	0.346
Non-smoking	0.367	0.201 – 0.668	0.001
Tumour stage III	1.092	0.597 - 2.000	0.775
Adenocarcinoma histology	0.706	0.426 - 1.171	0.177
sEGFR, per µg/l increment	0.958	0.930 - 0.986	0.004
Log CEA, per µg/l increment	1.507	1.111 - 2.043	0.008

Abbreviations: NSCLC, non-small cell lung cancer; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitors; sEGFR, soluble EGFR; CEA, carcinoembryonic antigen.

Early progression of disease or death within three months after the start of the EGFR-TKI treatment occurred in 67 of 102 patients (66%). High sEGFR (>55ug/L) was associated with a lower chance of early PD (Table 4: 57% vs 77%, p=0.04). No relation was detected between log CEA (or log CEA and sEGFR combined) and PD.

Table 4: Relation between pre-treatment sEGFR and CEA levels and early progressive disease in patients with advanced NSCLC after treatment with EGFR-TKIs. PD = Progressive Disease.

	PD < 3 months		
	N / total	%	P
sEGFR < 55 µg/l	33/43	77	
sEGFR > 55 µg/l	34/59	57	0.04
CEA < 12.6 µg/l	35/57	61	
CEA > 12.6 µg/l	30/43	70	0.26
sEGFR > 55 and CEA < 12.6	18/31	58	
Other combinations	47/69	68	0.23

Abbreviations: PD, progressive disease; NSCLC, non-small cell lung cancer; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitors; sEGFR, soluble EGFR; CEA, carcinoembryonic antigen.

DISCUSSION

In the present study, baseline sEGFR and CEA levels were measured in patients with advanced NSCLC before treatment with erlotinib or gefitinib. Our results suggest that higher sEGFR and lower CEA levels are related to prolonged survival in patients receiving EGFR-TKI treatment, indicating that the combination of sEGFR and CEA could be valuable for the selection of patients for EGFR-TKI treatment.

CEA is a member of the immunoglobulin superfamily and plays a role in cell-to-cell adhesion [31]. When CEA is overexpressed on the cell surface it is thought to play a role in tumorigenesis by disruption of cell polarity, inhibition of apoptosis

(anoikis) and inhibition of cell differentiation [32-34]. The overexpression of CEA has been found to be present in many types of carcinomas [35]. In non-small cell lung cancer, an elevated serum CEA level is generally considered to be a negative prognostic factor especially for adenocarcinoma[36]. Therefore, the finding of Okamoto et al. that a high CEA was predictive for good response to EGFR-TKI treatment, independent of histology, was highly surprising [22]. They did not have an explaining mechanism of action for this phenomenon, but hypothesized that an anti-apoptotic signal of the (mutant) EGFR may somehow elevate the expression level of CEA protein. In this study we could not confirm the results of Okamoto et al. On the contrary, we found that a low CEA level was independently associated with better outcome after treatment with EGFR-TKIs. This is in accordance with previous findings of the negative prognostic ability of CEA [36].

Our results suggest that serum levels of CEA and sEGFR are not (directly) regulated by the same mechanism of action, since both remained significant upon multivariate analysis. Only few studies are available on sEGFR in non-small cell lung cancer, mostly concerning the comparison of sEGFR levels in healthy individuals and lung cancer patients. Two studies found that patients with NSCLC had lower baseline sEGFR levels compared to healthy controls [18,37], whereas others did not detect significant differences [38,39]. However, up till now there are no data available on the prognostic value of serum sEGFR for NSCLC. Only one study investigated changes in sEGFR levels during EGFR-TKI treatment as a predictive marker for response to these inhibitors [21]. Responders showed a decrease in sEGFR levels at time of best response compared to baseline level, whereas non-responders showed an increase. A difference of -3.6 µg/l as a cut-off was found to identify responders at time of best response. However, a meaningful cut-off level for (pre-treatment) baseline levels could not be established, and therefore sEGFR was not considered to be a useful predictive marker. Unfortunately, in our study serum samples drawn during treatment were not available and we therefore could not validate these results.

The one-armed design and retrospective nature of our study prohibit clear differentiation between the prognostic and the predictive value of sEGFR and CEA. Interpretation of response data (progressive disease) remains difficult, but these data suggest at least some predictive potential for sEGFR. Higher levels of pre-treatment sEGFR in patients treated with EGFR-TKI's were associated with lower risk of progressive disease within 3 months.

The prognosis of advanced NSCLC after failure of second or third line treatment is generally only weeks to months. Intensive follow-up with additional imaging during this period is undesirable, and further efforts to evaluate response or progression-free survival are meaningless. The distinction between the prognostic value and the predictive value of these two markers remains important, since their potential predictive value may contribute to an adequate patient selection for expensive EGFR-TKI treatment. Therefore validation of this potential predictive value in a prospective controlled (two-arm) study is warranted.

In conclusion, these results suggest that sEGFR and CEA are markers of survival in patients treated with EGFR-TKIs. The potential predictive value of sEGFR needs confirmation in a prospective controlled trial.

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CONFLICT OF INTEREST

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8

LIGANDS OF EGFR AND THE INSULIN-LIKE GROWTH FACTOR FAMILY AS SERUM BIOMARKERS FOR RESPONSE TO EGFR-INHIBITORS IN PATIENTS WITH ADVANCED NSCLC

ABSTRACT

Introduction: The selection of non-small cell lung cancer (NSCLC) patients for epidermal growth factor (EGFR) inhibitor (EGFR-TKIs) therapy is suboptimal as tumor tissue is often unavailable. Ligands of EGFR, transforming growth factor-α (TGFa) and amphiregulin (ARG) and the insulin-like growth factor family have been associated with resistance to EGFR-TKIs. The aim of our study was to explore whether concentrations of these factors measured in serum were predictive of response to EGFR-TKIs.

Methods: We assessed serum levels of marker candidates using ELISA (TGFa and ARG) and chemiluminescent (insulin-like growth factor-1 (IGF1) and insulin-like binding protein-3 (IGFBP3)) assays in 61 advanced NSCLC patients treated with EGFR-TKIs and 63 matched advanced NSCLC control patients without EGFR-TKIs treatment. We dichotomized marker levels at the 20th, 50th or 80th percentile and evaluated whether the effect of EGFR-TKIs treatment on disease-specific survival (DSS) differed by marker level based on multivariate proportional hazards regression with an interaction term.

Results: The effect of EGFR-TKIs treatment on DSS showed a significant difference by TGFa and ARG (interaction p=0.046 and p=0.004, respectively). Low concentrations of TGFa and high concentrations of ARG were associated with a better DSS in EGFR-TKIs patients compared to control patients. Patients with high concentrations of IGFBP3 had significantly longer DSS, independent of treatment (HR 0.60 per 1mg/L, 95%CI 0.46-0.79).

Conclusion: Our results suggest that concentrations of TGFa and ARG measured in serum are predictive of EGFR-TKI response. The combination of these two biomarkers could be of value in the process of selecting patients for treatment with EGFR-TKIs.

INTRODUCTION

Non small cell lung cancer (NSCLC) is the leading cause of cancer mortality worldwide. Small molecule inhibitors competing with the adenosine triphosphate binding site of the epidermal growth factor receptor (EGFR) tyrosine kinase such as erlotinib and gefitinib (EGFR-TKIs), have proven their efficacy in the treatment of NSCLC [1-4]. These orally administered EGFR-TKIs showed rapid tumor responses and improvements in quality of life in patients with advanced NSCLC who were irresponsive to platinum-based chemotherapy. Unfortunately, the response rates of erlotinib and gefitinb are low in unselected NSCLC patients and many studies tested EGFR for its predictive potential. EGFR-mutations and amplification of EGFR were found to be predictive of response to EGFR-TKIs with response rates of up to 70% [5-11]. However, there are several reasons why selection based on EGFR-mutation or EGFR amplification status alone might not suffice. Firstly, stabilization of disease has not been correlated with the presence of EGFR mutations or amplification, although this treatment effect is considered beneficial in the management of NSCLC [6,12]. Secondly, no mutations were identified in 10-20% of patients with a partial response to gefitinib [5,6,13-16]. Lastly, to select patients based on EGFR mutations or amplification tumor tissue is required, which is often unavailable. Identifying new methods to select patients likely to respond to EGFR-TKIs therefore remains important.

Many studies have tested ligands of EGFR in relation to EGFR-TKI response. Of the EGFR-specific ligands, transforming growth factor alpha (TGFa) and amphiregulin (ARG) have been studied intensively *in vitro* and in patients in relation to EGFR-TKI treatment [17-21]. Gene expression microarray studies showed that both TGFa and ARG were overexpressed in tumors from NSCLC patients not responding to gefitinib [17]. Subsequently, Ishikawa et al. and Masago et al. found that serum measurements of TGFa and ARG were predictive of overall survival and tumor response in advanced NSCLC patients treated with gefitinib [18,21]. However, since a control group was missing, differentiation between therapy benefit and survival benefit remains unclear.

In addition to ligands of EGFR, insulin-like growth factor receptor type 1 (IGF1R) has been implicated in resistance to EGFR-TKI treatment [22-25]. Cross-talk between IGF1R and EGFR has been reported and overexpression of IGF1R has been correlated with decreased efficacy of EGFR targeting in a glioblastoma model [22]. IGF1R is mainly activated by its ligand insulin-like growth factor-1 (IGF1) [26-28]. The bioavailability of IGF1 is regulated by a family of IGF-binding proteins (IGFBP), particularly IGFBP3, a major serum binding protein for IGF1 [26-28]. Consequently, the IGF1:IGFBP3 ratio is commonly used as readout for the bioactivity of IGF1 [26-31].

Since IGF1R is activated by IGF1, whose bioavailability is regulated by IGF-binding protein 3 (IGFBP3), we hypothesized that serum concentrations of IGF1, IGFBP3 or the IGF1:IGFBP3 ratio might be predictive of response to EGFR-TKIs, in addition to levels of TGFa and ARG. The aim of this study therefore was to explore whether concentrations of TGFa, ARG, IGF1 or IGFBP3 measured in serum of advanced NSCLC patients were predictive of response to EGFR-TKIs.

We assessed serum levels of these candidate markers in patients treated with EGFR-TKI and a matched control group of untreated patients. We evaluated whether the effect of EGFR-TKI treatment on disease-specific survival (DSS) differed by marker level based on multivariate proportional hazards regression with an interaction term.

PATIENTS AND METHODS

Patient Selection

In this retrospective study, we studied patients with advanced NSCLC who had been enrolled between 2001 and 2005 in an Expanded Access Program. Patients had been treated on a compassionate use basis with gefitinib or erlotinib (EGFR-TKI) at the Netherlands Cancer Institute. Patients without brain metastases were eligible for this program in case of no response to conventional chemotherapy or unavailability of alternative treatment options [11]. Written informed consent was obtained from all patients. The inclusion criteria for our current study consisted of EGFR-TKI treatment for over 14 days and availability of serum collected within 100 days *before* start of treatment. To differentiate between prognostic and predictive value of the ligands a control group of patients was identified, not treated with EGFR-TKIs and matched for gender, age and histology to the EGFR-TKI treated patients. Controls were diagnosed between 1995 and 2006. In total 124 patients were included in this study which was designed following the REMARK guidelines [32]. The current translational study was approved by the Institutional Review board of the Netherlands Cancer Institute.

Treatment

Patients receiving gefitinib were treated with a daily dose of 250mg. Erlotinib was dosed at 150mg daily, dose reductions to 50mg occurred due to severe toxicity (n=2). Treatment with EGFR-TKIs was continued until disease progression or the occurrence of a serious adverse event.

Serum analyses

Serum was stored at -30°C. TGFa concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA-kit, DTGA00, R&D systems) according to manufacturer's instruction. A standard curve was prepared for each plate using human recombinant TGFa diluted in assay diluent (provided by the ELISA-kit) for reference. The minimum detection limit of the assay was 3.0 ng/L. For detection of ARG an ELISA research kit (DY262, R&D systems) was used according to manufacturer's instructions (validation experiments Appendix I). In short, 96-well clear flexible microtiter plates (DY990, R&D systems) were coated with 2 mg/L capturing antibody overnight. After washing with PBS (pH 7.4) containing 0.05% Tween20, wells were blocked with 300µl Reagent Diluent (DY995, R&D systems) for 1 hour and washed again. Subsequently, wells were incubated for 2 hours with serum samples. After washing, the wells were incubated for 2 hours with 100 µg/L biotinylated goat anti-human ARG (DY262,

R&D Systems) followed by washing and a 20 minute reaction with streptavidin-conjugated horseradish-peroxidase. After washing, 100µl substrate solution (DY 999, R&D systems) was added for 20 minutes. The color reaction was stopped by adding 50µl 2 N sulfuric acid. Color intensity was determined at a wavelength of 450 nm with reference wavelength of 540nm. The standard curve was drawn for each plate using recombinant ARG diluted in 10% fetal calf serum in PBS. Minimum detection limit of the assays for serum ARG was 3.0 ng/L (validation experiments Appendix I).

For the concentrations of IGF1 and IGFBP3 fully automated chemiluminescent immunometric technology was used (Immulite 2000®, Siemens Medical Solutions Diagnostics). To calculate the IGF1:IGFBP3 ratio, IGFBP3 (mg/L) was converted to molar concentrations with a conversion factor of 34.78. The normal range provided by the manufacturer for the age group 61-65 years was 9.75-27.56nmol/L for IGF1 and 3.2-6.6mg/L for IGFBP3.

Statistical analyses

Disease-specific survival (DSS) was defined as the time from start of treatment with the EGFR-TKI to death of disease, or end of follow-up. Patients who were still alive at the end of follow-up, lost to follow-up or who died due to non-NSCLC causes were censored at that time. For the control group DSS was calculated from the start of first treatment (radiotherapy or chemotherapy) *in* the Netherlands Cancer Institute (NKI) or date of first visit if no treatment was started within the NKI.

Differences in clinicopathological variables between the EGFR-TKI group and the control group were tested using Fisher's exact tests, Kruskal-Wallis tests, exact Mann-Whitney U tests and student T-tests. Patients with missing values for a variable were excluded from analyses involving that variable. Correlation between levels of candidate markers and age, and serum storage time were calculated using Spearman's rank and Pearson correlation analyses. Associations between marker candidates and gender, stage (III, IV), smoking status (smoker, non-smoker), performance status (WHO 0-1, 2-3) and histology (adenocarcinoma, other) were investigated by means of Mann-Whitney U tests and student T-tests.

We evaluated whether the effect of EGFR-TKI treatment on disease-specific survival (DSS), expressed as hazard ratio (HR), differed by candidate marker level based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders (gender, smoking, prior chemotherapy, stage, histology). Instead of searching for an optimal cut-off we a priori selected three alternative cut-offs (20th, 50th or 80th percentile) to dichotomize marker levels. For all cut-offs interaction terms were calculated using the multivariate model; the cut-off with the largest absolute interaction coefficient was evaluated for the proportional hazards assumption by adding interaction terms between the time-dependent natural logarithm of follow-up time plus one and the interaction between treatment and candidate markers (p-value between 0.33 and 0.92). No evidence for non-proportional hazards was found and the cut-offs of these candidate markers were further evaluated. Direct adjusted survival curves based on a multivariate Cox regression model including prior chemotherapy, performance

status and smoking and stratified for treatment were calculated using a SAS macro by Zhang et al. 2007 [33]. All other calculations were performed using the statistical package SPSS (version 15.0 for Windows).

RESULTS

Sixty-one EGFR-TKI treated patients were eligible for our current study (Figure 1). The control group consisted of 63 patients, matched for gender, age and histology as shown together with other clinical and pathological characteristics in Table 1. Patients treated with EGFR-TKIs were significantly more often never-smokers (p=0.03), had more stage IIIa/b disease (p<0.01, Table1) and presented more often with a poor performance status (p=0.05). Furthermore, this group had significantly more often received prior chemotherapy (p<0.01, Table1). All four marker candidates could be tested in all 124 patients. IGF1, IGFBP3 and the IGF1:IGFBP3 ratio showed a Gaussian distribution, while TGFa and ARG were lognormal distributed. Factors were not correlated with serum storage time (Supplementary Figure 1), age, stage, histology (data not shown) or prior chemotherapy (Supplementary Figure 2). Women had significantly higher concentrations of TGFa (Mann Whitney U, p=0.03), while patients with higher levels of IGFBP3 were significantly more often non-smokers (student T-test, p=0.03) and had a better performance status (student T-test p= 0.005, Supplementary Figure 2).

Smoking, prior chemotherapy and performance status were significantly associated with DSS (Supplementary Table 1) and were therefore included in multivariate analyses as potential confounders. The 20% cut-off yielded the largest

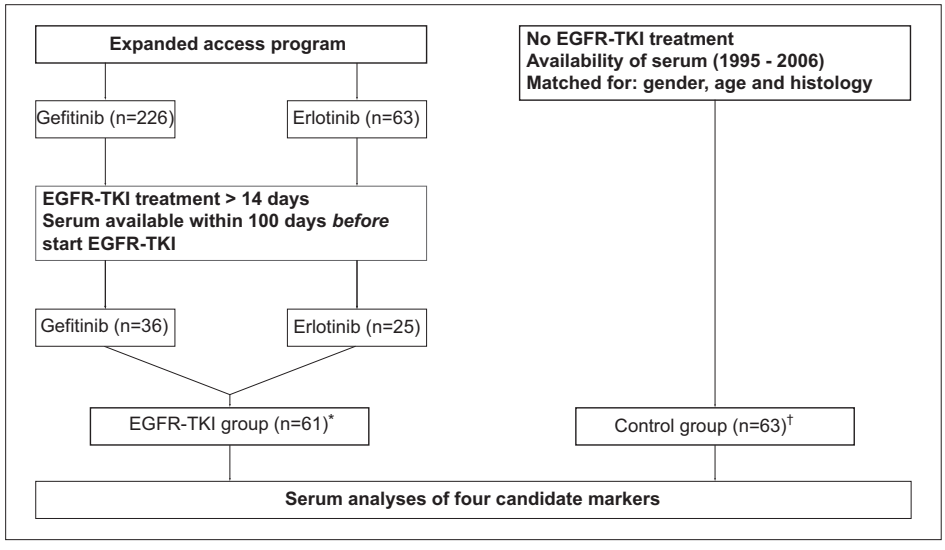

Figure 1. Flow chart of patient selection.
* Included in EGFR-TKI group: EGFR-TKIs treatment 12 days (n=1).
† Included control group: 9 days of EGFR-TKIs treatment before death of disease (n=1).

Table 1. Patient Characteristics.

	Total		Treatment				
			EGFR-TKI treated patients		Control patients		
	n	(%)	n	(%)	n	(%)	p-value
Total	124	(100)	61	(100)	63	(100)	
Gender							
Male	68	(55)	33	(54)	35	(56)	1.000*
Female	56	(45)	28	(46)	28	(44)	
Age at treatment							
< 65 years	73	(59)	34	(56)	39	(62)	0.584*
≥65 years	51	(41)	27	(44)	24	(38)	
Smoking							
Never smoked	16	(13)	12	(20)	4	(6)	0.029*
(former) smoker	102	(82)	44	(72)	58	(92)	
Unknown	6	(5)	5	(8)	1	(2)	
Performance Status							
0-1	86	(69)	37	(61)	49	(78)	0.052*
2-3	29	(23)	19	(31)	10	(16)	
Unknown	9	(7)	5	(8)	4	(6)	
Histology							
Large cell	31	(25)	13	(21)	18	(29)	0.673†
Squamous cell	20	(16)	9	(15)	11	(17)	
Adenocarcinoma	59	(48)	32	(52)	27	(43)	
Broncho Alveolair carcinoma	6	(5)	3	(5)	3	(5)	
Unknown	8	(6)	4	(7)	4	(6)	
Stage of disease							
IIIa/IIIb	20	(16)	16	(26)	4	(6)	0.003*
IV	104	(84)	45	(74)	59	(94)	
TNM T-status							
T1 - T2	46	(37)	16	(26)	30	(48)	0.356*
T3 - T4	34	(27)	16	(26)	18	(29)	
Tx & unknown	44	(35)	29	(48)	15	(24)	
TNM N-status							
N0 - N1	11	(9)	6	(10)	5	(8)	0.310*
N2 - N3	61	(49)	21	(34)	40	(63)	
Nx & unknown	52	(42)	34	(56)	18	(29)	
Prior Chemotherapy							
No	86	(69)	29	(48)	57	(90)	<0.001*
Yes	38	(31)	32	(52)	6	(10)	
Disease-specific Survival							
Range (days)	12 - 1638		16 - 1638		12 - 865		
Median (days)	151		115		171		0.156‡

Table 1. Patient Characteristics.

	Total		Treatment: EGFR-TKI treated patients		Treatment: Control patients		
	n	(%)	n	(%)	n	(%)	p-value
TGFa							
Range (ng/L)	0 - 135.0		0 - 135.0		0 - 73.8		
Median (ng/L)	10.5		11.1		9.6		0.383‡
ARG							
Range (ng/L)	0 - 2034.0		0 - 2034.0		0 - 1143.2		
Median (ng/L)	9.5		8.7		9.8		0.476‡
IGF1							
Range (nmol/l)	2.74 - 43.07		4.61 - 39.51		2.74 - 43.07		
Median (nmol/l)	17.55		17.35		17.60		0.815§
IGFBP3							
Range (mgl/L)	1.46 - 6.24		1.46 - 5.81		1.59 - 6.24		
Median (mg/L)	3.92		3.93		3.91		0.800§

p-value calculated using: *Fisher exact test; †Exaxt Kruskal-Wallis test; ‡Exact Mann-Whithney U test; §Student t-test. ***Abbreviations:*** TGFa, transforming growth factor-α; ARG, amphiregulin; IGF1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor binding protein-3.

interaction coefficient in the multivariate model for IGF1 and IGFBP3. Similarly, the median cut-off for ARG and the 80% cut-off for TGFa and IGF1:IGFBP3 ratio proved to be the largest (data not shown). These cut-offs were selected for further analyses.

The effect of EGFR-TKI treatment differed significantly between patients with high and low levels of TGFa (interaction p=0.05). Among patients with low levels of TGFa the risk of death was almost two-fold significantly decreased after EGFR-TKI treatment compared to the control group (Figure 2A, HR 0.55, 95%CI 0.32-0.96, p=0.04, Table 2), while in patients with high levels of TGFa this risk was non-significantly increased (Figure 2A, HR 1.51, 95%CI 0.58-3.91, p=0.40, Table 2).

ARG interacted significantly with the effect of EGFR-TKI treatment on DSS (interaction p<0.01). In patients with high levels of ARG, EGFR-TKI treatment significantly decreased the risk of death more than three-fold compared to the control patients (Figure 2B, HR 0.31, 95%CI 0.15-0.63, p<0.01, Table 2). No significant difference between both groups was found in patients with low ARG levels (Figure 2B, HR 1.14, 95%CI 0.59-2.17, p=0.70).

We observed a borderline significant benefit from EGFR-TKI treatment (interaction p=0.09) among patients, whose IGF1 levels exceeded the 20th percentile (Figure 2C, HR 0.48, 95%CI 0.27-0.83, p<0.01, Table 2) but not among others (Figure 2C, HR 1.27, 95%CI 0.45-3.53, p=0.65, Table 2).

EGFR-TKI treatment effects differed less strongly by levels of IGF1:IGFBP3 ratio or IGFBP3 and homogeneity was not rejected (Supplementary Table 2). Since IGFBP3 was significantly associated with performance status, a strong prognostic factor, we studied whether IGFBP3 was independently associated with

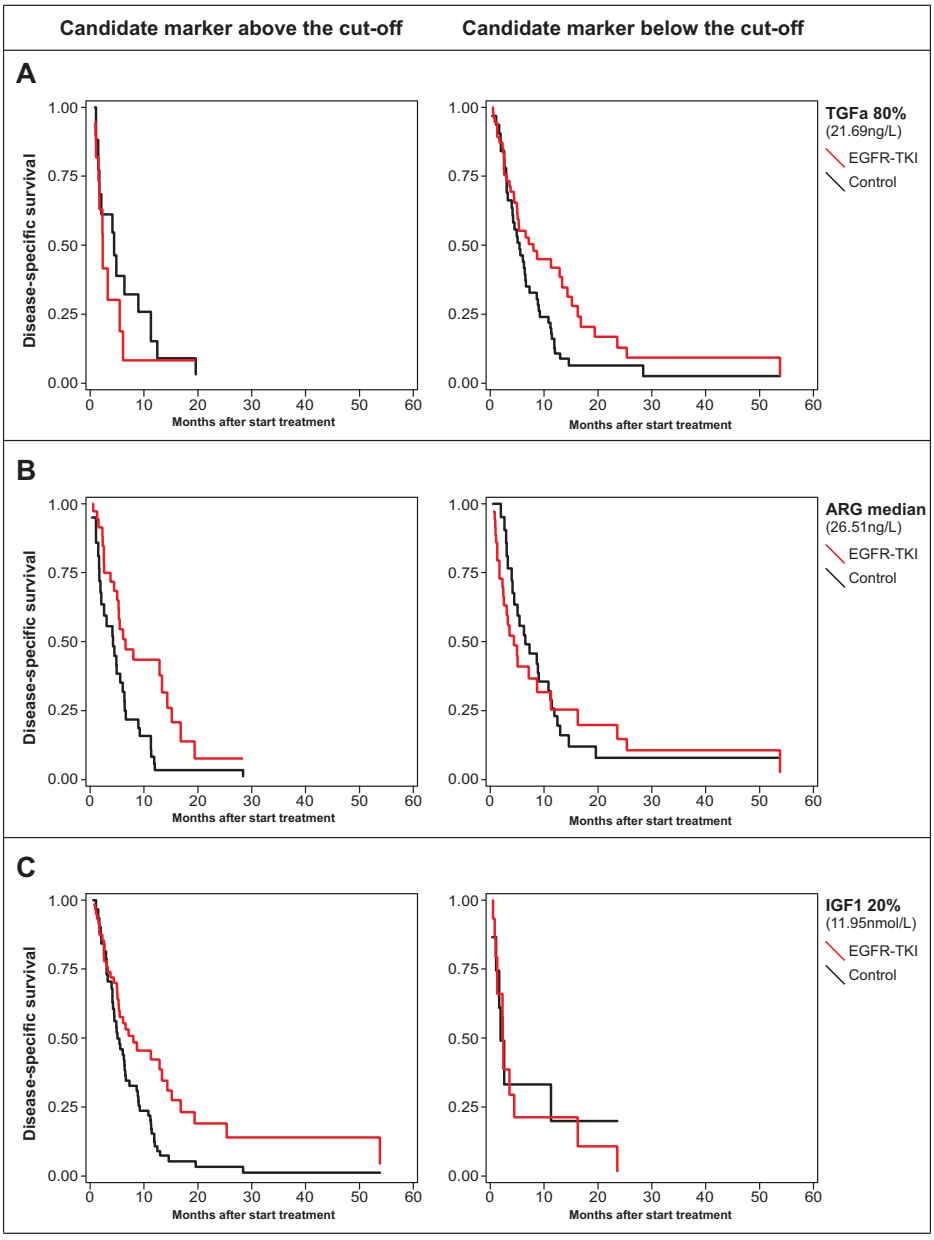

Figure 2. Relationship of candidate markers with outcome in EGFR-TKI treated patients and their matched controls. Direct adjusted survival curves based on a multivariate Cox regression model including prior chemotherapy, performance status and smoking and stratified for treatment. A) Disease-specific survival (DSS) according to TGFa levels above and below 80th percentile among patients treated with EGFR-TKIs and their matched controls. B) DSS according to ARG levels above and below the median among patients treated with EGFR-TKIs and their matched controls. C) DSS according to IGF1 above and below 20th percentile among patients treated with EGFR-TKIs and their matched controls.

Table 2. Multivariate Cox proportional-hazard regression analysis of the risk of death (disease-specific survival) per factor of interest.

	TGFa				ARG†				IGF1‡			
Variable	**No. Events / No. Patients**	**Hazard Ratio**	**95% CI**	**p-value**	**No. Events / No. Patients**	**Hazard Ratio**	**95% CI**	**p-value**	**No. Events / No. Patients**	**Hazard Ratio**	**95% CI**	**p-value**
Smoking												
(former) smoker	95 / 102	1.00			95 / 102	1.00			95 / 102	1.00		
Never smoked	13 / 16	0.76	0.38 – 1.52	0.440	13 / 16	0.59	0.29 – 1.16	0.127	13 / 16	0.59	0.30 – 1.16	0.123
Prior Chemotherapy												
No	79 / 86	1.00			79 / 86	1.00			79 / 86	1.00		
Yes	35 /38	2.97	1.68 – 5.23	<0.001	35 /38	3.47	1.98 – 6.09	<0.001	35 /38	3.78	2.17 – 6.61	<0.001
Performance Status												
0-1	78 / 86	1.00			78 / 86	1.00			78 / 86	1.00		
2-3	27 / 29	2.51	1.53 – 4.10	<0.001	27 / 29	2.97	1.76 – 5.00	<0.001	27 / 29	2.43	1.50 – 3.94	<0.001
Ligand concentration												
Low	90 / 99	1.00			56 / 62	1.00			22 / 25	1.00		
High	24 / 25	1.41	0.74 – 2.68	0.296	58 / 62	1.88	1.07 – 3.30	0.029	92 / 99	0.73	0.31 – 175	0.481
Ligand concentration high												
Control	13 / 14	1.00			31 / 33	1.00			49 / 53	1.00		
EGFR-TKI treatment	11 / 11	1.51*	0.58 – 3.91	0.399	27 / 29	0.31†	0.15 – 0.63	0.001	43 / 46	0.48‡	0.27 – 0.83	0.009
Ligand concentration low												
Control	44 / 49	1.00			26 / 30	1.00			8 / 10	1.00		
EGFR-TKI treatment	46 / 50	0.55*	0.32 – 0.96	0.035	30 / 32	1.14†	0.59 – 2.17	0.702	14 / 15	1.27‡	0.45 – 3.53	0.654

Homogeneity of both hazard ratios was rejected based on an interaction term with: * p= 0.046 (TGFa: cut-off at 80% (ligand low ≤21.69ng/L; ligand high>21.69ng/L)); †p=0.004 (ARG: cut-off at median (ligand low≤9.49ng/L; ligand high>9.49ng/L); ‡ Homogeneity of both hazard ratios was not rejected based on an interaction term with p=0.087 (IGF1: cut-off at 20% (ligand low≤11.95nmol/L; ligand high>11.95nmol/L)).
Abbreviations: TGFa, transforming growth factor-α; ARG, amphiregulin; IGF1, insulin-like growth factor-1.

DSS, regardless of treatment. We evaluated this factor as a continuous variable while adjusting for treatment. We observed a 40% decrease in risk per 1mg/L IGFBP3 increase (HR 0.60, 95%CI 0.46-0.79, p<0.001, Table 3).

Since the EGFR-TKI treated patients and the control patients were imbalanced for two important prognostic factors (i.e. prior chemotherapy and performance status), we subsequently repeated the analyses of TGFa, ARG and IGF1 in patients who had not received prior chemotherapy only (number of EGFR-TKI treated patients and controls reduced from 61 and 63 to 29 and 57, respectively) and in patients with a good performance status only (number of EGFR-TKI treated patients and controls reduced to 37 and 49, respectively). We observed very similar patterns of treatment-related hazard ratios by serum marker levels (Supplementary Table 3).

DISCUSSION

In this study we determined whether concentrations of TGFa, ARG, IGF1, IGFBP3 or the IGF1:IGFBP3 ratio measured in serum were predictive of EGFR-TKI response by comparing the disease-specific survival in patients treated with EGFR-TKIs compared to matched EGFR-TKIs untreated patients. We observed that patients with low serum concentrations of TGFa or high serum concentrations of ARG significantly benefited from EGFR-TKI treatment while there was no such evidence for patients with higher and lower respective values. Whether serum concentrations of IGF1 are predictive of response to EGFR-TKIs remains unclear, although our data suggest this may be the case.

Table 3. Multivariate Cox proportional-hazard regression analysis of death (disease-specific survival) according to IGFBP3, independent of treatment.

Variable	No. Events	Hazard Ratio	95% CI	p-value
Smoking				
(former) smoker	95	1.00		
Never smoked	13	0.88	0.43 – 1.79	0.726
Prior Chemotherapy				
No	79	1.00		
Yes	35	3.66	2.12 – 6.31	<0.001
Performance Status				
0-1	78	1.00		
2-3	27	2.04	1.26 – 3.31	0.004
Ligand concentration				
IGFBP3 per 1 mg/L	114	0.60	0.46 – 0.79	<0.001
Treatment				
Control	57	1.00		
EGFR-TKI treatment	57	0.56	0.34 – 0.94	0.027

Abbreviations: IGFBP3, insulin-like growth factor binding protein-3.

Our study confirms previous findings in which patients with high levels of TGFa do not benefit from EGFR-TKI treatment. More specifically, two Japanese groups found high levels of both TGFa and ARG to be associated with progressive disease and a worse overall survival after gefitinib treatment in NSCLC patients [18,21]. Since both studies lacked a control group, it was impossible to determine whether this difference in survival was due to therapy (prediction) or to tumor features (prognosis). Furthermore, both studies looked for an optimal threshold using internal data. Our results regarding ARG do not correspond with the findings mentioned above. Instead of a worse disease-specific survival, we observed a statistically significant benefit from EGFR-TKIs in patients with high serum ARG levels compared to the control group, but not among patients with low levels; this difference was statistically significant. This different direction of ARG has previously been observed. *In vitro,* primarily gefitinib sensitive head and neck cancer cell lines were shown to secrete amphiregulin [34]. Yonesaka et al. showed that high ARG expression as assessed by immunohistochemistry was associated with stable disease in NSCLC patients treated with erlotinib or gefitinib, while low expression was associated with progressive disease [35]. Furthermore, in patients with colorectal cancer treated with Cetuximab (monoclonal antibody against EGFR) high ARG expression by microarray analyses was associated with disease control (response and stable disease) and longer progression-free survival [36,37]. The discrepancy between our findings of ARG and both Japanese serum studies could be explained by the different ethnicity of the study populations. NSCLC patients from Asian origin are known to harbor EGFR mutations more frequently [38,39]. Subsequently, it is thought that these tumors may have a distinct pathogenesis in which tumors become completely dependent on the EGFR-pathway through mutations [38,39]. We speculate that high ARG levels might represent a different pathogenic pathway in NSCLC patients of Caucasian origin that leads to similar dependence on this pathway, albeit less complete. As a consequence treatment of patients with high ARG levels with EGFR-TKIs might lead to cell cycle arrest (i.e. stable disease) but not, as with EGFR mutations, to apoptosis (i.e. partial and complete response) as has been described in cell line studies [35,40]. Furthermore, this could explain the rapid progression of disease ("tumor flare") after discontinuation of EGFR-TKIs [41] as has also been documented in gastrointestinal stromal tumors after withdrawal of the BCR-ABL tyrosine kinase inhibitor imatinib [42].

In addition to ligands of EGFR, we studied components of the IGF1R-pathway. To the best of our knowledge this is the first study evaluating the relationship between benefit from EGFR-TKIs and IGF1, IGFBP3 and the IGF1:IGFBP3 ratio. IGF1 is known to stimulate cell proliferation and to inhibit apoptosis by binding to IGF1R, a receptor tyrosine kinase [27,43]. Activation of IGF1R leads to signaling of the proliferative Ras-Raf-MAPK pathway and of the pro-survival phosphoinositol-3 kinase (PI3K)-Akt pathway [27,43]. Failure to downregulate Akt characterizes insensitivity to EGFR-TKIs and has been shown to be mediated through PI3K signaling by IGF1R [22-25,44,45]. Furthermore, the combined use of anti-IGF1R and EGFR-TKIs has been shown to be more effective *in vitro* and *in vivo* than a single agent approach [25,46]. In our study, patients with especially low levels of IGF1 did not seem to benefit from EGFR-TKIs. We speculate that truly low levels of IGF1

are the result of a negative feedback-loop as the consequence of a constitutively active IGF1R-pathway in these patients. This negative regulation has not been documented but the opposite has in centennials, in whom a mutation in IGF1R was associated with reduced activity of IGF1R and high serum levels of IGF1 [47]. Consequently, tumors of patients with low IGF1 may be able to sustain the pro-survival signals through Akt activation by depending on the IGF1R-pathway for PI3K signaling instead of the EGFR-pathway. The only study reporting on the relation between the IGF1R-pathway, in the form of IGF1R expression assessed by immunohistochemistry, and response to gefitinib showed no association with gefitinib resistance [48]. These results cannot directly be compared to our results, since we studied different components of the IGF1R-pathway using different techniques.

Although studying prognostic factors was not an objective of our study, we found that IGFBP3 serum levels predicted disease-specific survival in advanced NSCLC patients regardless of treatment. The protective effect of high IGFBP3 levels could be explained by its ability to decrease the mitogenic action and inhibit the antiapoptotic effect of IGF1, but also by its IGF-independent inhibitory effect on cell growth [26,28,49-51]. Our results confirm findings of a previous study in which high IGFBP3 plasma levels were associated with a significantly longer overall survival in advanced NSCLC patients treated with irinotecan and cisplatin [52]. Furthermore, reduced IGFBP3 expression assessed by immunohistochemistry was associated with shorter disease-specific survival in patients with stage I NSCLC [53]. Use of serum IGFBP3 as a prognostic marker in stage I/II NSCLC would be appealing and warrants further study.

Our study was relatively small and therefore our evaluation could not attain the rigor of a training/validation study or a large randomized controlled trial. Furthermore, the EGFR-TKI group was not entirely homogeneous, since patients treated with gefitnib or erlotinib were included. However, these agents act via similar mechanisms, and sensitivity analyses by drug, although based on small numbers, were largely consistent with the combined results. Therefore, since this is an exploratory study of candidate markers we believe that we have assembled a sufficiently large data set to discover potentially predictive markers and have limited the number of cut-offs in order to avoid substantial overfitting. The retrospective nature of our study did not allow for a standardized measure for progression-free survival, which is considered to be more directly linked to response. This was due to the fact that imaging was not performed at set intervals. However, using data of documented radiological progression (or if unavailable, date of clinical progression) for progression-free survival resulted in very similar patterns of HRs for TGFa, ARG or IGF1 (data not shown). Furthermore, until now known markers predicting for response and progression-free survival, such as EGFR-mutations, have not been shown to predict for an overall or disease-specific survival benefit after EGFR-TKIs in randomized controlled trials [9,15,54], while this remains the ultimate goal in patient treatment. We therefore consider the presented data adequate to inform us whether and for which markers further studies are indicated. Although EGFR-TKIs response depends partly on presence of EGFR-mutations or EGFR amplifications, the two markers we identified could greatly

improve the prediction of EGFR-TKIs response in two ways. Firstly, not all patients who respond to EGFR-TKIs are identified by EGFR mutations or amplifications, resulting in withholding advanced NSCLC patients potential survival benefit from EGFR-TKIs (undertreatment). In this mostly palliative setting with limited treatment options, undertreatment would seem worse than overtreatment which can be justified to some extent. Consequently, limiting undertreatment by identification of a subgroup of patients resistant to EGFR-TKIs seems just as, or even more important. Secondly, serum measurements do not require tumor tissue, which is often unavailable, therefore greatly facilitating the prediction.

In conclusion, by using a matched control group we were able to evaluate EGFR-TKI treatment benefit by marker level. Our results suggest that concentrations of TGFa and ARG measured in serum are predictive of EGFR-TKI response. This is the first study in NSCLC patients of Caucasian origin in which this effect was evaluated and observed. The combination of these two biomarkers could be of value in the process of selecting patients for treatment with EGFR-TKI. The optimal cut-off for TGFa and ARG, the use of the combination of these two biomarkers as a predictive marker and its additive value to known clinical predictors for EGFR-TKI resistance warrants further investigation and validation, preferably in a prospective randomized controlled trial.

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CONFLICT OF INTEREST

The authors have no financial and/or personal relationships with commercial people or organisations that could influence or bias their work.

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Supplementary Table 1. Univariate Cox proportional-hazard regression analysis of death (disease-specific survival).

Variable	No. events	Hazard Ratio	95% CI	p-value
Gender				
Male	64			
Female	50	0.83	0.57 – 1.22	0.348
Age at treatment				
< 65 years	68			
≥65 years	46	0.75	0.51 – 1.09	0.133
Smoking				
(former) smoker	95			
Never smoked	13	0.50	0.27 – 0.93	0.028
Performance Status				
0-1	78			
2-3	27	1.98	1.26 – 3.11	0.003
Histology				
Adenocarcinoma	54			
Other	55	1.20	0.82 – 1.77	0.343
Stage of disease				
IIIa/IIIb	19			
IV	95	0.90	0.55 – 1.48	0.683
TNM T-status				
T1 - T2	39			
T3 - T4	34	1.06	0.66 – 1.69	0.811
TNM N-status				
N0 - N1	10			
N2 - N3	55	1.32	0.67 – 2.61	0.423
Prior Chemotherapy				
No	79			
Yes	35	2.04	1.35 – 3.09	0.001

Supplementary Table 2. Multivariate Cox proportional-hazard regression analysis of the risk of death (disease-specific survival) per factor of interest.

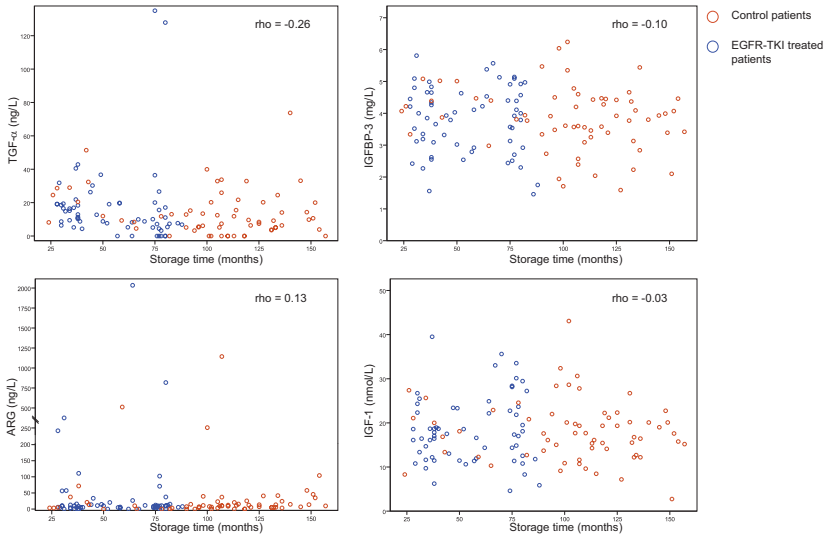
	IGFBP3*				IGB1:IGFBP3†			
Variable	**No. Events**	**Hazard Ratio**	**95% CI**	**p-value**	**No. Events**	**Hazard Ratio**	**95% CI**	**p-value**
Smoking								
(former) smoker	50	1.00			50	1.00		
Never smoked	64	0.75	0.39 – 1.47	0.406	64	0.65	0.33 – 1.29	0.218
Prior Chemotherapy								
No	95	1.00			95	1.00		
Yes	13	3.90	2.23 – 6.82	<0.001	13	3.17	1.82 – 5.53	<0.001
Performance Status								
0-1	79	1.00			79	1.00		
2-3	35	2.47	1.52 – 4.02	<0.001	35	2.20	1.32 – 3.65	0.002
Ligand concentration								
Low	23	1.00			89	1.00		
High	91	0.35	0.16 – 0.76	0.008	25	0.62	0.31 – 1.23	0.167
Ligand concentration high								
Control	48	1.00			12	1.00		
EGFR-TKI treatment	43	0.47	0.27 – 0.82	0.008	13	0.98	0.39 – 2.50	0.968
Ligand concentration low								
Control	9	1.00			45	1.00		
EGFR-TKI treatment	14	0.72	0.28 – 1.84	0.488	44	0.52	0.29 – 0.93	0.027

*IGFBP3: cut-off at 20% (ligand low ≤2.92mg/L; ligand high>2.92), interaction analysis p=0.400;
†IGF1:IGFBP3 ratio: cut-off at 80% (ligand low≤0.1696ng/L; ligand high>0.1696), interaction analysis p=0.233.
Abbreviations: IGF1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor binding protein-3.

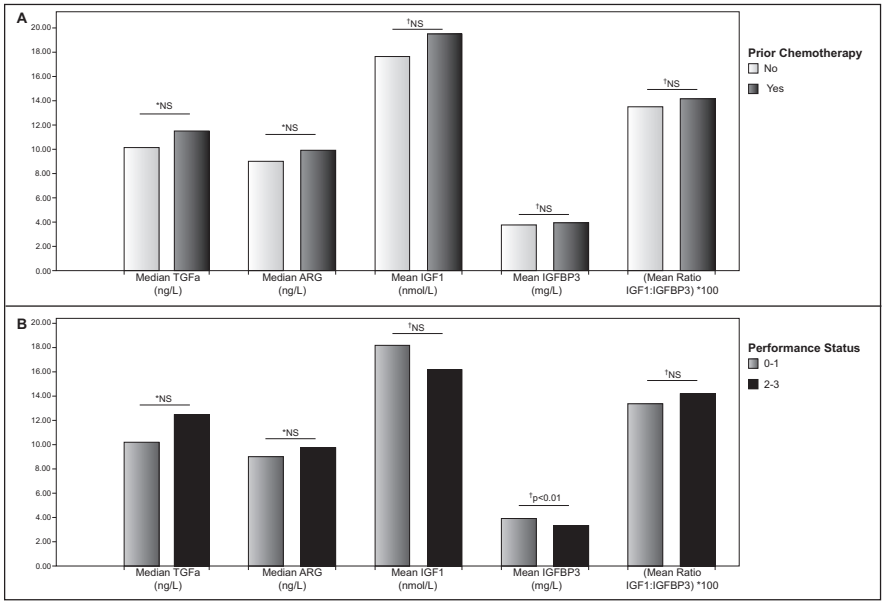
Supplementary Table 3. Multivariate Cox proportional-hazard regression analysis of the risk of death (disease-specific survival) per factor of interest specifically in patients with no prior chemotherapy and in patients with Performance Status 0-1.

	TGFa			
Variable	**HR patients with no prior Chemotherapy**	**p-value (no CT)**	**HR patients PS 0-1**	**p-value (PS 0-1)**
Ligand concentration				
Low	1.00		1.00	
High	1.44	0.274	1.34	0.398
Ligand concentration high				
Control	1.00		1.00	
EGFR-TKI treatment	3.16	0.057	0.85	0.788
Ligand concentration low				
Control	1.00		1.00	
EGFR-TKI treatment	0.51	0.049	0.47	0.020
	ARG			
Variable	**HR patients with no prior Chemotherapy**	**p-value (no CT)**	**HR patients PS 0-1**	**p-value (PS 0-1)**
Ligand concentration				
Low	1.00		1.00	
High	1.81	0.053	1.74	0.077
Ligand concentration high				
Control	1.00		1.00	
EGFR-TKI treatment	0.41	0.041	0.30	0.004
Ligand concentration low				
Control	1.00		1.00	
EGFR-TKI treatment	0.87	0.747	0.78	0.500
	IGF1			
Variable	**HR patients with no prior Chemotherapy**	**p-value (no CT)**	**HR patients PS 0-1**	**p-value (PS 0-1)**
Ligand concentration				
Low	1.00		1.00	
High	0.82	0.677	0.76	0.532
Ligand concentration high				
Control	1.00		1.00	
EGFR-TKI treatment	0.46	0.029	0.37	0.003
Ligand concentration low				
Control	1.00		1.00	
EGFR-TKI treatment	1.16	0.805	1.14	0.819

All analyses shown were adjusted for smoking, and performance status or prior chemotherapy in respectively the analysis of no prior chemotherapy selected patients and the analysis of performance status 0-1 selected patients.
Abbreviations: HR, hazard ratio; CT, chemotherapy; PS, performance status;transforming growth factor -α; ARG, amphiregulin; IGF1, insulin-like growth factor-1.



Supplementary Figure 1. Influence of the serum storage duration on serum marker levels. Scatter plots of all concentrations of serum marker candidates by their duration of storage time by -30°C.



Supplementary Figure 2. Ligand concentrations per prior chemotherapy and per performance status. **(A)** Association of ligand concentrations and prior chemotherapy. **(B)** Association of ligand concentrations and performance status. Because TGFa and ARG showed no Gaussian distribution the median instead of the mean is depicted. * Exact Mann Whitney U test. † Student T-test. *Abbreviations*: NS, non significant; TGFa, transforming growth factor-alpha; ARG, amphiregulin; IGF1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor binding protein-3.

APPENDIX: VALIDATION AMPHIREGULIN DY262, R & D SYSTEMS

Table 1: Recovery linearity of amphiregulin: dilution in reagens diluent of R&D

Sample	Dilution	Measured Concentration (ng/L)*	Calculated Concentration (ng/L)	recovery linearity (%)
32423	0x	>1000	>1000	NA
32423	3x	562.6	1687.7	NA
32423	5x	330.4	1652.0	NA
9184	0x	220.9	220.9	100.0
9184	5x	25.6	127.9	57.9
9184	10x	10.8	107.7	48.8
28489	0x	117.3	117.3	100.0
28489	3x	43.3	130.0	110.8
28489	5x	25.9	129.7	110.6
24227	0x	69.9	69.9	100.0
24227	2x	39.8	79.7	114.0
24227	4x	18.1	72.3	103.4
24227	5x	11.7	58.4	83.5
24227	8x	10.1	80.9	115.7
24227	10x	8.5	84.9	121.4
24227	16x	4.0	64.2	91.8
24227	20x	3.7	73.5	105.2
4529	0x	23.3	23.3	100.0
4529	5x	<3.0	Min	NA
4529	10x	<3.0	Min	NA
21701	0x	12.0	12.0	100.0
21701	3x	4.22	12.7	106.0
21701	5x	<3.0	Min	NA
28362	0x	11.9	11.9	100.0
28362	2x	4.4	8.7	73.2
28362	4x	<3.0	Min	NA
28362	8x	<3.0	Min	NA
37107	0x	<3.0	Min	NA
37107	2x	<3.0	Min	NA
37107	4x	<3.0	Min	NA
37107	8x	<3.0	Min	NA
37107	16x	<3.0	Min	NA

* Detection limit: 3.0 ng/L. *Abbreviations:* Min, minimum under detection limit; NA, not applicable.

Table 2: Recovery, by spiking 200 ng/L recombinant amphiregulin in undiluted and diluted serum samples.

Sample	Dilution	Unspiked concentration (ng/L)*	Expected concentration (ng/L)	Observed spiked concentration (ng/L)	Recovery %
9184	0x	220.9	420.9	429.0	101.9
9184	5x	25.6	225.6	227.1	100.7
9184	10x	10.8	210.8	216.2	102.6
4529	0x	23.3	223.3	214.9	96.2
4529	5x	<3.0	201.0	181.5	90.3
4529	10x	<3.0	200.0	199.2	99.6
7571	0x	<3.0	201.8	153.5	76.1
7571	5x	<3.0	200.0	167.7	83.8
7571	10x	<3.0	200.0	181.9	90.9

* Detection limit: 3.0 ng/L.

Results

All samples were analyzed in duplicate on the same plate. Minimum detection limit of the assays for serum ARG was 3.0 ng/L. The interassay variability of this ELISA was 20% (concentrations in the low range) and 8.5% (concentrations in the high range). The results shown above are a representation of all the validation tests we have run. Using reagens diluent of R&D, the recovery linearity of the assay ranged between 49% and 121%, with an average of 105% of the expected concentration (Appendix Table 1). However, when samples were diluted using 10% fetal calf serum/PBS no signal intensity of endogenous amphiregulin could be measured for 15 out of 18 samples, while in 3 samples a signal was present (data not shown). The recovery was determined by spiking in 200ng/L of recombinant amphiregulin into serum samples. The range was between 76% and 102%, with an average of 93.6% of the expected concentration when reagens diluent of R&D was used (Appendix Table 2).

Conclusion

Although the recovery linearity of reagens diluent had a wide range, this was partly due to the samples with high concentrations of amphiregulin (>220ng/L) in which low recovery linearity percentages arose. However, the second dilution step of these higher concentrated samples did seem to be linear with the first dilution step. The results with fetal calf serum dilutions were unexpected. We suspect that the endogenous 'free' amphiregulin is bound by binding proteins in the FCS. We did not perform any additional experiments to prove this hypothesis, since this was beyond the scope of our research. Given the above findings we proceeded the serum analyses for amphiregulin as described in the methods section and as described many times in literature [17-18-21-36-55-56].

9

DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Adjuvant systemic therapy is one of the cornerstones in the treatment of most cancer types. Current clinical guidelines regarding systemic therapies largely depend on large randomized controlled trials, in which modest absolute survival benefits lead to a change in treatment protocols. This strategy seems justified especially since a 5% improvement in 10-years survival was deemed worthwhile by 70 to 90% of breast cancer patients who had experienced adjuvant chemotherapy [1,2]. Consequently, administering adjuvant systemic therapy results in a balance between prolonging life of a relatively small subgroup at the expense of toxic side-effects without any benefit for the vast majority (overtreatment). Adequate selection prior to systemic therapy of patients likely to benefit from a specific adjuvant systemic regimen would improve the toxicity-benefit relation and would avoid overtreatment of the majority. Identifying markers capable of this selection, *i.e.* predictive markers, would enable patient-tailored treatment and is the rational for this thesis. In this concluding chapter we evaluate what the previous chapters might have contributed to the field of predictive markers and present the foremost questions resulting from our studies which remain unanswered.

High dose chemotherapy and breast cancer

Whether systemic treatment benefits outweigh the risks of treatment depends largely on two factors: one, the magnitude of the benefit, and two, the degree and scale of the side-effects. In case of high dose (consisting mostly of alkylating agents) chemotherapy in breast cancer patients, one of the regimens studied in this thesis (**chapters 3 – 5**), it has been argued that the reduced risk of recurrence of approximately 13%, in the absence of significantly reduced risk of death does not outweigh treatment harm when compared to less toxic treatment schedules [3]. However, in this thesis we found that by using a predictive marker (BRCA-like^{CGH}) a subgroup could be identified whose relative risk of recurrence was reduced with ~80% after treatment with high-dose cyclophosphamide, thiotepa and carboplatin (**chapters 3&4**). We speculate that the good outcome observed after high-dose chemotherapy in patients with a BRCA-like^{CGH} tumor might be due to the underlying molecular biology. The array comparative genomic hybridization (aCGH) classifiers determining BRCA-like^{CGH} status were constructed on breast tumors of *BRCA1*- and *BRCA2*-mutation carriers, likely to be deficient in homologous recombination. BRCA-like^{CGH} status might therefore also enrich for homologous recombination deficient tumors, albeit via different mechanisms besides mutations. In the absence of homologous recombination and subsequently error-free double-strand break (DSB) repair, alkylating and platinum compounds causing DSBs may have targeted the Achilles' heel of these tumors. Unfortunately, this remains mere speculation since we miss an essential link (figure 1): the specific mechanisms that would result in homologous recombination deficiency. Although ~60% of the BRCA1-like^{CGH} tumors could be explained by either *BRCA1*-mutations or *BRCA1*-promoter methylation, we did not explain the presence of this aCGH profile for the remaining 40%. Extrapolating these findings we speculate that ~30% of the BRCA2-like^{CGH} tumors might be explained

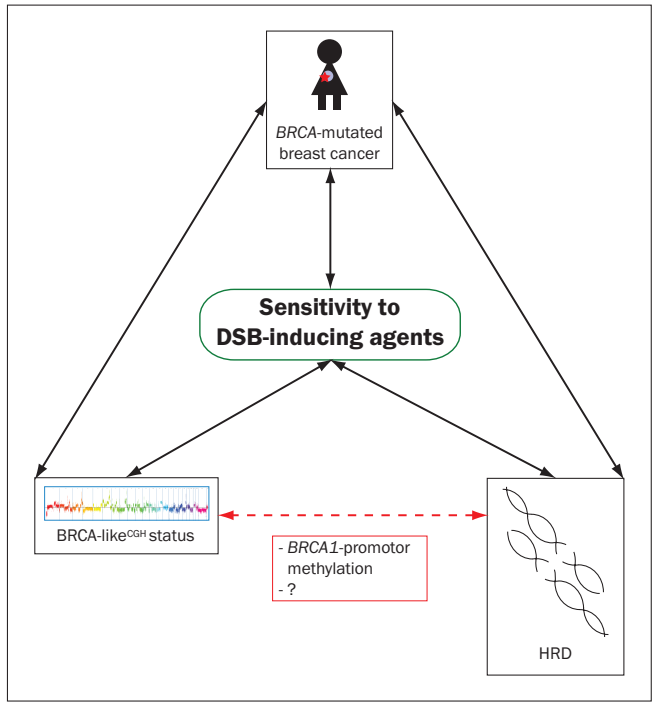


Figure 1. Missing link BRCA-like^{CGH} status and homologous recombination deficiency. There are a number of links between *BRCA*-mutated breast cancer, homologous recombination deficiency (HRD) and the predictive marker: BRCA-like^{CGH} status. 1) *BRCA1* and *BRCA2* play a role in DNA repair through homologous recombination; mutations in *BRCA1/2* have been associated with homologous recombination deficiency (HRD). 2) *BRCA*-mutated breast cancer and cancer cells displaying HRD have both been associated with sensitivity to double-strand break (DSB)-inducing agents. 3) BRCA-like^{CGH} status is based on the construction of genomic profiles of *BRCA*-mutated breast cancer and has been shown to be associated with benefit to DSB-inducing agents. However, it remains unclear whether BRCA-like^{CGH} status is directly associated with HRD.

by a *BRCA2*-mutation, leaving a large number unexplained. Currently, using next-generation massively parallel sequencing (MPS) techniques, genes involved in homologous recombination are being studied in the DNA of BRCA-like^{CGH} breast tumors versus non-BRCA-like^{CGH} breast tumors to identify potential mechanisms leading to homologous recombination deficiency and thereby benefit to DSB-inducing agents. However, whether this benefit might be due to the combination of three DSB-inducing agents (cyclophosphamide-thiotepa-carboplatin), the dosing of this regimen or a combination of the two has not been clarified. The question therefore remains: Does dose matter?

Dose intensity in breast cancer

In the late seventies and early eighties it was postulated that higher dosed alkylating agents with autologous stem-cell support would result in greater cytotoxicity

also for relatively resistant tumor cells. This hypothesis was based on preclinical findings in which a steep dose response curve was seen for alkylating agents without a plateau-phase as was seen for non-alkylating agents [4]. Although phase I and II studies looked promising, two recent meta-analyses of 15 randomized controlled trials (RCTs) in the adjuvant setting and six RCTs in the metastatic setting comparing adjuvant high-dose chemotherapy with stem-cell support with standard chemotherapy in breast cancer patients, showed no significant benefit for overall survival (OS) [3,5]. However, there are a number of reasons why high dose chemotherapy might be beneficial in the clinical setting, despite the results of these RCTs.

Firstly, both meta-analyses showed a significantly improved progression-free or recurrence-free survival (PFS, respectively RFS) for the high-dose arm.

Secondly, all RCTs were performed in the general breast cancer population, while the results of this thesis suggest that only subgroups might have benefit from high dose chemotherapy (**chapter 3 – 5**). Evidence that specific subgroups benefit from high dose chemotherapy has also been observed in these RCTs; multiple studies have found that patients of younger age [6-12] and premenopausal patients [11,13] have a better RFS after high dose chemotherapy. Furthermore, HER2-negative [8,11] and triple-negative (TN) disease [14] have both been associated with a better outcome after high-dose chemotherapy, while HER2-positive patients did not derive benefit [8]. These findings are confirmed in either one of the two meta-analyses [3,5]; however with regard to TN disease, which was confirmed in the adjuvant meta-analysis, it was stated that this finding was spurious, since patients with hormone-receptor negative tumors with unknown HER2-status showed less treatment benefit than those with hormone-receptor negative tumors and known HER2 status [3]. However, this might be due to the fact that in the current meta-analysis only 7% of the HER2-known patients were HER2-positive. This is substantially less than the reported 15-20% in the general breast cancer population [15]; it could therefore well be that the HER2-unknown group of the meta-analysis will contain more HER2-positive tumors than the tested patients and as such will not have benefited from high-dose chemotherapy. Similarly, in the metastatic meta-analysis it was shown that younger age and premenopausal status was associated with a significantly better OS after high dose when compared to standard dose [5]. The size of this effect was not published as the authors thought the subsets were too small and associations too weak. However as the authors mention, the advantage of meta-analyses is being able to identify subsets of patients who might benefit and therefore it is regrettable that treatment effect was not stated. Interestingly, all of the above-mentioned subgroups have been associated with *BRCA1*- or *BRCA2*-mutated breast cancer [16,17], and except for HER2-negativity (as our studies were performed in HER2-negative patients only) all were significantly associated with BRCA-like^{CGH} status. These subsets could therefore be enriched for the same subgroup, patients with a potential homologous recombination deficient tumor, and therefore show benefit to high dose alkylating chemotherapy.

Thirdly, in a preclinical study nimustine, a bifunctional alkylator, was found to be specifically lethal to *BRCA2*-mutated cells in a pharmaceutical screen [18]. Using nimustine in genetically engineered mouse models for breast cancer resulted

in substantial benefit for *BRCA1*-associated breast cancer compared to non-*BRCA1*-associated breast cancer (unpublished data, courtesy of dr. S Rottenberg, Figure 2A). Lowering the dose of nimustine resulted in a significant shorter time to relapse in mice with these *BRCA1*-associated breast cancers (Figure 2B). Interestingly, time to relapse with 50% of the maximum tolerated dose (MTD) for nimustine was similar to time to relapse for the 100% MTD of cisplatin [19], suggesting that higher dose results in survival benefit and bifunctional alkylators might be more on target than platinum-based agents. This could be due to the ability of nimustine to induce a higher amount of DSBs in the DNA as is implied by the higher level of γH2AX staining, present for a longer time period in nimustine treated tumors compared to carboplatin treated tumors (figure 2C, upper panel), thereby resulting in a higher percentage of apoptosis (Figure 2C lower panel).

Fourthly, in the clinical setting it is hard to elucidate the effect of dose intensification, since all high dose RCTs compared different high-dose regimens with different standard chemotherapy arms. This is reflected by the broad range of dose-intensities over all trials [3,5]. Results are therefore difficult to interpret; especially considering that in five of the 15 adjuvant RCTs the standard dose arm reached a higher total dose intensity than their respective high dose arms (*i.e.* 2474 patients out of 6210 patients in the meta-analysis of adjuvant RCTs, 39%) [3]. However, adjusting for (total) dose intensity in multivariate analyses involving all RCTs resulted in a significant reduced risk for death in both the metastatic and adjuvant setting per unit increase of dose-intensity, suggesting that higher dose does result in survival benefit.

Lastly, toxicity can be seen as read-out of efficacy of chemotherapy to induce damage in dividing cells. As such, leucocyte nadir has been associated with a better outcome [20,21]. Furthermore, tailoring 5-fluorouracil – epirubicin - cyclophosphamide (FEC) on hematologic toxicity for every individual patient resulted in a higher total dose intensity and better outcome than the high dose arm in a Swedish RCT [22,23]. This suggests not only that dose might matter, since taking into account individual pharmacokinetics ensured an effective dose for every individual, but also that dose intensity might not have to reach myelo-ablative levels to be effective. The concept of higher, non-myeloablative dose is further supported by findings in the B-25 trial in which breast cancer patients younger than 50 and with 4-9 positive lymph nodes showed a significantly better disease-free survival with highly intensified cyclophosphamide (without stem cell support) compared to moderately intensified cyclophosphamide-based chemotherapy [24]. Similarly, a recent adjuvant trial showed improved outcome after intensified, non-myeloablative dose dense epirubicin, paclitaxel and cyclophophamide [25].

This is of course all circumstantial evidence and the issue regarding dose intensity and potential benefit in a pre-specified subgroup, such as BRCA-like^{CGH} status, remains unsolved. To answer this question the results regarding the BRCA-like^{CGH} status and *Xist* expression levels (**chapter 3 – 5**) should first be validated retrospectively in a RCT; preferably one which has included a fair number of patients and in which the total dose-intensity is reasonably higher than its control arm (for example the Pegase 01 trial) [26]. Secondly, a prospective trial should be carried out which for example compares a DSB-inducing regimen

with intensified levels *and* stem cell support to the same DSB-inducing regimen with non-myeloablative intensified dose levels in marker-positive breast cancer patients (BRCA-like^{CGH} or low *Xist* levels); while in marker-negative (non-BRCA1-like^{CGH} or high *Xist* levels) patients the same non-myeloablative intensified DSB-inducing regimen is compared to standard taxane-based chemotherapy.

Extrapolating knowledge on a cancer-wide scale

Although, historically it is common to study one cancer type and apply the knowledge obtained only on an intra-cancer-type scale, this thesis argues in favor of applying this knowledge inter-cancer-type wide (**chapters 1&2**). This could accelerate the process of identifying predictive biomarkers and as such personalize medicine.

Applying knowledge obtained in breast cancer studies to other cancer types

The above findings could therefore very well be applicable to other cancer-types. For example, we tested the aCGH BRCA1-classifier in a small number of ovarian cancer patients (data not shown), but found no significant association with survival. This could be due to sample size, inability to differentiate between prognosis and prediction, or it could be that *BRCA1*-associated ovarian cancers display different genomic aberrations than *BRCA1*-associated breast cancers. Currently, DNA of ovarian cancers of *BRCA1/2*-mutation carriers is being collected to study whether a classifier can be made to distinguish them from non-carriers and test whether this classifier can predict platinum response in sporadic ovarian cancer patients. Similar efforts are undertaken for other less obvious cancer types such as gastric cancer and pancreatic cancer, which are both associated with an increased risk in *BRCA2*-mutation carriers [27,28]. Ideally, a general *BRCA*-associated genomic profile will be developed which is not specific for a certain cancer type but for *BRCA*-associated, or even homologous recombination deficient cancer. In this respect, it would be interesting in the future to compare breast, ovarian, gastric and pancreatic *BRCA*-associated genomic classifiers and try to denominate common overlapping regions, which as such could indicate general *BRCA*-deficiency or even homologous recombination deficiency. This strategy could also be undertaken for cancers without a *BRCA*-association, as for example non-small cell lung cancer (NSCLC), in which high *BRCA1* expression has been associated with a poor outcome after platinum-based chemotherapy [29,30]. Currently, two RCTs in the metastatic and adjuvant setting randomize NSCLC patients according to *BRCA1* expression levels to docetaxel, or cisplatin combinations (BREC, NCT00617656, respectively SCAT, NCT00478699). It would be interesting to study in these trials whether a general genomic profile or gene expression profile can be constructed to differentiate between high and low *BRCA1* expression, which would select a larger subgroup benefitting from DSB-inducing agents.

Vice versa, it would be interesting to study cancer types with a high level of sensitivity to DSB-inducing agents for associations with homologous recombination deficiency. Examples of these cancers would be germ cell tumors, which are known to be highly sensitive to DSB-inducing agents. Moreover, a recent study showed that salvage high dose platinum-based chemotherapy might

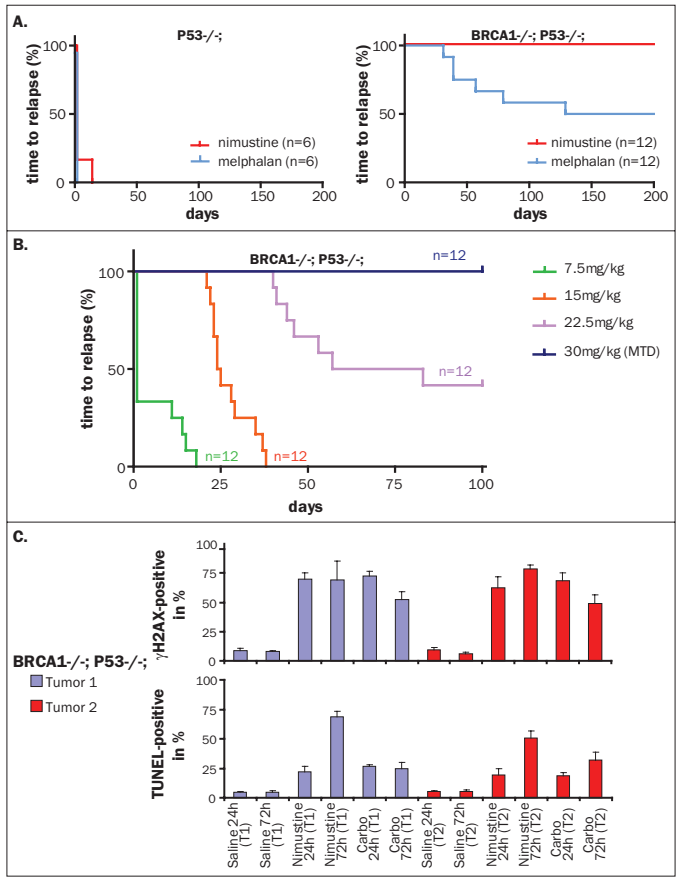


Figure 2. Response to different doses of nimustine, a bifunctional alkylating agent in a *Brca1*-associated, or of *p53*-deficient only mouse model for breast cancer (unpublished data, courtesy of dr. S Rottenberg). Two mouse models for breast cancer were studied: a conditional, tissue-specific deletion of either *p53* and *Brca1*, or with *p53* only. Four individual BRCA1-/-;P53-/- deficient donor tumors were transplanted into three mice per treatment group and three individual P53-/- donor tumors were transplanted into two mice each. The Kaplan Meier (KM) curves shown display time until relapse of tumors that shrank at least by 50% in volume (v=length*width2*0.5). A) All were treated with a one-time dose of 10mg melphalan per kg intraperitoneally (i.p.) or 30mg nimustine per kg i.p. (both the maximum tolerated dose, MTD). The KM curves show a significant benefit of both alkylators for mice with *Brca1*$^{-/-}$ associated breast cancer but not for mice with *P53*-deficient breast cancer. B) The curve shows response to different doses of nimustine with respect to time to relapse. It is noteworthy that the 50% relapse-free survival of the MTD of carboplatin is of similar duration after the 50% decreased dose of nimustine. C) Two tumors were transplanted into six different animals each; tumors were harvested either 24 hours or 72 hours after injection with saline (control), the MTD of carboplatin (100mg carboplatin per kg i.p.) or the MTD of nimustine. Tumors were assessed with immunohistochemistry for their expression of either γH2AX, a marker for the presence of DSBs in the DNA, or TUNEL, an apoptosis marker, showing more DSBs being present 72 hours after nimustine than carboplatin; and consequently showing more apoptosis in the tumor 72 hours after nimustine than carboplatin which could explain the longer relapse-free survival after treatment with nimustine. [Unpublished data, courtesy of dr. S Rottenberg]

result in a better outcome compared to standard chemotherapy for germ-cell tumors [31]. Interestingly, germ cell cancer has been associated with a numerical increase of the X-chromosome [32]. Although *XIST* was not found to be associated with silencing of X-linked genes [32], it would be interesting to test whether *XIST* expression is also associated with sensitivity to DSB-inducing agents in this tumor type. Moreover, our findings regarding *XIST* (**chapter 5**) might be an indirect read-out of an X-linked mechanism which studying germ cell cancers might help elucidate.

Applying knowledge obtained in NSCLC patients to other cancer types

Similarly, it would be interesting to see whether our findings in NSCLC regarding soluble EGFR (sEGFR), CEA and ligands of EGFR (**chapters 7&8**) can be extrapolated to other cancer types. In a small neoadjuvant study we investigated whether EGFR ligand levels would also predict response to trastuzumab in HER2-positive breast cancer patients, but found no association (data not shown). This could be explained by small numbers, but also by the fact that HER2 dimerization is ligand independent and activation might mainly depend on homodimerization through the high level of HER2 amplification and less on heterodimerisation with other HER-members. However, it would be interesting to see whether levels of sEGFR or ligands of EGFR, such as transforming growth factor alpha (TGFa) or amphiregulin (ARG), would predict tamoxifen resistance, since premenopausal patients with high EGFR expression were shown to have a decreased RFS after tamoxifen compared to no treatment [33]. Fitting with this finding was the observation that estrogen-receptor (ER)-positive breast cancer patients resistant to tamoxifen showed benefit to gefitinib [34]. Similarly, it would make sense to study whether sEGFR, TGFa and ARG levels in serum would predict for sensitivity to the EGFR/HER2-inhibitor, lapatinib; especially since it was recently found that low serum TGFa levels were associated with a better response to lapatinib in HER2-positive breast cancer patients [35], which is in line with our results (**chapter 8**). Similar studies could be performed to investigate the relation of these serum markers with response to pertuzumab, an agent inhibiting dimerization of HER2 with other HER-members.

Besides breast cancer, EGFR seems to play a role in colon cancer, since patients are effectively treated with a monoclonal antibody against EGFR, namely cetuximab. High gene expression levels of ARG were found to predict for a better response and longer RFS in patients with metastatic colorectal cancer treated with cetuximab in two independent studies [36,37]. However, in both studies a control arm lacking cetuximab was missing and whether ARG expression levels are predictive or prognostic can therefore not be distinguished. It would therefore be appealing to test gene expression levels of TGFa and ARG and serum levels of both and of sEGFR in patients with colorectal cancers participating in a RCT in which cetuximab is included in one of the treatment arms.

In NSCLC patients a number of questions still remain with regard to our findings (**chapters 7&8**); firstly, the results need to be validated in a randomized controlled setting in which confounding variables are equally distributed over both arms. Furthermore, from a biological perspective it would be interesting

to study sEGFR, CEA and EGFR ligand levels with regard to its NSCLC gene expression levels and with regard to EGFR- and KRAS-mutation status.

FUTURE PERSPECTIVES: ORGAN-RELATED DISEASE FOR THE SURGEON, DISEASE OF ONCOGENIC PATHWAYS FOR THE MEDICAL ONCOLOGIST

Currently, cancer is divided by organ where it originated from. This is mainly due to surgery being the mainstay of cancer treatment. The last decade has shown us that even within one cancer type a high level of heterogeneity exists, which also influences the response to systemic therapies. The studies described in this thesis advocate an approach which uses molecular aberrations in well-known oncogenic pathways as starting point for biomarker discovery. This approach revealed a homogeneous subset of tumors within the heterogeneous total. Moreover, lymph node metastases were found to be remarkably similar to their primary tumor (**chapter 6**), suggesting that molecular aberrations found in the primary tumor might also be present in metastatic lesions in the absence of therapy.

More importantly, since oncogenic pathways are not organ-specific and theoretically may be activated in every cancer cell, biomarkers discovered according to this approach might be used in multiple cancer types. Furthermore, targeted therapies developed to target these pathways may be applied to multiple cancer types thereby improving cost-effectiveness and clinical implementation. In the future, oncologists might treat cancer patients based on the evaluation of activation of for example five oncogenic pathways out of a list of 50 known, testable pathways, prioritizing on organ of origination, instead of one general clinical guideline per cancer type. These developments will largely depend on smart clinical trials implementing both predictive markers, prognostic factors and the search for new predictive biomarkers in its design, thereby ultimately increasing survival of cancer patients.

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10 SUMMARY

SUMMARY

The majority of breast and lung cancer patients are treated with adjuvant systemic therapy. However, a large proportion of patients needlessly receives this therapy as they would have been cured by locoregional therapy (*e.g.* surgery and, or radiotherapy) alone. More specifically, in breast cancer between ~42% and 47% of the patients will not have a recurrence the following 15 years, even when systemic therapy is omitted [1]. There has been progress in the breast cancer field to more adequately identify those patients with a low likelihood of recurrence (prognosis) using gene expression signatures, such as the 70-gene profile (mammaprint®) [2]. However, even if only those patients with a high risk of recurrence received systemic therapy, many patients would still not benefit from standard chemotherapy regimens; for example treating breast cancer patients with standard chemotherapy decreases 15-year recurrence rates absolutely by only ~4 to 12% [1]. Therefore, being able to predict who is likely to respond to a specific therapeutic (prediction) would not only prevent unnecessary treatment of a large fraction of patients, but it would also result in less cancer mortality as patients would ideally receive therapy to which they respond from the start, saving precious time. Until now decisions regarding chemotherapy are based on large randomized controlled trials; however results of these trials do not reflect whether a single individual will benefit (**chapter 1**). This thesis focuses on the search of new predictive markers in breast (**chapter 2 – 5**) and lung cancer (**chapter 7&8**) using knowledge of well known molecular defects in both cancer types (**chapter 1**) to individualize treatment.

We used a hypothesis-driven strategy that exploited the concept that DNA repair pathways are required for error-free repair of DNA lesions caused by chemotherapeutics. Defects in these repair pathways therefore might not only induce cancer, they might also offer a potential target for these chemotherapeutics. The best known example of this concept is probably the hypersensitivity to agents inducing double-strand breaks (DSBs) in the DNA, in patients with defects in their homologous recombination repair pathway, the pathway responsible for error-free repair of DSBs. Hereditary breast and ovarian cancer caused by heterozygous germline mutations in *BRCA1*- or *BRCA2* display this hypersensitivity as both these genes are part of the homologous recombination pathway. In **chapter 2** we presented an overview of the hallmarks of homologous recombination deficiency, namely genomic instability and hypersensitivity to DNA damage and review the efforts already undertaken to identify patients benefitting from DSB-inducing agents in both breast and ovarian cancer, as knowledge obtained in one cancer type might very well be applicable to the other cancer type.

BRCA1-mutated breast cancers have been shown to have characteristic copy number gains and losses which can be visualized by array comparative genomic hybridization (aCGH) [3-6]. These copy number aberrations (CNAs) are so specific that the familial cancer clinic was able to develop an aCGH classifier that determines the likelihood (defined as a probability score) of patients being *BRCA1* -mutation carriers [3-5]. In **chapter 3** we evaluated whether this classifier would be able to select sporadic breast cancer patients benefitting from DSB-inducing therapy, since we

hypothesized that this classifier could also identify other causes of *BRCA1*-loss besides mutations. We first optimized the aCGH BRCA1-classifier for identification of benefit to DSB-inducing agents, as this classifier was originally constructed on a separate dataset to identify *BRCA1*-mutation carriers (chapter 3, Appendix B). For this purpose, we studied metastatic breast cancer (MBC) patients treated with high dose (HD), DSB-inducing, chemotherapy consisting of carboplatin, thiotepa and cyclophosphamide (CTC) and determined the cut-off of the BRCA1-probability score on the highest positive predictive value for progression-free survival exceeding 24 months. This optimized aCGH BRCA1-classifier scored 41% (16/39) of the MBC patients as BRCA1-like^{CGH}. To validate this optimized classifier and test its predictive potential we next studied 230 stage-III, HER2-negative breast cancer patients, who were part of a controlled trial and had been randomized between adjuvant HD-chemotherapy and conventional anthracycline-based chemotherapy (consisting of 5-fluorouracil – epirubicin – cyclophosphamide) [7]. We found that HD-chemotherapy improved recurrence-free survival (RFS) in patients with BRCA1-like^{CGH} tumors (multivariate HR: 0.12, p<0.01), but not for patients with non-BRCA1-like^{CGH} tumors (multivariate HR: 0.78. p=0.25). This difference in treatment benefit was significantly different between both groups (p-interaction<0.01). Similar results were found for overall survival (OS) and when restricting the analysis to the triple-negative (TN) subgroup, as this subgroup was associated with BRCA1-like^{CGH} status (34/41 vs 26/189, p<0.01). To ascertain that BRCA1-like^{CGH} status was not equivalent to mutation status, we screened for *BRCA1*-mutations and found 13 mutations of which eight scored as BRCA1-like^{CGH}. Furthermore, we assessed *BRCA1*-promoter methylation and found 12 tumors to be methylated all scoring as BRCA1-like^{CGH}. These data support our hypothesis that the classifier also identifies patients with *BRCA1*-loss due to other causes than mutations and the significant test for interaction suggests BRCA1-like^{CGH} status is a predictive marker.

The predictive marker studied in **chapter 3** was strongly associated with TN status and subsequently did not select many ER-positive patients with potential benefit to DSB-inducing agents. However, ~70% of the *BRCA2*-mutated breast cancers are estrogen (ER)- or progesterone (PR)-positive [8]. In **chapter** 4, we therefore studied, the predictive potential of adding an aCGH BRCA2-classifier, previously defined to identify *BRCA2*-mutation carriers [9], to the BRCA1-classifier. We show that 81 out of 249 breast tumors of patients, who participated in the same randomized controlled trial as in **chapter 3**, display a BRCA1-like and/or BRCA2-like CGH pattern (defined as BRCA-like^{CGH} status). The effect of HD-chemotherapy differed significantly between patients with a BRCA-like^{CGH} and non-BRCA-like^{CGH} tumor on RFS as well as OS (p-interaction<0.01 for both). The risk of death decreased five-fold in patients with a BRCA-like^{CGH} tumor after HD-chemotherapy compared to conventional chemotherapy (multivariate HR for OS: 0.19, p<0.01), while no superior effect was seen in Non-BRCA-like^{CGH} patients (multivariate HR for OS: 0.90, p=0.71). Half of all BRCA-like^{CGH} tumours were ER-positive, making this the first study reporting on a potential marker for sensitivity to DSB-inducing agents within the sporadic ER-positive breast cancer population. This study warrants validation in a prospective randomized controlled

trial. Currently, one such study is being conducted in the neoadjuvant setting in TN breast cancer patients (NCT01057069) and studies in the metastatic, TN setting are being developed. Furthermore, these studies only show an association with benefit to DSB-inducing agents and specific genomic profiles but do not give any insights into the causes of these genomic profiles. At this time, tumors of these studies are being analyzed with high-throughput sequencing of mainly DNA-repair genes to elucidate the molecular biology behind these associations.

Another approach to find predictive markers is to correlate response to therapy with data of genome-wide strategies such as gene expression profiling. In **chapter 5** we describe two interesting findings using such an approach to find predictive markers for docetaxel and cisplatin response in a genetically engineered mouse model for *BRCA1*-associated breast cancer. Firstly, a low expression of *XIST* was associated with i) high sensitivity to cisplatin in mice, ii) subsequently with benefit to HD-chemotherapy (containing carboplatin) in 60 human breast cancer patients in terms of RFS, iii) with BRCA1-like^{CGH} status in human breast cancers, and iv) with *BRCA1*-promoter methylation in human breast cancers. Secondly, we studied markers able to predict poor response to docetaxel in this model. Previously, increased expression of *Abcb1*, encoding a P-glycoprotein transporting docetaxel out of cells, was found to cause docetaxel resistance in this model [10]. Suprisingly, we did not find *Abcb1* to be elevated when the RNA of the tumors was analyzed on gene expression arrays. However, when *Abcb1* RNA was directly analyzed with RT-Multiplex Ligation-dependent Probe Amplification (RT-MLPA), we did find that five out of 22 poorly responding tumors contained sufficiently high *Abcb1* RNA levels to explain resistance. This significant result was completely missed when these five tumors were part of the complete set of 22 poor responders, clearly illustrating why this genome-wide approach might not be suitable in heterogeneous populations. The mechanism behind why tumors with low *XIST* levels are sensitive to cisplatin remains unclear, however this marker might predict benefit to DSB-inducing agents in breast cancer patients. Potentially this could also include patients with a *BRCA1*-mutated tumor as this result was found in a *BRCA1*-deficient background. Currently, *XIST* expression is being analyzed in a larger number of patients to validate this result and investigate the association with the aCGH BRCA2-classifier.

The search for predictive markers most often is performed in the primary breast tumor, including above chapters. However, it remains unclear to what extent micrometastatic tumor cells, for example lymph node (LN) metastases, having already metastasized locally, resemble their primary breast tumors and as such will respond to the systemic therapy chosen based on these predictive markers. In **Chapter 6**, we investigated the genetic difference between primary breast cancers and their paired LN metastases using a high resolution aCGH platform over all patients and by TN and luminal subtype, since we speculated that the genetic instability of TN tumors might result in an increase in genetic aberrations in LN metastases, simply due to random occurrence in this subgroup. We found that all LN metastases, except for two, closely resembled their primary tumor with no significant regional differences overall or within specific subtypes, suggesting a strong clonal relationship. The two discrepancies were explained

by poor hybridization quality and, interestingly, the presence of two histological components (biphasic) in one tumor. The number of genetic aberrations between primary tumor – LN pair did not differ between TN and luminal tumors, indicating that regardless of complexity of genomic aberrations, aberrations are likely to be functional and not random. Finally, the BRCA1-classifier could be used on both primary tumors and on LN metastases as test results were concordant with exception of the biphasic tumor. These findings support the use of the primary tumor characteristics to guide adjuvant systemic chemotherapy in breast cancer patients, since primary tumors and their subsequent LN metastases seem remarkably similar, at least prior to treatment.

In the second part of this thesis we searched for predictive markers for epidermal growth factor receptor (EGFR), tyrosine-kinase inhibitor (TKI) therapy, such as erlotinib and gefitinib in non-small cell lung cancer (NSCLC) patients. In **chapter 7**, we analyzed serum of 102 advanced NSCLC patients who all received erlotinib or gefitinib and studied the association of soluble EGFR (sEGFR) and carcinoembryogenic antigen (CEA) with OS. We found that increasing sEGFR levels were independently associated with a better OS (multivariate HR per ug/L increase: 0.96, p=0.004). Similarly decreasing levels of the logarithm of CEA was associated with an increase in OS (multivariate HR per log ug/L increase: 1.51, p=0.008). Unfortunately, this study did not include a control group not treated with EGFR-TKIs and therefore sEGFR and CEA could still be a prognostic rather than a predictive marker.

In **chapter 8** we therefore matched 64 EGFR-TKI naïve, NSCLC patients on gender, age and histology to 61 EGFR-TKI (erlotinib or gefitinib) treated patients with advanced NSCLC. In this chapter we explored whether serum concentrations of ligands of EGFR, transforming growth factor alpha (TGFa) or amphiregulin (ARG), or ligands of the insulin-like growth factor (IGF) receptor, with which EGFR has crosstalk, namely IGF1 and IGF-binding protein 3 (IGFBP3) were predictive of EGFR-TKI benefit with regard to disease-specific survival (DSS). We found that low concentrations of TGFa were correlated with a better DSS in EGFR-TKIs patients compared with control patients (multivariate HR: 0.55, p=0.04), while high concentrations were associated with a non-significantly worse DSS (multivariate HR: 1.51, p=0.40). This difference was significantly different with a p-interaction of 0.05. Similar results were obtained with ARG, in which high concentrations of ARG were associated with a better DSS in EGFR-TKI patients compared to control patients (multivariate HR: 0.31, p=0.001) and this effect of EGFR-TKIs treatment on DSS also showed a significant difference by ARG (p-interaction: 0.004). These results suggest that concentrations of TGFa and ARG measured in serum might be predictive of EGFR-TKI benefit. Next to these potential predictive markers, we found IGBP3 to be a potential prognostic marker as increase in serum concentrations was associated with significantly longer DSS irrespective of treatment (multivariate HR per 1mg/L increase: 0.56, p=0.027). Due to the case-control set-up of our study, patients were not as well matched as in a randomized controlled trial. Furthermore, due to relative small numbers the optimal cutoff for TGFa and ARG and its additive value to known clinical predictors for EGFR-TKI sensitivity could not be determined. Currently, we are trying to validate the

most promising results regarding sEGFR, TGFa and ARG, in a small neoadjuvant study in which NSCLC patients received erlotinib prior to surgery in which serum concentrations will be matched to PET-response data. However, it would also be interesting to test these findings preferably in a prospective randomized controlled setting of advanced NSCLC patients.

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11

SAMENVATTING
LIST OF PUBLICATIONS
DANKWOORD
CURRICULUM VITAE

NEDERLANDSE SAMENVATTING

De meerderheid van de borst- en longkanker patiënten wordt behandeld met adjuvante systemische therapie. Voor een groot deel van de patiënten is deze therapie echter onnodig, aangezien ze genezen zouden zijn door louter locoregionale therapie (zoals een operatie, en of radiotherapie). Om precies te zijn: in afwezigheid van systemische therapie zullen tussen de ~ 42% en 47% van de borstkanker patiënten geen recidief krijgen in de 15 jaar na ontstaan van de tumor [1]. Er is vooruitgang geboekt bij de adequatere identificatie van borstkankerpatiënten met een lage recidief kans (prognose) met behulp van genexpressieprofielen, zoals het 70-gen profiel (MammaPrint ®) [2]. Maar zelfs als alleen patiënten met een hoog risico op recidief systemische therapie ontvangen, hebben nog steeds veel patiënten geen baat bij standaard chemotherapie; zo vermindert standaard chemotherapie bij borstkanker patiënten de recidief kans absoluut met slechts ~4 tot 12% [1]. Derhalve zou het kunnen voorspellen welke patiënten zullen reageren op specifieke middelen (predictie) niet alleen onnodige behandeling van een groot deel van de patiënten voorkomen, maar zou het ook kunnen leiden tot een lagere kankersterfte. Dit mede omdat patiënten in dat geval de therapie zouden krijgen waarop zij vanaf het begin reageren, wat kostbare tijd zou schelen. Tot op heden worden beslissingen aangaande systemische therapie gebaseerd op grote gerandomiseerde, gecontroleerde studies. De resultaten van deze studies geven echter geen antwoord op de vraag wat de baat is voor een individuele patiënt (**hoofdstuk 1**). Dit proefschrift richt zich op de zoektocht naar nieuwe predictieve (voorspellende) markers in borstkanker (**hoofdstuk 2 - 5**) en longkanker (**hoofdstuk 7 & 8**); hierbij wordt gebruik gemaakt van kennis van bekende moleculaire defecten in beide vormen van kanker (**hoofdstuk 1**), met als doel behandeling te individualiseren.

We hebben een hypothese-gedreven strategie gebruikt die uit gaat van het fenomeen dat DNA-reparatie routes nodig zijn voor een foutloze reparatie van DNA-beschadigingen veroorzaakt door chemotherapie. Defecten in deze reparatie routes veroorzaken mogelijk niet alleen kanker, maar ze zouden ook een potentieel doelwit voor chemotherapie kunnen zijn. Het bekendste voorbeeld van dit fenomeen wordt waarschijnlijk gevonden in patiënten met defecten in hun homologe recombinatie DNA reparatie route, de route die verantwoordelijk is voor foutloze reparatie van dubbelstrengs breuken in het DNA, aangezien zij deze niet kunnen repareren. Erfelijke borst- en eierstokkanker veroorzaakt door heterozygote kiembaan mutaties in een tweetal genen, te weten *BRCA1* of *BRCA2*, tonen deze overgevoeligheid aangezien beide genen deel uit maken van de homologe recombinatie DNA reparatie route. In hoofdstuk 2 wordt een overzicht gegeven van de kenmerken van homologe recombinatie defecten, te weten instabiliteit van het genoom en overgevoeligheid voor bepaalde DNA-schade. Ook komen in hoofdstuk 2 de eerdere pogingen aan bod om patiënten te identificeren die baat hebben bij dubbelstrengs breuken-inducerende stoffen in zowel borst- als eierstokkanker. Dit wordt in beide typen kanker bekeken, aangezien kennis verkregen in de ene soort kanker heel goed van toepassing zou kunnen zijn op de andere soort kanker.

Borstkanker met een *BRCA1* mutatie kent specifieke DNA veranderingen resulterend in verlies van DNA (deleties) of ongewenste toename aan DNA (zoals amplificaties) die kunnen worden gevisualiseerd door array comparative genomic hybridization (aCGH) [3-6]. Deze DNA veranderingen zijn zo specifiek dat de polikliniek familiaire tumoren in staat is geweest om een aCGH profiel te ontwikkelen die de kans bepaalt (gedefinieerd als een kans score) dat patiënten drager zijn van *BRCA1*-mutaties [3-5]. In hoofdstuk 3 wordt geëvalueerd of dit profiel in staat zou zijn tot het selecteren van patiënten zonder familiaire aanleg voor borstkanker, die desondanks baat van dubbelstrengs breuken-inducerende therapie zouden kunnen hebben. De veronderstelling daarbij was dat dit profiel naast mutaties ook andere oorzaken van *BRCA1*-verlies zou kunnen identificeren en daarmee gevoeligheid voor dubbelstrengs breuken-inducerende therapie vaststelt. In deze studie hebben we eerst de aCGH BRCA1-classifier geoptimaliseerd voor de identificatie van baat bij dubbelstrengs breuken-inducerende stoffen, aangezien deze classifier oorspronkelijk is vervaardigd op basis van een aparte dataset om *BRCA1*-mutaties te identificeren (hoofdstuk 3, Bijlage B). Hiervoor bestudeerden we gemetastaseerde borstkanker patiënten die behandeld waren met hoge dosis, dubbelstrengs breuken-inducerende, chemotherapie bestaande uit carboplatine, thiotepa en cyclofosfamide (CTC). Voor deze optimalisatie hebben we een cut-off bepaald van de kans score voor het aanwezig zijn van BRCA1 mutaties, welke gebaseerd was op de hoogste positieve voorspellende waarde voor progressievrije overleving van meer dan 24 maanden. Deze geoptimaliseerde aCGH BRCA1-classifier deelde 41% (16/39) van patiënten met gemetastaseerde borstkanker in als BRCA1-like^{CGH}. Om deze geoptimaliseerde classifier te valideren en de voorspellende mogelijkheden te testen, hebben we vervolgens 230 fase-III, HER2-negatieve borstkankerpatiënten bestudeerd, die deel uitmaakten van een gecontroleerde studie en die willekeurig waren verdeeld over adjuvante hoge dosis chemotherapie en conventionele op anthracycline gebaseerde chemotherapie (bestaande uit 5-fluorouracil - epirubicine - cyclofosfamide) [7]. Wij concludeerden dat hoge dosis-chemotherapie de recidief-vrije overleving verbeterde bij patiënten met BRCA1-like^{CGH} tumoren (multivariate HR: 0,12, p <0,01), en niet bij patiënten met niet-BRCA1 -like^{CGH} tumoren (multivariate HR:. 0,78 p = 0,25). Dit verschil in baat bij behandeling was significant tussen beide groepen (p-interactie <0,01). Vergelijkbare resultaten werden gevonden voor de totale overleving en bij het beperken van de analyse tot de triple-negatieve subgroep, aangezien deze subgroep werd geassocieerd met een BRCA1-like^{CGH} status (34/41 vs 26/189, p <0,01). Om vast te stellen of de BRCA1-like^{CGH} status gelijk was aan mutatie status, hebben we gescreend op BRCA1-mutaties. We vonden 13 mutaties waarvan er acht gescoord zijn als BRCA1-like^{CGH}. Daarnaast hebben we *BRCA1*-promotor methylering onderzocht, waaruit bleek dat 12 tumoren met een BRCA1-like^{CGH} profiel *BRCA1*-promotor methylatie vertoonden, maar geen mutatie. Deze gegevens ondersteunen onze hypothese dat de classifier ook patiënten identificeert met *BRCA1*-verlies als gevolg van andere oorzaken dan mutaties. Daarnaast suggereert de significante interactie dat BRCA1-like^{CGH} status een predictieve marker is.

De in hoofdstuk 3 bestudeerde predictieve marker werd sterk geassocieerd met triple-negatieve tumor status en selecteerde als gevolg niet veel oestrogeenreceptor (ER)-positieve patiënten met potentiële baat van dubbelstrengs breuken-inducerende stoffen, zoals CTC. Belangrijk is dat ~70% van de *BRCA2*-gemuteerde borstkankers oestrogeen- of progesteron-positief zijn [8]. In hoofdstuk 4 hebben we daarom gekeken naar de predictieve waarde van het toevoegen van een aCGH BRCA2-classifier, waarvan eerder is vastgesteld dat deze *BRCA2*-mutatiedragers identificeert [9], aan de BRCA1-classifier. We laten zien dat 81 van 249 borsttumoren van patiënten, die in dezelfde gerandomiseerde gecontroleerde studie hebben deelgenomen als in hoofdstuk 3, een BRCA1-achtig en / of BRCA2-achtig DNA patroon vertonen bij aCGH analyse (gedefinieerd als BRCA-like^{CGH} status). Het effect van hoge dosis-chemotherapie (CTC) verschilde significant tussen patiënten met een BRCA-like^{CGH} en niet-BRCA-like^{CGH} tumor met betrekking tot recidief-vrije overleving, als ook tot totale overleving (p-interactie <0,01 voor beide). Het risico op overlijden verminderde vijfmaal bij patiënten met een BRCA-like^{CGH} tumor na hoge dosis-chemotherapie in vergelijking met conventionele chemotherapie (multivariate HR voor totale overleving: 0,19, p <0,01), terwijl er geen significant effect werd gezien in niet-BRCA-like^{CGH} patiënten (multivariate HR voor totale overleving: 0,90, p = 0,71). De helft van alle BRCA-like^{CGH} tumoren was ER-positief. Dit is de eerste studie die rapporteert over een mogelijke marker voor de gevoeligheid voor dubbelstrengs breuken-inducerende stoffen binnen de non-familiaire ER-positieve borstkanker populatie. Dit onderzoek rechtvaardigt validatie in een prospectieve gerandomiseerde gecontroleerde studie. Momenteel wordt een dergelijke studie uitgevoerd in een neoadjuvante setting bij triple-negatieve borstkanker patiënten (NCT01057069). Tegelijkertijd worden studies bij gemetastaseerde triple-negatieve borstkanker patiënten ontwikkeld. Echter onze studie heeft alleen een associatie met baat bij dubbelstrengs breuken-inducerende stoffen en specifieke genomische profielen getoond, maar geeft geen enkele inzicht in de oorzaken van deze genomische profielen. Op dit moment worden borst tumoren geanalyseerd met high-throughput sequencing van voornamelijk DNA-herstel genen om de moleculaire biologie achter deze associaties te verduidelijken.

Een andere benadering om voorspellende markers te vinden is om de respons op de therapie te correleren met gegevens van genoom-brede strategieën, zoals gen expressie profilering. In hoofdstuk 5 beschrijven we twee interessante bevindingen welke met behulp van een dergelijke benadering predictieve markers voor docetaxel en cisplatine respons heeft opgespoord, in een genetisch gemanipuleerd muis-model voor BRCA1-geassocieerde borstkanker. Ten eerste werd een lage expressie van het XIST-gen geassocieerd met i) hoge gevoeligheid voor cisplatine bij muizen, ii) vervolgens met baat bij hoge dosis-chemotherapie (met carboplatine) in 60 borstkanker patiënten wanneer werd gekeken naar recidief-vrije overleving, iii) met BRCA1-like^{CGH} status in borstkanker, en iv) met BRCA1-promotor-methylering in borstkanker. Ten tweede werden markers bestudeerd welke resistentie tegen docetaxel voorspelde in dit muis model. Eerder werd gevonden dat resistentie tegen docetaxel in dit muis model geassocieerd was met een verhoogde expressie van *Abcb1*, een gen verantwoordelijk voor

P-glycoproteine dat docetaxel cellen uit kan transporteren [10]. In tegenstelling tot onze verwachtingen, vonden we geen verhoogde *Abcb1* waarden bij het bestuderen van RNA van deze tumoren met behulp van gen expressie arrays. Bij het direct analyseren van het *Abcb1* RNA met RT-Multiplex Ligation-dependent Probe Amplification (RT-MLPA) vertoonden echter vijf van de 22 docetaxel resistente tumoren wel genoeg verhoogde *Abcb1* waarden om resistentie te verklaren. Dit significante resultaat werd in zijn geheel gemist wanneer deze vijf tumoren onderdeel vormden van de complete groep van 22 resistente tumoren, wat duidelijk aangeeft waarom genoom-brede aanpak niet geschikt zou zijn bij een heterogene populatie. Het mechanisme achter het waarom van de associatie tussen lage *XIST*-gen niveaus en gevoeligheid voor cisplatine blijft onduidelijk, maar deze marker zou baat bij dubbelstrengs breuken-inducerende stoffen kunnen voorspellen bij borstkankerpatiënten. Mogelijk zou dit ook kunnen bij patiënten met een *BRCA1*-gemuteerde tumor aangezien dit resultaat werd gevonden in een *BRCA1*-deficiënte omgeving. Op dit moment wordt *XIST* expressie geanalyseerd bij een groter aantal patiënten om dit resultaat te valideren en de associatie met de aCGH BRCA2-classifier te onderzoeken.

Het zoeken naar predictieve markers wordt meestal uitgevoerd in de primaire borsttumor, net als in de eerdere hoofdstukken. Het blijft echter onduidelijk in hoeverre micrometastatische tumorcellen, bijvoorbeeld lymfeklieren metastasen welke al lokaal zijn uitgezaaid, lijken op hun primaire borsttumoren. Daarmee is ook onduidelijk in hoeverre lymfklier metastatische tumor cellen zullen reageren op de systemische therapie die op basis van deze predictieve markers wordt gekozen. In hoofdstuk 6 onderzochten we het genetische verschil tussen primaire borstkankers en de bijbehorende lymfeklier metastasen met behulp van een hoog resolutie aCGH platform. Dit onderzochten we bij alle patiënten, maar ook per triple-negatief en luminaal subtype, omdat we er van uit gingen dat de genetische instabiliteit van triple-negatieve tumoren zou kunnen resulteren in een toename van DNA-veranderingen in lymfeklier metastasen, simpelweg op basis van willekeur in deze subgroep. We hebben gevonden dat alle lymfeklier metastasen, op twee na, zeer veel op hun primaire tumor leken zonder significante regionale verschillen of verschillen tussen specifieke subtypes, wat wijst op een sterke klonale relatie. De twee verschillen worden verklaard door slechte kwaliteit van hybridisatie en, interessant genoeg, de aanwezigheid van twee histologische componenten (bifasisch) in één tumor. Het aantal DNA-veranderingen tussen de primaire tumor en de bijbehorende lymfeklier metastase verschilde niet tussen triple-negatieve en luminale tumoren, wat aangeeft dat ongeacht de complexiteit van de DNA-veranderingen deze waarschijnlijk functioneel zijn en niet willekeurig. Ten slotte kon de BRCA1-classifier gebruikt worden op zowel primaire tumoren als lymfeklieren metastasen aangezien de test resultaten met elkaar in overeenstemming waren, met uitzondering van de bifasische tumor. Deze bevindingen ondersteunen het gebruik van primaire tumor kenmerken om adjuvante chemotherapie bij patiënten met borstkanker te bepalen, omdat primaire tumoren en hun bijbehorende lymfeklieren metastasen opmerkelijk gelijk lijken, in ieder geval voorafgaand aan de behandeling.

In het tweede deel van dit proefschrift hebben we gezocht naar predictieve markers voor epidermale groeifactor receptor (EGFR), tyrosine-kinase-inhibitor therapie, zoals erlotinib en gefitinib bij niet-kleincellige longkanker patiënten. In hoofdstuk 7, hebben we het serum geanalyseerd van 102 patiënten met gevorderd niet-kleincellige longkanker die allemaal erlotinib of gefitinib toegediend hadden gekregen. In deze groep werd de associatie tussen soluble-EGFR (sEGFR) en tussen carcinoembryogeen antigeen (CEA) met totale overleving bestudeerd. We hebben geconcludeerd dat toegenomen hoeveelheden sEGFR geassocieerd waren met een betere totale overleving (multivariate HR per µg / L stijging: 0,96, p = 0,004). Ook afnemende hoeveelheden van CEA (concentratie omgezet in logarithme) werden geassocieerd met een toename van de totale overleving (multivariate HR per log µg / L stijging: 1,51, p = 0,008). Helaas kent deze studie geen controlegroep van patiënten die geen behandeling met epidermale groeifactor receptor-tyrosine-kinase-inhibitors had ontvangen. Daarom zouden sEGFR en CEA nog steeds slechts overleving kunnen voorspellen (prognostische marker) in plaats van therapie respons (predictieve marker).

In hoofdstuk 8 hebben we daarom 64 patiënten met een EGFR-tyrosine-kinase-inhibitor naïef, niet-kleincellig longcarcinoom gekoppeld op basis van geslacht, leeftijd en histologie aan 61 patiënten met gevorderd niet-kleincellig longkanker die wel met EGFR-tyrosine-kinase-inhibitor (erlotinib of gefitinib) behandeld waren. In dit hoofdstuk hebben we onderzocht of serum concentraties van liganden van EGFR (transforming growth factor-alfa of amphiregulin), of liganden van de insuline-achtige groei factor (IGF)-receptor waarmee EGFR interactie heeft (namelijk IGF1 en IGF-bindend eiwit 3), voorspellend waren voor baat bij EGFR-kinase-inhibitor in relatie tot ziekte-specifieke overleving. Uit deze studie bleek dat lage concentraties van transforming growth factor-alfa correleerden met een betere ziekte-specifieke overleving in patiënten behandeld met EGFR-tyrosine-kinase-inhibitors in vergelijking met controle patiënten (multivariate HR: 0,55, p = 0,04), terwijl hoge concentraties werden geassocieerd met een niet-significante slechtere ziekte-specifieke overleving (multivariate HR: 1,51 , p = 0.40). Dit verschil was significant met een p-interactie van 0,05. Vergelijkbare resultaten werden verkregen met amphiregulin, waarbij hoge concentraties amphiregulin geassocieerd waren met een betere ziekte-specifieke overleving bij patiënten behandeld met EGFR-tyrosine-kinase-inhibitor vergeleken met controle patiënten (multivariate HR: 0,31, p = 0,001). Dit effect van EGFR-tyrosine-kinase-inhibitor behandeling op ziekte-specifieke overleving toonde ook een significant verschil voor amphiregulin (p-interactie: 0,004). Deze resultaten suggereren dat de concentraties van transforming growth factor-alfa en amphiregulin in het serum voorspellend zouden kunnen zijn voor baat bij EGFR-tyrosine-kinase-inhibitor therapie. Naast deze potentiële predictieve markers, was onze bevinding dat IGF-bindend eiwit 3 een potentiële prognostische marker is aangezien verhoging van serumconcentraties werd geassocieerd met een significant langere ziekte-specifieke overleving, ongeacht de behandeling (multivariate HR per 1 mg / L stijging: 0,56, p = 0,027). Als gevolg van de case-control opzet van onze studie zijn kenmerken van patiënten niet zo goed verdeeld over de EGFR-tyrosine kinase inhibitor groep en de controle groep als in een gerandomiseerde

gecontroleerde studie. Daarnaast kon als gevolg van relatief kleine aantallen de optimale cutoff voor transforming growth factor-alfa en amphiregulin en hun toegevoegde waarde met betrekking tot bekende klinische voorspellers voor EGFR-kinase-inhibitor gevoeligheid niet worden bepaald. Op dit moment proberen we de meest veelbelovende resultaten aangaande sEGFR, transforming growth factor-alfa en amphiregulin te valideren in een kleine neoadjuvante studie. In deze studie hebben niet-kleincellig longkanker patiënten erlotinib ontvangen voorafgaand aan de operatie, waarbij serum marker concentraties zullen worden gemeten en gekoppeld aan PET-respons data. Het zou echter ook interessant zijn om deze bevindingen te testen in een bij voorkeur prospectieve gerandomiseerde gecontroleerde omgeving van patiënten met gevorderd niet-kleincellig longkanker.

BEKNOPTE LEKEN SAMENVATTING

Borst- en longkanker patiënten worden behandeld met antikankermiddelen op basis van effectiviteitstudies in de gehele groep patiënten. Deze studies geven echter geen inzicht in de effectiviteit per individu. In dit proefschrift is de ontwikkeling van testen beschreven welke vooraf voorspellen of iemand wel of geen baat heeft bij bepaalde behandelingen. Hiermee zouden patiënten vanaf het begin de juiste behandeling kunnen krijgen, waarmee kostbare tijd bespaard wordt en uiteindelijk sterfte zal verminderen.

Recentelijk is aangetoond dat vooral patiënten met erfelijke borstkanker, met een mutatie in de genen BRCA1 of BRCA2, veel baat hebben bij alkylerende middelen. Een verklaring hiervoor is dat borsttumoren met deze mutaties de DNA-schade, veroorzaakt door alkylerende middelen, niet goed meer kunnen repareren en hierdoor afsterven. Daarnaast is het bekend dat borsttumoren van patiënten met deze mutaties een zeer specifiek patroon van DNA-veranderingen vertonen.

Wij hebben gevonden dat dit patroon van DNA-veranderingen niet alleen aanwezig is in patiënten met erfelijke borstkanker, maar in 15-25% van de gehele borstkanker populatie. Patiënten met tumoren met deze DNA-veranderingen bleken vijf keer minder kans te hebben op sterfte na hoge-dosis, alkylerende chemotherapie dan na standaard chemotherapie.

In longkanker zijn tumoren met een EGFR-mutatie extra gevoelig voor erlotinib of gefitinib, middelen welke EGFR remmen. In dit proefschrift bleek dat patiënten met vergevorderde longkanker met in het bloed verlaagde concentraties van transforming growth-factor alfa of verhoogde concentraties van amphireguline, beide eiwitten sturen EGFR aan, meer baat hadden van erlotinib of gefitinib.

Bevestiging van deze vindingen is vereist voor introductie in de klinische praktijk.

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Something was bound to go right today. All these broken pieces fit together to make a perfect picture

Adapted from Daybreak by Snow Patrol

DANKWOORD

Albert Einstein schreef: "Logic will get you from A to Z; imagination will get you everywhere". Hoewel ik toch een redelijk grote fantasie en verbeelding bezit, had ik me vijfeneenhalf jaar geleden (en eigenlijk tot voor kort) niet kunnen indenken dat het einde nu bijna inzicht is. Het afgelopen jaar realiseer ik me steeds beter wat een prachtig waardevol gegeven het is om de tijd, vrijheid en ruimte te hebben om je te verdiepen en vast te bijten in interessante vraagstukken. En dat in een omgeving gevuld met een heleboel mensen die je aanmoedigen, je uitdagen, je met hun verbluffende hoeveelheid kennis enthousiasmeren, je nieuwe inzichten geven, je op koers houden in tijdelijk noodweer en met wie je vooral ook veel lacht en deelt. Het eind van dit proefschrift wil ik dan ook gebruiken om alle mensen te bedanken die dit fantastische, indrukwekkende, soms wat bizarre, maar vooral leerzame avontuur voor mij mogelijk hebben gemaakt.

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CURRICULUM VITAE

Marieke Vollebergh was born at the 23rd of October 1982 in Velsen. After graduating from grammar school at the Gymnasium Felisenum in Velsen-Zuid, Marieke commenced her medical school at the University of Utrecht. During her studies, Marieke extended her skills in medicine both by creating knowledge and sharing knowledge: she performed academic research at the Dutch Cancer Institute into predicting response to high dose chemotherapy in metastatic breast cancer, and she taught students of medicine at the University of Utrecht in both practical skills and theory.

After obtaining her medical degree, Marieke started her PhD research at the Dutch Cancer Institute / Antoni van Leeuwenhoek Hospital in January 2007. Supervised by dr. Sabine Linn and prof. dr. Sjoerd Rodenhuis, Marieke performed research into the identification of predictive markers for systemic treatment in breast and lung cancer patients.

In February 2011, Marieke started her residency in Pulmonology at the Academical Medical Centre Amsterdam, supervised by prof. dr. E.H.D. Bel. Marieke spends year one and two of this trajectory as resident in Internal Medicine at Tergooiziekenhuizen Hilversum and Blaricum, supervised by dr. S. Lobatto.