Predicting therapy resistance and toxicity in breast cancer patients

Annelot Geerke Jantine van Rossum

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Predicting therapy resistance and toxicity in breast cancer patients

Voorspellen van therapieresistentie en toxiciteit in borstkankerpatiënten

(met een samenvatting in het Nederlands)

Proefschrift

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Annelot Geerke Jantine van Rossum

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1

General introduction

INTRODUCTION

Breast cancer is one of the most common causes of cancer worldwide.[1] In 2016, 1.7 million people were diagnosed with breast cancer and 545,000 people died of the disease. Despite advances in diagnostics, risk assessments, and therapeutic strategies, there are still many challenges to overcome. This thesis focusses on finding predictive biomarkers to improve efficacy and minimize toxicity of systemic treatment for breast cancer. First, we will discuss systemic therapies for early breast cancer that require biomarkers in order to tailor treatment. Secondly, an overview of clinically relevant prognostic and predictive biomarkers is given. Lastly, we give two examples of biomarker breast cancer trials in the metastatic setting.

Adjuvant chemotherapy

Chemotherapy as addition to locoregional treatment aims to eradicate micrometastases in order to prevent the occurrence of distant metastatic lesions.[2] Over the past decades the combination of drugs, the dose of chemotherapeutic agents and the schedule of administration have been optimized. Also, the timing of chemotherapy has changed. While chemotherapy given after surgery, adjuvant chemotherapy, was the standard, an increased rate of patients receives chemotherapy before surgery nowadays, called neoadjuvant chemotherapy.[3] Currently used therapies are multidrug regimens. Here we discuss two chemotherapeutic classes that have substantially contributed to improved outcome. Understanding their mechanism of action is pivotal to find predictive biomarkers. Also, we discuss alternative ways of scheduling chemotherapy to increase survival rates.

Chemotherapeutic agents

Anthracyclines act through different mechanisms to eradicate tumor cells. First, anthracyclines inhibit topoisomerase 2 (TOP2).[4] At the site of DNA loops or entanglements, TOP2 cuts both DNA strands to allow realignment of the DNA. Inhibiting TOP2 leads to DNA double strand breaks. Secondly, anthracyclines are known to form free radicals that disrupt DNA strands, leading to more DNA damage.[5] Under normal circumstances, DNA double strand breaks are repaired starting with phosphorylation of histone variant H2AX. This elicits the DNA repair response. However, anthracyclines are also thought to promote histone eviction from the DNA, including H2AX.[6] Absence of H2AX at the site of the DNA double strand breaks hampers the DNA repair response and increases the amount of DNA damage. When abundant enough, the DNA damage

caused by anthracyclines leads to apoptosis of tumor cells and, subsequently, to shrinkage of the tumor.

The first anthracycline-based regimen consisted of 4 cycles of doxorubicin combined with cyclophosphamide (AC). Four cycles of AC appeared to be equally effective as six cycles of the established combination of cyclophosphamide, methotrexate and 5-fluorouracil (CMF).[7] Significantly improved survival rates of anthracycline-based regimens compared with standard chemotherapy were observed when doxorubicin or epirubicin was combined with cyclophosphamide and 5-fluorouracil (FAC, CAF, FEC, CEF). A meta-analysis of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) showed that 6 cycles of anthracycline-based chemotherapy established a significant absolute 15-year reduction in breast cancer mortality of 3-10% compared with no adjuvant chemotherapy[8] and 10-year reduction in breast cancer mortality of 4% compared with CMF.[7] In addition, a higher cumulative dose of anthracyclines has been associated with improved outcome.[7] However, previous attempts to raise the cumulative dose were limited by increased rates of adverse events, particularly congestive heart failure.[9-11] Although cardiac toxicity may have long-term adverse effects and impair quality of life, it is observed in a minority of patients treated with anthracyclines. Therefore, the efficacy of anthracyclines is still thought to outweigh its potential harms.[12]

The chemotherapeutic class of taxanes are known to interfere with microtubules.[13] Microtubules consist of tubulin heterodimers of alpha and beta subunits. The maintenance of microtubules is dynamic: tubulin dimers are constantly bound and released to allow microtubules to undergo conformational changes. Microtubules are involved in many cellular processes, including adaptations in cell shape and intracellular transport.[14] Taxanes stabilize microtubules by inhibiting the release of tubulins, thereby hampering the conformational changes of the microtubules required for their functions, which in turn leads to apoptosis and tumor shrinkage. Also, during mitosis microtubules form the intracellular structure to pull the chromosomes out of the metaphase plate to the two centrosomes at either end of the cell. When stabilized microtubules are not able to bind all chromosomes during mitosis, the cell cycle will arrest. Even if the cell is able to escape this arrest via mitotic slippage, it may result in ongoing cell survival with considerable DNA rearrangements, senescence or cell death.[15]

Taxanes have further improved survival of early breast cancer patients when added to anthracycline-based chemotherapy.[7,16,17] Four large adjuvant trials showed that a taxane-and-anthracyline-based regimen improved disease free survival (DFS) and

overall survival (OS) compared with anthracycline-based chemotherapy.[18-21] An update on the BCIRG001 at 10 years follow up confirmed the superior survival after 6 cycles of TAC compared with 6 cycles of FAC.[22] However, adding a taxane to anthracycline-based chemotherapy also caused additional toxicity. Peripheral neuropathy is a common, possibly irreversible and long-lasting side effect of taxanes.[23,24] Therefore, predictive biomarkers are needed to assess which patients will benefit from the addition of a taxane. In chapters 2 and 3 we aim to find biomarkers for the efficacy and toxicity, respectively, of taxane-based treatment.

Treatment schedules

To determine the optimal chemotherapy schedule several aspects should be taken into account: the number of cycles, concurrent or sequential administration of chemotherapeutic agents, and dose intensification.

Dose intensity is defined as the total dose of drug given per body surface area per unit of time, denoted as mg/m² per week.[25] One could increase the dose intensity of a treatment by shortening the interval between each dose, known as dose densification, or by giving a higher dose, called dose escalation. Dose escalation is based on the log-kill model[26], which suggests that a certain dose of chemotherapy would kill the same amount of cells regardless of the size of the tumor. Increasing the dose would therefore result in an increased amount of tumor cells killed. However, the effect is limited. Two clinical trials showed that increasing the dose beyond an upper limit of 60 mg/m² doxorubicin and 600 mg/m² cyclophosphamide every 21 days does not result in additional survival gain.[19,27] Dose densification is founded on the Norton-Simon hypothesis[28,29], which assumes Gompertzian growth of a tumor. According to this model, tumor growth increases with the size of the tumor to reach a plateau at a certain volume. Norton and Simon hypothesized that the rate of regression of a tumor is proportional to the growth of a tumor. Shortening the interval between chemotherapy cycles will give the tumor less time to regrow and the tumor will therefore shrink in size (Figure 1).

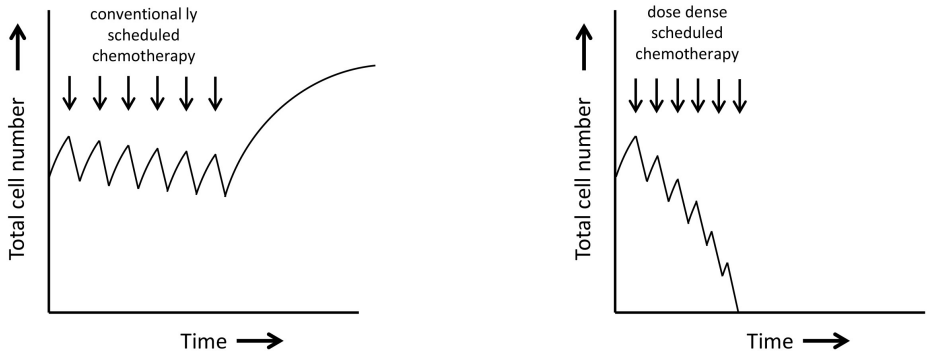

Figure 1. The association between time and tumor volume during chemotherapy administration. Conventionally scheduled chemotherapy is given every 3 weeks, dose dense scheduled chemotherapy every 2 weeks. With conventionally scheduled chemotherapy the tumor can regrow between chemotherapy cycles and eventually escape treatment. With dose dense scheduled chemotherapy the tumor has less time to regrow, which leads to tumor shrinkage.

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Three meta-analyses on dose dense chemotherapy had the same conclusion: dose dense administration of adjuvant chemotherapy improved DFS and disease specific survival of early breast cancer patients.[30-32] However, a separate analysis on anthracycline-based regimens on the one hand and taxane-and-anthracyline-based chemotherapy on the other is lacking. In chapter 1 of this thesis we investigate whether dose dense scheduling of anthracycline-based chemotherapy is equally effective as adding a taxane to conventionally scheduled anthracycline-based chemotherapy. We directly compare 6 cycles of dose dense scheduled (given every 2 weeks) doxorubicin and cyclophosphamide (ddAC) and 6 cycles conventionally scheduled (given every 3 weeks) docetaxel, doxorubicin and cyclophosphamide (TAC) as adjuvant treatment for early breast cancer. In chapter 2, we aim to improve survival at the individual level by identifying a gene expression profile that predicts which patients derive survival benefit from ddAC and which patients from TAC.

The risk of increased toxicity often hampers dose escalation. However, when chemotherapeutic agents are given sequentially instead of concurrently, drug dose may be increased without causing additional toxicity. Further increase of the dose intensity can be achieved by scheduling a dose escalated, sequential regimen in a dose dense manner. An example of a dose intensified, sequentially given chemotherapy regimen is discussed in chapter 4. Previously, analyses of the German Adjuvant Intergroup Node-

positive Study 2 (GAIN-2)[33] showed that 3 x 3 cycles of dose intensified, sequentially given epirubicin, paclitaxel and cyclophosphamide (ETC) resulted in similar DFS and OS compared with 4 cycles concurrently given epirubicin and cyclophosphamide followed by 10 cycles weekly paclitaxel and 4 cycles capecitabine (EC-TX) as adjuvant treatment for primary breast cancer.[34]

The BRCA1 protein plays an important role in the repair of DNA double strand breaks via the error free homologous recombination (HR) pathway. If the BRCA1 protein is inactive due to for instance a mutation in the *BRCA1* gene or due to hypermethylation of the promotor[35], genomic instability arises. This can result in a distinct pattern of DNA copy number gains and losses, which is called a *BRCA1*-like profile.[36,37] Previous studies have shown that patients with a *BRCA1*-like tumor have a lower risk of recurrence when treated with myeloablative, high-dose platinum or alkylating drugs compared with conventional chemotherapy.[38,39] Our hypothesis is that the *BRCA1*-like profile also predicts survival benefit of dose intensified, non-myeloablative alkylating chemotherapy, such as the ETC arm in the GAIN-2 study. In chapter 4, we investigate the predictive value of the *BRCA1*-like profile for survival benefit of ETC in TNBC patients of the GAIN-2 study cohort.

Biomarkers for therapy benefit

Every tumor is unique, employing its own pathways to grow and proliferate. Therefore, a tailored treatment strategy should be used to optimize efficacy. Prognostic and predictive biomarkers can help to make tailored treatment decisions. However, there is an important difference on how to use them.

Prognostic markers

Prognostic markers are used to identify who to treat.[40] Every tumor has a likelihood of metastasizing to distant sites. Systemic therapy as addition to locoregional treatment aims to prevent the occurrence of metastases. Therefore, the risk of a patient to develop distant lesions determines who needs treatment. Biomarkers that adequately estimate this risk can be used to select patients who should receive chemotherapy, endocrine therapy, or both.

Over the years, several patient-related and tumor-related factors have been used as prognostic biomarkers. Patient-related factors include age at diagnosis, menopausal status and WHO performance status. Tumor-related characteristics comprise tumor size,

involvement of locoregional lymph nodes, presence or absence of distant metastases, histologic grade and expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Some of these features are highly correlated.[41,42] Clinicopathologic factors are combined in various risk assessment tools, including the Nottingham Prognostic Index (NPI)[43], the St. Gallen Classification[44], PREDICT[45], and Adjuvant![46]. The NPI comprises a simple model based on tumor size, number of tumor positive lymph nodes and histologic grade. Although updated over the years[47], the only validated version of the prediction is the original classification. PREDICT and Adjuvant! were both based on data of large patient cohorts and included mainly the same characteristics, including age, tumor size, number of positive lymph nodes, histologic grade and ER status. However, while Adjuvant! has taken comorbidity into account, PREDICT has been updated to correct for HER2 status and proliferation marker Ki67[48]. The St. Gallen Classification initially accounted for the same characteristics as PREDICT and Adjuvant! and added Ki67 as a predictor.[49,50] Although applied in several classifications, the use of Ki67 is under debate. The interobserver variability and the lack of consensus on the recommended cut-off[51] hamper the unequivocal use of this marker in the clinic.[52,53]

Although the afore mentioned clinicopathologic classifications estimate the prognosis of patients in general quite well, miscalculations have been observed in subgroups of patients.[54-56] Moreover, classifications based on clinicopathological features do not take the molecular complexity of breast cancer into account. Therefore, new prognostic signatures were developed based on gene expression (RNA) data, genomic (DNA) data or both. Importantly, these signatures add prognostic information to the known clinicopathologic features instead of replacing them.

Prognostic gene expression signatures can be divided into intrinsic signatures and outcome-based signatures. Whereas intrinsic gene expression signatures group patients based on shared molecular features, outcome-based gene expression signatures define patient groups based on the association between the expression of genes and outcome of the disease. A widely-used intrinsic gene expression-based signature is the PAM50 classification.[57,58] It defines five breast cancer subtypes (luminal A and B, HER2 enriched, basal and normal-like) that show overlap with clinicopathological features.[59] Luminal A and B tumors are both associated with ER positivity. Luminal B tumors differ from luminal A tumors with regard to size and grade.[60] The majority of the HER2 enriched tumors shows protein overexpression of HER2. Basal tumors generally lack expression of ER, PR and HER2. In addition, the gene expression-based IntClust classification distinguishes 10 subtypes, each harboring distinct oncogenic drivers.[61,62]

Other gene expression signatures were built with genes selected for their association with survival, such as the 70-gene MammaPrint[63] and 21-gene OncotypeDx[64]. The MammaPrint was developed in 117 patients with early breast cancer, leading to a 'low risk' or 'high risk' classification. Prognostic value was confirmed after median follow-up of 18.5 years.[65] In addition, the MammaPrint was validated in retrospective studies of specific patient subgroups (lymph node negative[66], lymph node positive[67], older patients[68] and HER2 positive breast cancer[69]) and prospectively in the RASTER study[70,71]. Also, the MINDACT trial showed that the genomic MammaPrint signature adds information to clinicopathologic classifier Adjuvant!.[72] In patients who had a high clinicopathologic risk and a low genomic risk, 5-year distant metastasis-free survival was similar for the subgroup that did receive chemotherapy and the subgroup that did not receive chemotherapy, indicating that patients with a low genomic risk may forego chemotherapy. The recurrence score of OncotypeDx was tested on 668 ER-positive, lymph node negative breast cancer patients, leading to a 'low-risk', 'intermediate-risk' or 'high-risk' classification, and was validated in another 651 patients[73]. Whereas high-risk patients would benefit from (neo)adjuvant chemotherapy and low-risk patients would not due to the low baseline likelihood of developing a recurrence[73], it was unclear whether the intermediate-risk group would need chemotherapeutic treatment. The TAILORx study assessed the added value of chemotherapy for the intermediate-risk patients. Patient who were treated with endocrine treatment or chemotherapy and endocrine treatment had similar DFS and OS, except for the patients of 50 years of age or younger who did derive some benefit of adjuvant chemotherapy.[74]

Due to their added value to clinicopathologic factors, the PAM50 classifier, MammaPrint signature and the OncotypeDx signature were included in the ASCO guidelines[75,76] and are currently used in the clinic.

Predictive markers

Predictive markers are used to determine how to treat.[40] If a patient requires systemic treatment according to the risk of developing distant metastases, the next step is to choose the therapy that is most effective and causes least side effects. Predicting survival benefit or toxicity from a particular treatment, or from one treatment over another is pivotal to tailor therapy. However, finding clinically valid predictive biomarkers is challenging.[77] In order to be successful, a biomarker study needs to comply with certain conditions.

First, the investigated cohort must consist of treated and untreated or differently treated patients[78-80], as is the case in a randomized clinical trial. The need for treated and untreated or differently treated patients is illustrated in figure 2. The cohort is split into a biomarker negative and a biomarker positive subgroup. The prognostic effect is the survival difference between the untreated subgroups. The difference in survival between the treated and the untreated patients within each biomarker subgroup is the effect of the treatment. The predictive value of the biomarker can be derived from the difference in treatment effects in the marker positive and the marker negative subgroup. Secondly, the design of the study should aim at finding biomarkers.[80] A randomized clinical trial that aims to find a predictive biomarker, thereby taking the treatment effect in the biomarker subgroups into account, provides the highest level of evidence, while an exploratory, retrospective analysis has considerably less value. Thirdly, the treatment groups should be balanced for known prognostic characteristics, which is often secured in a randomized clinical trial. Given their effect on survival, these characteristics might interfere with the association between the predictive biomarker and survival. Finally, a refined and robust method to measure or determine the biomarker is needed.

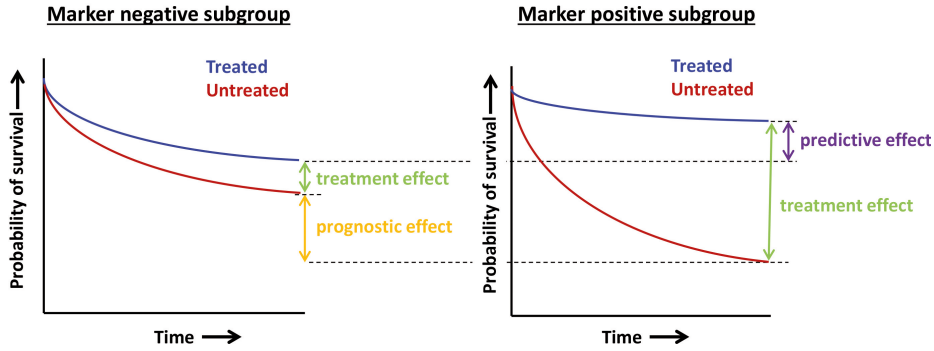

Figure 2. Prognostic and predictive effect of a biomarker. A biomarker splits a cohort of treated and untreated patients into a marker negative subgroup and a marker positive subgroup. The survival difference of the untreated cohorts is the prognostic value of the biomarker (depicted in orange). The effect of the treatment is defined as the difference between the treated and the untreated patients within the biomarker subgroups (green). The predictive value of the biomarker is the differential treatment effect between the biomarker subgroups (purple).

Many reports on predictive biomarkers for efficacy of chemotherapy[79,81-89], endocrine therapy[78,90-92], and targeted therapies[93-97] exist. However, very few predictive biomarkers have made it to the clinic. To date, the only clinically implemented predictive biomarkers are ER expression for endocrine therapy and HER2 overexpression for anti-HER2-therapies. The search for biomarkers may have been hampered by imperfect trial design.[80] Single arm studies are suited to investigate the potential predictive capacity of a biomarker. However, it is impossible to distinguish the prognostic from predictive effect in these studies. Moreover, finding biomarkers is hardly ever the primary aim of a trial. Most randomized trials are designed to compare efficacy of two treatments. Biomarkers analyses are done as a post-hoc investigation or as a secondary objective. Depending on the size of the biomarker subgroups and of the treatment effect within the subgroups, the number of patients in the cohort is usually insufficient to find a predictive biomarker. Prospective biomarker trials are scarce, but pivotal in order to get biomarkers to the clinic. Furthermore, chemotherapy and endocrine therapy for early breast cancer is applied to a large group of patients, including patients who might not need extensive systemic therapy in order to prevent disease recurrence due to the natural course of their disease.[98] Besides overtreatment, it causes statistical challenges in biomarker investigations. This issue may be solved by selecting only those patients who are at risk of developing a disease recurrence and therefore need adjuvant chemotherapy and/or endocrine therapy. Although the risk can be assessed by several tools, it remains challenging in practice.

In addition to biomarkers for treatment efficacy, biomarkers that predict toxicity may be of additional value in treatment decision making. Predicting toxicity becomes particularly important when two treatments are equally effective. Numerous associations between chemotherapy toxicity and genetic variants have been described.[99-106] To our knowledge, only one biomarker based on genetic variants in dihydropyrimidine dehydrogenase (DPD) is currently used in the clinic to screen patients for chemotherapy-related toxicity[107]. The conditions for studies on biomarkers for survival benefit apply to a large extend also to studies on biomarkers for treatment toxicity. However, randomized clinical trials are generally not designed to find biomarkers for toxicity. If incorporated at all, it is a secondary objective or it is analyzed in a post-hoc manner. Validation in independent cohorts is therefore crucial for putative toxicity biomarkers to make their way to the clinic.

Targeted therapy for metastatic breast cancer

Every tumor has its own mechanisms to grow and proliferate. Whole genome sequencing of 560 breast tumors identified 93 protein-coding genes harboring a potential driver mutation.[108] Targeting a driver mechanism may improve survival of a subgroup of breast cancer patients whose tumor relies on this mechanism. Numerous therapies that target specific mechanisms are currently under development. In this thesis we introduce two targeted agents.

PI3K inhibitor taselisib

Seventy percent of all breast cancer patients is diagnosed with ER-positive disease and is treated with endocrine therapy. Although the use of adjuvant endocrine therapy has prolonged breast cancer specific survival[109], endocrine treatment resistance is an important problem that leads to incurable metastatic disease. Mechanisms that underlie endocrine treatment resistance have been studied intensively, indicating an important role for the phosphatidylinositol-3-kinase(PI3K) pathway.[110] Two randomized clinical trials in metastatic breast cancer patients showed that mammalian target of rapamycin (mTOR) inhibitor everolimus in combination with endocrine therapy results in better outcome compared with endocrine therapy alone.[111,112] However, a considerable amount of patients encountered toxicity, including stomatitis, rash, diarrhea, pneumonitis and hyperglycemia.[111,112] Moreover, inhibition of mTOR could lead to activation of upstream PI3K pathway component protein kinase B (Akt)[113,114], causing resistance via other pathways.

To improve on the toxicity profile and prevent resistance via other pathways, a selective, β-isoform sparing inhibitor of PI3K, taselisib, has been developed. Out of three classes, class IA PI3Ks are most involved in cancer progression. They consist of a p110 catalytic and a p85 regulatory subunit[115] of which the p110α isoform is associated with oncogenic transformation[116]. The p110α isoform is encoded by the *PIK3CA* gene. Activating mutations in *PIK3CA* are common in ER-positive breast cancer: 25% of ductal breast cancers and 40-45% of lobular breast cancers harbors a mutation in *PIK3CA*.[117,118]

Preclinical and clinical work on taselisib has shown promising safety and efficacy data. Taselisib appeared to have superior efficacy in *PIK3CA* mutant cancer cell lines and xenograft models.[119] A phase I dose escalation study of taselisib single agent in solid tumors showed encouraging antitumor activity as well as downregulation of PI3K pathway components.[120] A phase 1b study of 6 mg taselisib QD (capsule formulation;

equivalent to 4 mg tablet formulation) combined with letrozole indicated that the combination was well-tolerated, that there were no drug interactions, and that the overall response rate was 38% in patients with *PIK3CA* mutant breast cancer and 9% in patients with *PIK3CA* wildtype breast cancer.[121] A single arm study of taselisib combined with fulvestrant showed similar objective response rates.[122] In chapter 5 we report on the phase 1b POSEIDON study in which taselisib is combined with tamoxifen. We evaluate toxicity, efficacy and potential predictive biomarkers for this combination treatment.

VEGF-A inhibitor bevacizumab

Angiogenesis, the formation of new blood vessels, is pivotal for tumor cells to grow and proliferate. Xenograft models of invasive breast cancer showed that new blood vessels were formed in all models with invasive breast cancer, while angiogenesis was not observed in normal tissue models.[123] Moreover, microvascular density in the primary tumor has been associated with presence of metastases[124,125] and survival[126,127].

The process of angiogenesis is mediated by several factors, including vascular endothelial growth factors (VEGFs).[128] The group of VEGFs consists of four variants (VEGF-A, VEGF-B, VEGF-C and VEGF-D) that interact with three tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR-3).[129] VEGF-A is pivotal for angiogenesis by inducing endothelial cell division, promoting endothelial cell survival, and increasing vascular permeability.[130] Also, VEGF-A was found at higher levels in breast cancer patients than in healthy women[131,132] and it has been associated with survival.[133] Given its crucial role in tumor angiogenesis VEGF-A has become the therapeutic target of monoclonal antibody bevacizumab.[130]

Bevacizumab appeared most valuable as add-on to chemotherapy. A phase 3 study showed that bevacizumab combined with capecitabine resulted in increased response rates compared with capecitabine alone.[134] Convincing evidence on the value of bevacizumab, however, was obtained from a large phase 3 trial in which patients were randomized between paclitaxel combined with bevacizumab and paclitaxel only as first line treatment for metastatic breast cancer. Bevacizumab addition led to substantially longer progression free survival (PFS; 11.8 vs 5.9 months; HR 0.88).[135] Based on these data the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) decided to approve bevacizumab in combination with paclitaxel as treatment for metastatic breast cancer. However, results of subsequent trials (AVADO, RIBBON-1) were less persuasive[136,137], which even led to withdrawal of the FDA approval.

Despite the moderate effect of bevacizumab observed in a general population of metastatic breast cancer patients, there might be a subgroup of breast cancer patients that will derive PFS and OS benefit. Although previous work indicated that TNBC expresses higher levels of VEGF-A compared with non-TNBC[138], a large trial showed that bevacizumab addition to chemotherapy for this subgroup did not result in survival benefit.[139] Instead, retrospective analyses of the AVADO trial cohort indicated that high plasma VEGF-A and VEGFR-2 levels could predict which patients would derive benefit from bevacizumab addition.[94,139] However, the prospective MERiDiAN trial could not confirm the predictive value of VEGF-A for survival benefit of bevacizumab.[93] In chapter 6 of this thesis we report on the prospective biomarker Triple-B trial in which the predictive potential of plasma VEGFR-2 levels for survival benefit of bevacizumab is investigated.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to identify predictive biomarkers for efficacy and toxicity of systemic treatment for breast cancer. **Chapters 1** until **4** focus on biomarkers for adjuvant chemotherapy. Two targeted therapies for metastatic disease are discussed in **chapter 5** and **6**.

Biomarkers for chemotherapy in the adjuvant setting

Taxane addition and dose dense scheduling of adjuvant anthracycline-based chemotherapy have improved breast cancer specific survival substantially. However, it is unknown which patients will benefit from taxane addition and which patients from dose dense scheduled chemotherapy. In **chapter 1** we report on the survival of early breast cancer patients who were randomized between 6 cycles adjuvant dose dense scheduled doxorubicin and cyclophosphamide (ddAC) and 6 cycles adjuvant docetaxel, doxorubicin and cyclophosphamide (TAC) in the MATADOR trial. In **chapter 2** we discuss the primary objective of the MATADOR trial. We aim to identify a gene expression profile that predicts benefit of either dose dense or taxane-based chemotherapy. In addition, other biomarkers for efficacy of either of the two treatments are described. In **chapter 3**, we focus on clinical parameters and single nucleotide polymorphisms (SNPs) that predict toxicity of the treatments in the MATADOR trial. In **chapter 4** we report on the predictive capacity of the *BRCA1*-like profile in the German Adjuvant Intergroup Node positive study 2 (GAIN-2) in which patients were randomized between 3 x 3 cycles of dose intensified, sequentially given epirubicin, paclitaxel and cyclophosphamide (ETC) and 4 cycles of epirubicin and cyclophosphamide followed by 10 cycles weekly paclitaxel and 4 cycles capecitabine (EC-TX).

Biomarkers for targeted therapy in the metastatic setting

In **chapter 5** we describe the results of the phase 1b part of the POSEIDON study in which PI3K inhibitor taselisib was combined with tamoxifen in metastatic, ER positive breast cancer patients. Data on toxicity, preliminary efficacy, and predictive biomarkers derived from tumor tissue and circulating tumor DNA are discussed. Finally, in **chapter 6** we report on the interim analysis of toxicity and efficacy of two chemotherapeutic regimens ± bevacizumab as first line treatment for triple negative breast cancer in a prospective biomarker trial, the Triple-B study. Also, we report on the potential predictive value of plasma VEGFR-2 level for survival benefit of bevacizumab addition.

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Adjuvant dose-dense doxorubicin-cyclophosphamide versus docetaxel-doxorubicin-cyclophosphamide for high-risk breast cancer: first results of the randomised MATADOR trial (BOOG 2004-04)

A.G.J. van Rossum, M. Kok, E. van Werkhoven, M. Opdam, I.A.M. Mandjes,
A.E. van Leeuwen - Stok, H. van Tinteren, A.L.T. Imholz, J.E.A. Portielje, M.M.E.M. Bos,
A. van Bochove, J. Wesseling, E.J. Rutgers, S.C. Linn, H.M. Oosterkamp,
on behalf of the MATADOR Trialists' Group

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ABSTRACT

Background: Dose-dense administration of chemotherapy and the addition of taxanes to anthracycline-based adjuvant chemotherapy have improved breast cancer survival substantially. However, clinical trials directly comparing the additive value of taxanes with dose-dense anthracycline-based chemotherapy are lacking.

Patients and methods: In the multicentre, randomised, biomarker-discovery Microarray Analysis in breast cancer to Tailor Adjuvant Drugs or Regimens (MATADOR) trial, patients with pT1-3, pN0-3 breast cancer were randomised (1:1) between six adjuvant cycles of doxorubicin 60 mg/m² and cyclophosphamide 600 mg/m² every 2 weeks (ddAC) and six cycles of docetaxel 75 mg/m², doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m² every 3 weeks (TAC). The primary objective was to discover a predictive gene expression profile for ddAC and TAC benefit. Here we report the preplanned secondary endpoints recurrence-free survival (RFS) and overall survival (OS).

Results: Between 2004-2012, 664 patients were randomised. At 5 years, RFS was 87% (95% confidence interval [CI] 83%–91%) in the ddAC-treated patients and 88% (84–92%) in the TAC-treated subgroup (hazard ratio [HR] 0.89, 95% CI 0.62-1.28, P=0.53). OS at 5 years was 93% (90%–96%) in the ddAC-treated and 94% (91%–97%) in the TAC-treated patients (HR 0.89, 95% CI 0.57-1.39, P=0.61). Anaemia was more frequent in ddAC-treated patients (62/327 patients [18.9%] versus 15/319 patients [4.7%], P<0.001) and diarrhoea (21 [6.4%] versus 53 [16.6%], P<0.001) and peripheral neuropathy (15 [4.6%] versus 46 [14.4%], P<0.001) were observed more often in TAC-treated patients.

Conclusions: With a median follow-up of 7 years, no significant differences in RFS and OS were observed between six adjuvant cycles of ddAC and TAC in high-risk breast cancer patients.

Trial registration numbers: ISRCTN61893718 and BOOG 2004-04

INTRODUCTION

Adjuvant chemotherapy for early breast cancer aims to eradicate micrometastases to improve survival. Anthracycline-containing regimens have increased breast cancer survival substantially.[1]

Incorporation of taxanes into anthracycline-based schedules has further improved efficacy of adjuvant chemotherapy. Compared with six cycles of 5-fluorouracil-doxorubicin-cyclophosphamide, six cycles of adjuvant docetaxel-doxorubicin-cyclophosphamide (TAC) significantly improved overall survival (OS) from 81% to 87% in node-positive breast cancer.[2] The addition of four cycles of a taxane to a fixed anthracycline-based regimen, thereby extending treatment duration, also improved breast cancer specific survival (BCSS).[1]

Dose-dense scheduling of chemotherapeutic agents accounted for another important step forward. Dose densification is defined as the shortening of the interval between cycles, giving the tumour less time to regrow between treatment cycles. Three meta-analyses showed that adjuvant dose-dense chemotherapy improves disease-free survival (DFS) and OS of breast cancer patients compared with conventionally scheduled chemotherapy regimens.[3-5]

Knowing that both the addition of a taxane and dose-dense scheduling increase efficacy of adjuvant chemotherapy, it is unclear which of these strategies gives the largest benefit for an individual patient. Two studies compared a taxane-based, dose-dense regimen directly with conventional dosed anthracycline-based treatment, resulting in a minor survival advantage for dose-dense-treated patients compared with conventionally treated patients.[6,7] However, to date, no randomised trial has directly compared a taxane-containing, conventionally scheduled treatment with a non-taxane-containing, dose-dense regimen. Here, we report the results of the preplanned secondary analyses of a randomised, biomarker discovery trial comparing six cycles of dose-dense-administered AC (ddAC) with six cycles of adjuvant TAC. The primary objective of this trial was to investigate whether a gene expression profile could be identified that could predict who should receive ddAC and who should receive TAC for the best outcome. Application of such a classifier would then lead to a better outcome for the whole group, than when all patients would have received one of these regimens that would have turned out best for the average patient.

MATERIALS AND METHODS

Study design and patients

The Microarray Analysis in breast cancer to Tailor Adjuvant Drugs Or Regimens (MATADOR, ISRCTN61893718) study is a multicentre, randomised, open-label, phase III trial primarily designed to identify a gene expression profile that can predict survival benefit of ddAC or TAC. Women with a pathologically confirmed T1-T3, N0-3b adenocarcinoma of the breast without signs of distant metastases were considered eligible. The study was amended to also include N0 patients from June 2008 onwards (Amendment 2). Adequate bone marrow, liver and renal functions were required. Main exclusion criteria were prior systemic treatment for cancer, history of breast cancer and other cancers (except for curatively treated non-melanoma skin cancer, in situ carcinoma of the cervix and ipsilateral ductal carcinoma in situ), and significant cardiac, neurological or psychiatric disorders. With trastuzumab not being part of the study treatment and accumulating evidence showing that concurrent trastuzumab and chemotherapy appeared superior compared with sequential scheduling, patients with human epidermal growth factor receptor 2 (HER2)-positive disease were considered ineligible after 2007 (Amendment 2).

The study protocol and amendments were approved by the ethical committee of the Netherlands Cancer Institute and the institutional review boards of the participating centres. The study was performed in accordance with Good Clinical Practice guidelines and with the Declaration of Helsinki (version 17C). All patients provided written informed consent.

Randomization and treatment

Patients were initially randomised among four treatments: four or six cycles of ddAC or four or six cycles of TAC. With emerging evidence that six cycles of fluorouracil-doxorubicin-cyclophosphamide (FAC) resulted in better outcomes than six cycles of cyclophosphamide-methotrexate-fluorouracil (CMF)[8], with six cycles of CMF being equally effective as four cycles of AC[9], randomisation was limited to the six cycle regimens (Amendment 1). By then, five patients had received four cycles of ddAC and five patients received four cycles of TAC. Randomisation (1:1) was performed centrally at the Netherlands Cancer Institute using the automated ALEA system (FormsVision BV, the Netherlands).

Patient received either six cycles of doxorubicin 60 mg/m² plus cyclophosphamide 600 mg/m² every 2 weeks or six cycles of docetaxel 75 mg/m², doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m² every 3 weeks. Granulocyte colony-stimulating factor (pegfilgrastim 6 mg) was given to all patients the day after chemotherapy administration. Prophylactic antibiotics were not standard of care in the study.

Randomisation was stratified by the menopausal status, type of surgery, sequence of adjuvant therapy, tumour size and lymph node status according to AJCC staging, hormone receptor status, HER2 status and treatment centre using Pocock's minimisation technique.

Dose reductions and interruptions were allowed in case of adverse events grade III or higher according to common toxicity criteria for adverse events (CTCAE) version 3.0, except for peripheral neuropathy that required dose reduction of docetaxel at grade II. Adjuvant radiotherapy and/or endocrine therapy were initiated according to the Dutch guidelines on breast cancer treatment (www.oncoline.nl).

Assessments

Patients were assessed for relapse of disease at regular intervals for 10 years. Evaluation included physical examination and yearly mammography. Adverse events grade II and higher were reported using the CTCAE v3.0.

Histological grade according to the modified Bloom-Richardson classification[10], and morphology were assessed locally. Tissue microarrays (3 cores of 0.6 mm per patient) were constructed and stained for oestrogen receptor (ER), progesterone receptor (PR) and HER2. According to the Dutch guidelines, ER and PR staining of 10% or more and HER2 score of 3+ or more were scored as positive. In case of a 2+ HER2 score, an in situ hybridisation assay was performed. Central assessment of ER, PR and HER2 was used. If tumour tissue was unavailable, local assessment was used. Breast cancer subtype was defined as (1) ER and/or PR-positive and HER2-negative; (2) HER2-positive, regardless of ER and PR status or (3) triple negative.

Objectives and endpoints

The primary objective of the trial was to generate a gene expression profile predictive of DFS benefit of either dose-dense chemotherapy or a docetaxel-containing schedule. DFS was defined as the interval between randomisation and locoregional or distant

relapse, second primary cancer, or death by any cause. Because a second primary cancer could not directly be attributed to failure of eradicating micrometastases with systemic treatment, the study protocol was amended (Amendment 3) to change the primary endpoint to recurrence-free survival (RFS). RFS was defined as the interval between randomisation and locoregional or distant relapse or death by any cause.[11]

The secondary objective was to compare the efficacy of TAC and ddAC. End-points included RFS, distant recurrence-free interval (DRFI), defined as the time from randomisation until distant relapse or breast cancer-related death, OS and BCSS. Also, we evaluated the patients who received at least one cycle of the allocated treatment for toxicity during follow-up.

Statistics

The primary endpoint of the trial was the gain in RFS attributed to the genetic profile. This gain was defined as the improvement of RFS at 5 years with the treatment strategy using the profile, over the strategy in which all patients would get the same treatment (either ddAC or TAC), whichever would appear better from the direct comparison (which was the secondary objective). It was calculated that if the profile would be developed using data from 400 patients, the standard error of the estimate of the gain would be less than 2.5%. The sample size of the study was set at 660 so that 1/3 of the data could be used as a validation cohort, allowing for 10% early dropout. For the direct comparison of the arms (the secondary objective), 192 RFS events were required to obtain 80% power to detect a difference of a hazard ratio (HR) of 0.67. During the course of the study, it became clear that the event rate was lower than expected. Therefore, an amendment was made to the protocol. At the time of this amendment, RFS 87 events were observed, and it was calculated that with a two-sided significance level of $\alpha = 0.025$ (to account for a final analysis after 10 years of follow-up), the smallest difference that could be detected with 80% power was an HR ratio of approximately 0.50. Results from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) overview[1] suggested that the benefit of taxanes diminishes after 5 years; so waiting for more events would not provide much more information about sensitivity to treatment with taxanes. Therefore, the analysis after 5-year follow-up was added to the amendment (Amendment 3). In addition, it was decided to use a cross-validation method instead of separation in a development and a validation cohort as this may result in a better profile and more precise estimates of its predictive accuracy

The database was closed on 14 November 2017. We compared the categorical clinicopathological characteristics of the two treatment groups using a Chi-square or Fisher's exact test.

Efficacy analyses were performed in the intention-to-treat (ITT) population, including all patients who were allocated to one of the two treatment arms. RFS, DRFI, OS and BCSS of the two treatments were estimated using the Kaplan-Meier method and compared with a logrank test. Multivariable Cox proportional hazards models were generated to correct for known prognostic factors. Exploratory subgroup analyses on RFS and OS, including interactions, were performed using Cox regression models. Additionally, efficacy analyses were performed in the per-protocol treated (PPT) subgroup. The PPT population consisted of patients who received at least one treatment of ddAC or TAC. Patients were excluded if they were randomised to and received four cycles of chemotherapy, if they randomised for ddAC and were treated with an adjuvant taxane outside the scope of this study or if they had HER2-positive disease.

Observed toxicity was evaluated in all patients who received at least one cycle of the allocated treatment and was compared using a Chi-square or Fisher's exact test.

All p-values were two-sided, and values below 0.05 were considered significant, except for the comparison of ddAC with TAC for the RFS efficacy end-point, where the threshold was set at 0.025 (two-sided). Statistical analyses were performed using SPSS 22 and R 3.3.1.

RESULTS

Between 2004 and 2012, 664 patients were enrolled and randomised in 29 centres throughout the Netherlands (ITT population). Toxicity analysis was performed in 646 patients. The PPT population consisted of 614 patients (Figure 1).

The treatment groups were well balanced regarding prognostic clinicopathologic characteristics (Table 1). Mean age was 51.1 years (standard deviation 8.0). Five hundred thirty-one of 664 patients (80%) had lymph node-positive disease and 108 patients (16.3%) had triple negative breast cancer (TNBC). Twenty-one patients with HER2-positive disease were included of whom 14 were treated with trastuzumab.

Figure 1. CONSORT diagram. A = doxorubicine; C = cyclophosphamide; T = docetaxel; dd = dose-dense; HER2 = human epidermal growth factor receptor 2; ITT = intention-to-treat; PPT = per-protocol treated

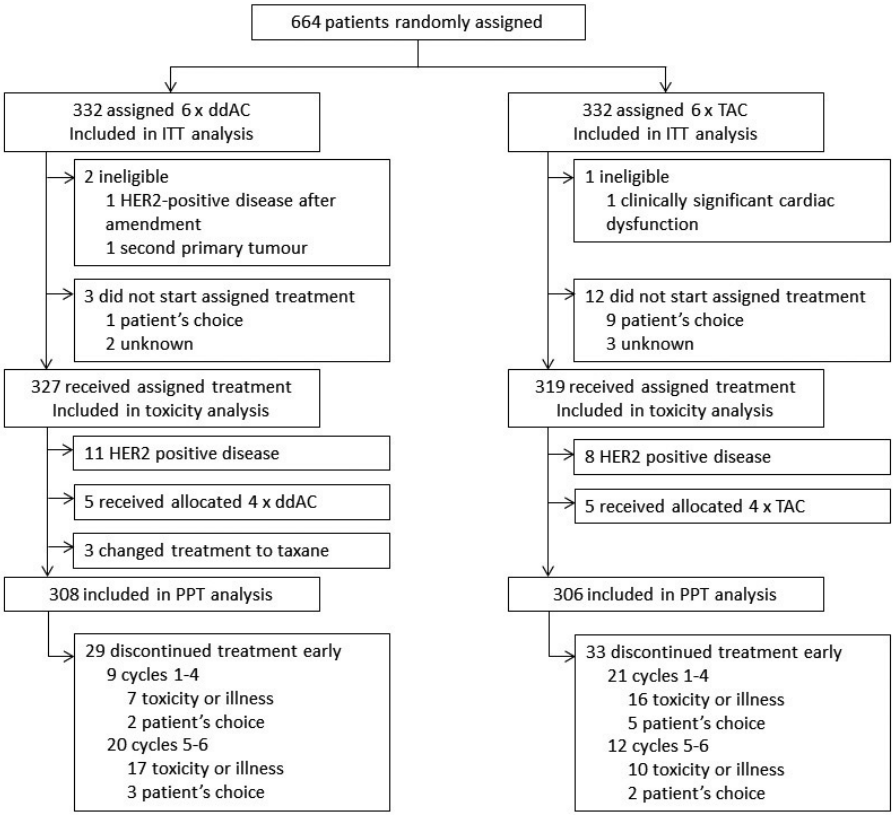


Table 1. Baseline characteristics of intention to treat population. A = doxorubicin; C = cyclophosphamide; T=docetaxel dd=dose-dense; * Pearson Chi-square test or Fisher's exact test (2-sided), missing values excluded; † According to AJCC staging 6th edition; ‡ Grading according to the modified Bloom-Richardson grading system; § ER and PR nucleic staining of 10% staining or more was scored as positive, HER2-score of 3+ was considered positive, in case of a 2+ HER2-score, an in situ hybridization assay was performed; Subtypes were defined as 1. estrogen receptor (ER) and/or progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-negative; 2. HER2-positive, regardless of ER or PR status; 3. Triple (ER, PR, HER2) negative

		6x ddAC *N*=332		6x TAC *N*=332		p-value
Age groups (%)	< 50 years	143	(43.1)	154	(46.4)	0.435
	≥ 50 years	189	(56.9)	178	(53.6)	
Surgery (%)	breast conserving surgery	180	(54.2)	169	(50.9)	0.538
	mastectomy	151	(45.5)	158	(47.6)	
	missing	1	(0.3)	5	(1.5)	
Endocrine treatment (%)	no	54	(16.3)	59	(17.8)	0.641
	yes	278	(83.7)	268	(80.7)	
	missing	0	(0)	5	(1.5)	
T stage† (%)	T1	158	(47.6)	155	(46.7)	0.654*
	T2	156	(47.0)	152	(45.8)	
	T3	16	(4.8)	19	(5.7)	
	T4	2	(0.6)	0	(0)	
	missing	0	(0)	6	(1.8)	
N stage† (%)	N0	65	(19.6)	63	(19.0)	0.889
	N1	208	(62.7)	200	(60.2)	
	N2	44	(13.3)	45	(13.6)	
	N3	15	(4.5)	19	(5.7)	
	missing	0	(0)	5	(1.5)	
Grade‡ (%)	good	32	(9.6)	35	(10.5)	0.796
	intermediate	151	(45.5)	138	(41.6)	
	poor	139	(41.9)	137	(41.3)	
	missing	10	(3.0)	22	(6.6)	
Histology (%)	ductal	270	(81.3)	257	(77.4)	0.507
	lobular	47	(14.2)	46	(13.9)	
	other	13	(3.9)	19	(5.7)	
	missing	2	(0.6)	10	(3.0)	
Subtype§ (%)	ER and/or PR-positive, HER2-negative	266	(80.1)	269	(81.0)	0.800
	HER2-positive	12	(3.6)	9	(2.7)	
	triple negative	54	(16.3)	54	(16.3)	

Efficacy

At the time of the analyses, the ITT population had a median follow up of 7 years. Two hundred eighty (84.3%) of 332 patients completed six cycles ddAC at the planned dose, 271 (81.6%) of 332 patients received six full cycles of TAC treatment (P=0.41).

The estimated 5-year RFS rate was 86.9% (95% CI 83.3-90.6) in the ddAC-treated patients and 87.9% (84.4-91.5) in the TAC-treated subgroup, which was not significantly different (HR 0.89, 95% CI 0.62-1.28, P=0.53; Figure 2a), neither after adjustment for known prognostic factors (Supplementary table S1). The same holds true for DRFI (Supplementary figure S1 and table S2). Of note, although not shown here, similar results were obtained using DFS as primary endpoint.

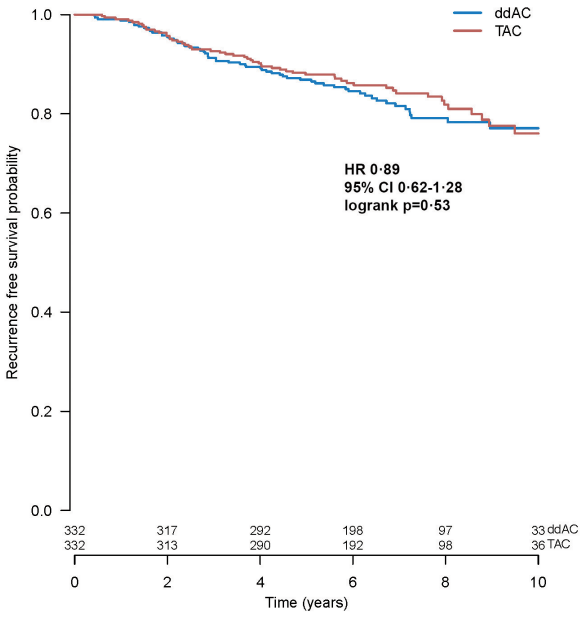
The 5-year OS did not significantly differ between the two treatment arms: 92.6% (95% CI 89.8-95.5) in the ddAC-treated subgroup and 93.8% (91.1-96.5) in the TAC-treated patients (HR 0.89, 95% CI 0.57-1.39, P=0.61; Figure 2b), neither when adjusted for known prognostic factors (Supplementary table S3). No difference was observed for BCSS between ddAC and TAC (Supplementary figure S2 and tables S4).

In the exploratory subgroup analyses, the interaction between age as a dichotomous variable and treatment showed a trend for OS ($P_{interaction}$ =0.040; Figure 3) with a numerical survival benefit for patients younger than 50 years when treated with ddAC (HR 1.72, 95% CI 0.79-3.73) and for patients who were 50 years or older when treated with TAC (HR 0.62, 95% CI 0.35-1.11). The interaction was not significant for RFS ($P_{interaction}$=0.084; Supplementary figure S3).

Fifty patients were excluded from the PPT analyses (Figure 1). Similar to the ITT population, RFS and OS were not significantly different between the ddAC-treated patients and the TAC-treated patients (Supplementary figures S4a-b).

Figure 2. Recurrence free survival (a) and overall survival (b) of the intention-to-treat population. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense; HR= hazard ratio; 95% CI=95% confidence interval.

a



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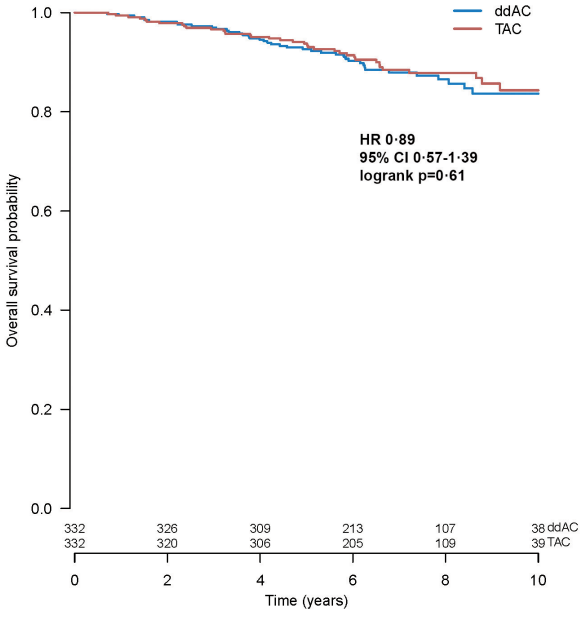
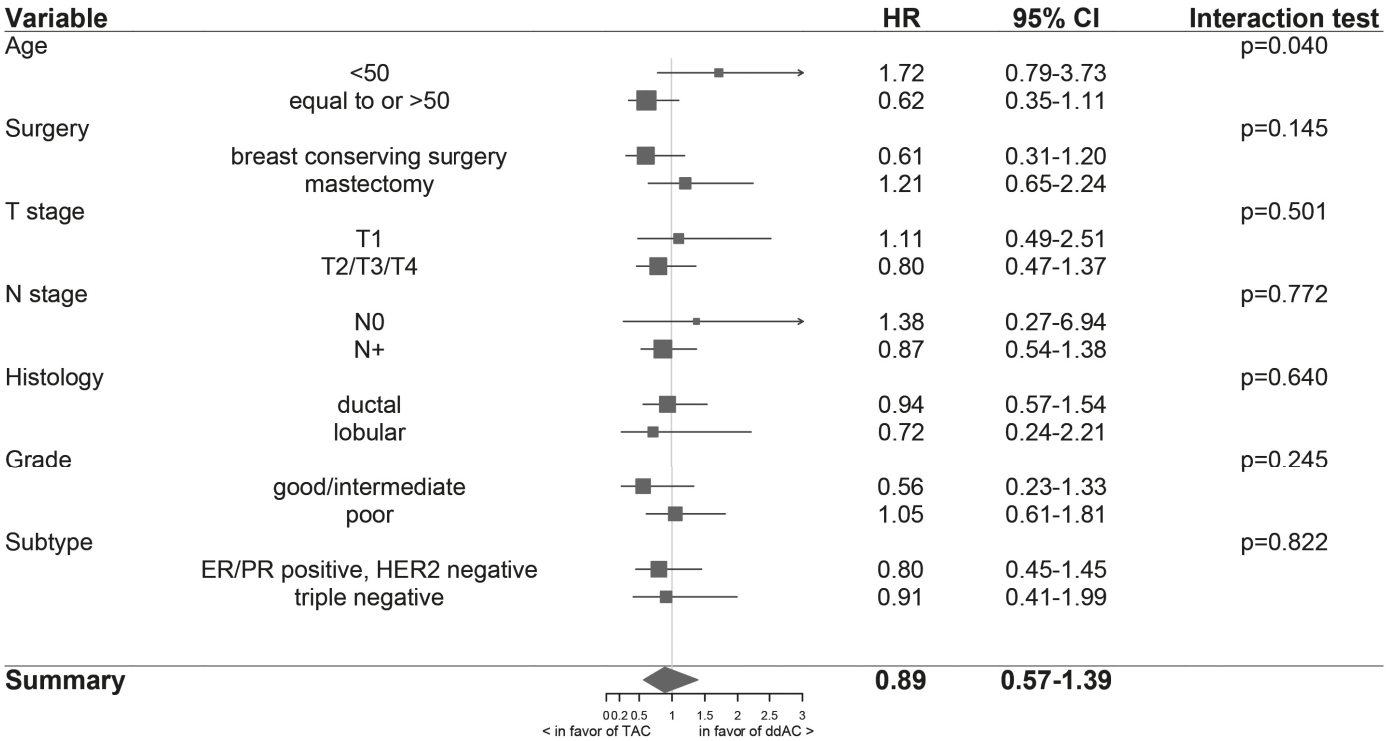


Figure 3. Forest plot of treatment effect on overall survival in subgroups. T stage and N stage are based on the TNM classification 2002. A = doxorubicin; C = cyclophosphamide; T = docetaxel; dd = dose-dense; HR = hazard ratio; 95% CI = 95% confidence interval; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; Subtypes were defined as 1. ER and/or PR positive and HER2 negative; 2. Triple (ER, PR, HER2) negative.



Toxicity

The observed adverse events (grade II and higher) of the two treatments are distinct (Table 2). Importantly, anaemia was more frequent in ddAC-treated patients (62 [18.9%] of 327 patients versus 15 [4.7%] of 319 patients, P<0.001) and diarrhoea (21 [6.4%] versus 53 [16.6%], P<0.001) and peripheral neuropathy (15 [4.6%] versus 46 [14.4%], P<0.001) were observed more often in TAC-treated patients. Regarding severe adverse events, acute myeloid leukaemia (AML) occurred twice in both treatment groups. One ddAC-treated patient developed myelodysplastic syndrome (MDS). Cardiac failure grade III or IV was observed in one ddAC-treated patient and in two TAC-treated patients. Toxicity of ddAC and TAC treatment in the context of drug metabolism-related polymorphisms was reported elsewhere.[12]

Table 2. Most frequent toxicities (grade 2 or higher) for ddAC treated patients and TAC treated subgroup. A = doxorubicin; C = cyclophosphamide; T=docetaxel dd=dose-dense *Pearson Chi-square test 2-sided

Side effects	ddAC n=327 (%)		TAC n=319 (%)		p-value*
Anemia	62	(18.9)	15	(4.7)	**<0.001**
Leukocytopenia	30	(9.2)	20	(6.3)	0.167
Fatigue	117	(35.8)	109	(34.2)	0.668
Diarrhea	21	(6.4)	53	(16.6)	**<0.001**
Nausea	65	(20.0)	52	(16.3)	0.238
Vomiting	35	(10.7)	21	(6.6)	0.063
Febrile neutropenia	36	(11.0)	40	(12.5)	0.546
Peripheral neuropathy	15	(4.6)	46	(14.4)	**<0.001**

DISCUSSION

Here we present the first direct comparison of efficacy of six cycles of ddAC and six cycles of TAC as adjuvant treatment for breast cancer as a secondary analysis of a randomised biomarker discovery trial. With a median follow-up of 7 years, ddAC and TAC were not significantly different regarding the survival end-points in our study. This is in line with the Oxford Overview meta-analysis[1] that contains more than 14,000 patients for the specific comparison between taxanes given concurrently with anthracyclines versus a non-taxane-containing regimen with a less than two times increased dose of non-taxane chemotherapy and with the CALGB40101 trial[13]. Interestingly when compared with the previously mentioned meta-analysis data, the survival rates in our cohort were remarkably high, particularly in this high-risk patient population in which 80.0% of the patients had lymph node-positive disease.

Several factors might have contributed to the relatively high survival rates of our cohort compared with previously reported outcomes in older studies. First, patients with HER2-positive disease were excluded after the introduction of trastuzumab. In older cohorts that included the HER2-positive tumours that were not treated with anti-HER2-based therapy, the survival was less favourable.[14,15] Also stage migration, also known as the Will Rogers phenomenon, might play a role. Improved diagnostics and new technologies, as shown previously for 18F-fluorodeoxyglucose positron emission tomography combined with computed tomography[16], lead to more accurate identification of (distant) metastases. Patients who would have been diagnosed with stage III disease in the past and treated with adjuvant systemic therapy are nowadays diagnosed with stage IV disease.[17] The taxane plus anthracycline trials reported in the Oxford Overview meta-analysis enrolled patients between 1994 and 2005, almost a decade earlier than inclusion of patients in the current trial (2004-2012). Interestingly, the MINDACT trial (2007-2011) was executed in the same time period in Europe, and our relatively favourable survival data resemble the survival data of the high-risk patients included in MINDACT who received adjuvant chemotherapy.[18]

The primary objective of this trial is to generate a predictive gene expression profile, which is currently being explored. Because the sample size was calculated for the primary end-point, the study may be underpowered for the secondary objective, particularly with the unexpected low number of events observed. However, because chemotherapy displays the largest survival effect in the first years after diagnosis and the carry-over effect diminishes after 7 years for taxanes and even earlier for anthracycline-based regimens[1], it seems relevant to report these results now.

The enrolment period from 2004 until 2012 was relatively long. The novel design of a biomarker study required some adjustments of daily clinical practice. To ensure sufficient quality of the RNA, the ability to freeze tumours was a requirement for hospitals to participate in the trial. At the start of this trial, only a few hospitals had the logistics in place to freeze tumours after surgery. Given the speedy accrual of other biomarker-based trials that started a couple of years later, such as but not limited to the MINDACT trial, developments in molecular diagnostics have resulted in logistics for frozen tumours in the majority of hospitals nowadays. Also, emerging evidence caused a shifting landscape of potential adjuvant systemic treatment regimens, compromising the accrual. Nevertheless, the primary objective of this trial is still a valid and clinically relevant aim.

In this trial, we evaluated three variables: (1) the time between cycles (2 weeks versus 3 weeks), (2) the different dosage of doxorubicin (60 mg/m² versus 50 mg/m²) and cyclophosphamide (600 mg/m² versus 500 mg/m²) and (3) the taxane addition. The number of variables makes it difficult to assess to what extent a specific factor contributes to the efficacy of these regimens. The lack of superiority of TAC over ddAC could be due to the somewhat higher dosed doxorubicin and cyclophosphamide in the ddAC arm compared with TAC, thereby increasing the dose intensity defined as mg/m² per time interval. The dose-dense schedule further increases the dose intensity without increasing the toxicity.[19] Dose intensification of doxorubicin and cyclophosphamide seems, therefore, equally effective as the addition of docetaxel to these agents after a median follow-up of 7 years in our cohort.

The unplanned subgroup analysis provided some evidence of an interaction between age and treatment, with a numerical OS benefit for younger patients (< 50 years) when treated with ddAC compared with TAC and for older patients (≥ 50 years) when treated with TAC compared with ddAC. These results are in line with a previous report on improved survival after dose-dense chemotherapy compared with standard-interval chemotherapy in young breast cancer patients.[20] Also, higher survival rates are observed in older patients treated with taxane-containing regimens compared with patients of the same age treated with non-taxane-based regimens.[1,21] Although one might expect ddAC to be more efficacious in relative aggressive tumours that are more prevalent in younger patients[19,22], we did not observe an association between the grade and age in our population, nor did we find a significant interaction between the grade and treatment effect. Currently ongoing gene expression analyses might provide hints on the biology that could be driving this.

The regimens used in our cohort displayed distinct toxicity profiles, which are in line with previous studies on dose-dense chemotherapy[4,13] and reports on taxane-based treatments[23,24]. AML and MDS were observed in 2 (0.6%) of 327 ddAC-treated patient and 2 (0.6%) of 319 TAC-treated patients. Previous anthracycline-based studies have shown a similar probability of AML and MDS of 0.55% at 8 years of follow-up.[25] Compared with the BCIRG 001 trial[24], cardiac failure was uncommon in our study population (1 ddAC-treated patient [0.3%], 2 TAC-treated patients [0.6%]). However, longer follow-up is needed to assess the long-term toxicity of these regimens. Because these toxicities are associated with anthracyclines in a dose-dependent manner, four courses of anthracycline-based chemotherapy, followed by taxanes may be the preferred regimen in the absence of predictive biomarkers for regimen-specific efficacy. Predicting sensitivity for toxicity, for instance by screening for genetic polymorphisms, may help to tailor treatment.[12,26] In addition, treatment duration might be important for some patients. For these patients, a 12-week during schedule might be more attractive than an 18-week during schedule.

Our data show that the 5-year survival of high-risk breast cancer patients is excellent after adjuvant treatment with six cycles of TAC or six cycles of ddAC and that distinct toxicity profiles and treatment durations characterise these schedules. Although the preferred adjuvant schedule may shift towards dose-dense sequential chemotherapy[5], knowledge about 'second best' schedules with their own characteristics may help to search for alternative regimens if required. In addition, predictive biomarkers are warranted to further improve well-informed treatment decisions. Therefore, we aim to develop a gene expression profile predictive for treatment efficacy of either ddAC or TAC.

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SUPPLEMENTARY MATERIAL

Table S1. Multivariable Cox regression model of recurrence free survival on intention-to-treat population. Of note, the proportional hazards assumption seemed violated for lymph node status. The hazard ratio is given as usual, which can be interpreted as a weighted average across the follow up time (weighted by the number of patients at risk). HR = hazard ratio; 95% CI = 95% confidence interval

Variable		HR	95% CI	p-value
Treatment	ddAC	reference		
	TAC	0.85	0.58-1.23	0.39
Age	< 50 years	reference		
	≥ 50 years	1.11	0.76-1.63	0.60
T stage	T1	reference		
	T2-4	**1.81**	**1.21-2.72**	**<0.01**
N stage	N0	reference		
	N+	**3.86**	**1.77-8.43**	**<0.01**
Histologic grade	good	reference		
	intermediate	1.82	0.72-4.64	0.21
	poor	**3.42**	**1.35-8.68**	**0.01**
Molecular subtype	ER /PR-positive, HER2-negative	reference		
	HER2-positive	1.63	0.70-3.84	0.26
	triple negative	1.78	0.75-4.22	0.19
Type of surgery	breast conserving surgery	reference		
	mastectomy	0.93	0.64-1.36	0.71
Adjuvant endocrine therapy	no	reference		
	yes	1.00	0.43-2.31	1.00

Table S2. Multivariable Cox regression model of distant recurrence free interval of intention-to-treat population. Of note, the proportional hazards assumption seemed violated for T stage. The hazard ratio is given as usual, which can be interpreted as a weighted average across the follow up time (weighted by the number of patients at risk). HR = hazard ratio; 95% CI = 95% confidence interval

Variable		HR	95% CI	p-value
Treatment	ddAC	reference		
	TAC	1.04	0.67-1.60	0.88
Age	< 50 years	reference		
	≥ 50 years	0.88	0.57-1.37	0.58
T stage	T1	reference		
	T2-4	**2.01**	**1.24-3.26**	**<0.01**
N stage	N0	reference		
	N+	**4.76**	**1.71-13.19**	**<0.01**
Histologic grade	good	reference		
	intermediate	2.05	0.62-6.78	0.24
	poor	**4.76**	**1.46-15.55**	**0.01**
Molecular subtype	ER /PR-positive, HER2-negative	reference		
	HER2-positive	1.68	0.65-4.35	0.28
	triple negative	1.48	0.55-3.96	0.44
Type of surgery	breast conserving surgery	reference		
	mastectomy	0.82	0.52-1.29	0.39
Adjuvant endocrine therapy	no	reference		
	yes	1.40	0.53-3.69	0.50

Table S3. Multivariable Cox regression model of overall survival of intention-to-treat population. HR = hazard ratio; 95% CI = 95% confidence interval

Variable		HR	95% CI	p-value
Treatment	ddAC	reference		
	TAC	0.84	0.53-1.33	0.45
Age	< 50 years	reference		
	≥ 50 years	1.52	0.94-2.48	0.09
T stage	T1	reference		
	T2-4	**1.88**	**1.12-3.15**	**0.02**
N stage	N0	reference		
	N+	**3.21**	**1.27-8.11**	**0.01**
Histologic grade	good	reference		
	intermediate	2.43	0.57-10.42	0.23
	poor	5.76	1.37-24.28	**0.02**
Molecular subtype	ER /PR-positive, HER2-negative	reference		
	HER2-positive	2.21	0.84-5.79	0.11
	triple negative	2.41	0.89-6.55	0.08
Type of surgery	breast conserving surgery	reference		
	mastectomy	1.15	0.71-1.85	0.56
Adjuvant endocrine therapy	no	reference		
	yes	1.12	0.43-2.89	0.82

Table S4. Multivariable Cox regression model of breast cancer specific survival of intention-to-treat population. Of note, the proportional hazards assumption seemed violated for lymph node status. The hazard ratio is given as usual, which can be interpreted as a weighted average across the follow up time (weighted by the number of patients at risk). HR = hazard ratio; 95% CI = 95% confidence interval

Variable		HR	95% CI	p-value
Treatment	ddAC	reference		
	TAC	0.81	0.47-1.42	0.47
Age	< 50 years	reference		
	≥ 50 years	0.98	0.56-1.72	0.95
T stage	T1	reference		
	T2-4	**2.34**	**1.23-4.45**	**0.01**
N stage	N0	reference		
	N+	**5.79**	**1.38-24.29**	**0.02**
Histologic grade	good	reference		
	intermediate	2.84	0.37-21.90	0.32
	poor	**9.24**	**1.24-68.90**	**0.03**
Molecular subtype	ER /PR-positive, HER2-negative	Reference		
	HER2-positive	2.04	0.67-6.20	0.21
	triple negative	2.21	0.72-6.78	0.17
Type of surgery	breast conserving surgery	reference		
	mastectomy	1.07	0.61-1.90	0.81
Adjuvant endocrine therapy	no	reference		
	yes	1.36	0.46-4.00	0.58

Figure S1. Distant recurrence free interval of the intention-to-treat population. A=doxorubicin; C=cyclophosphamide; T=docetaxel; dd=dose-dense; HR= hazard ratio; 95% CI=95% confidence interval

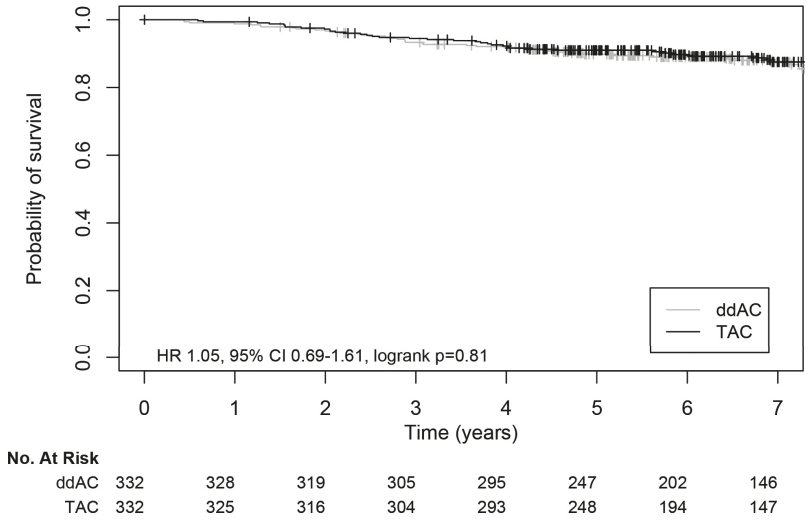


Figure S2. Breast cancer specific survival of the intention-to-treat population. A=doxorubicin; C=-cyclophosphamide; T=docetaxel; dd=dose-dense; HR= hazard ratio; 95% CI=95% confidence interval.

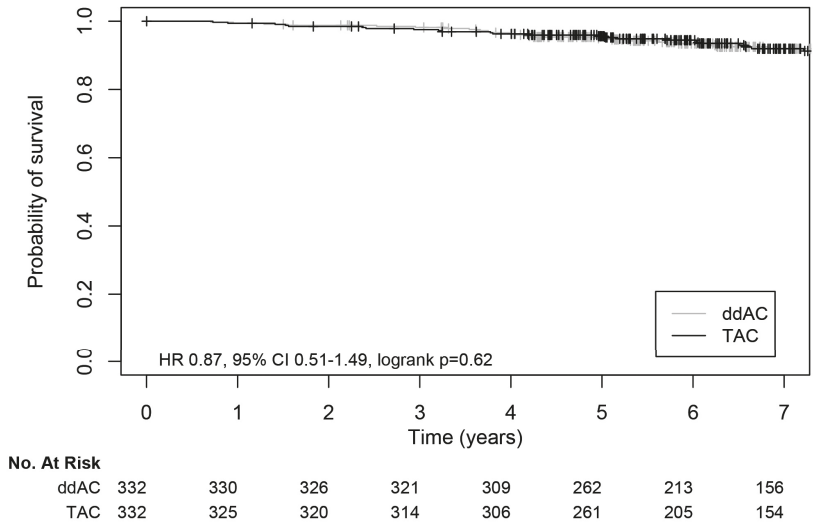


Figure S3. Forest plot of treatment effect on recurrence free survival in subgroups. T stage and N stage are based on the TNM classification 2002. A = doxorubicin; C = cyclophosphamide; T = docetaxel; dd = dose-dense; HR = hazard ratio; 95% CI = 95% confidence interval; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; Subtypes were defined as 1. estrogen receptor (ER) and/or progesterone receptor (PR) positive, human epidermal growth factor receptor 2 (HER2)-negative; 2. Triple (ER, PR, HER2) negative.

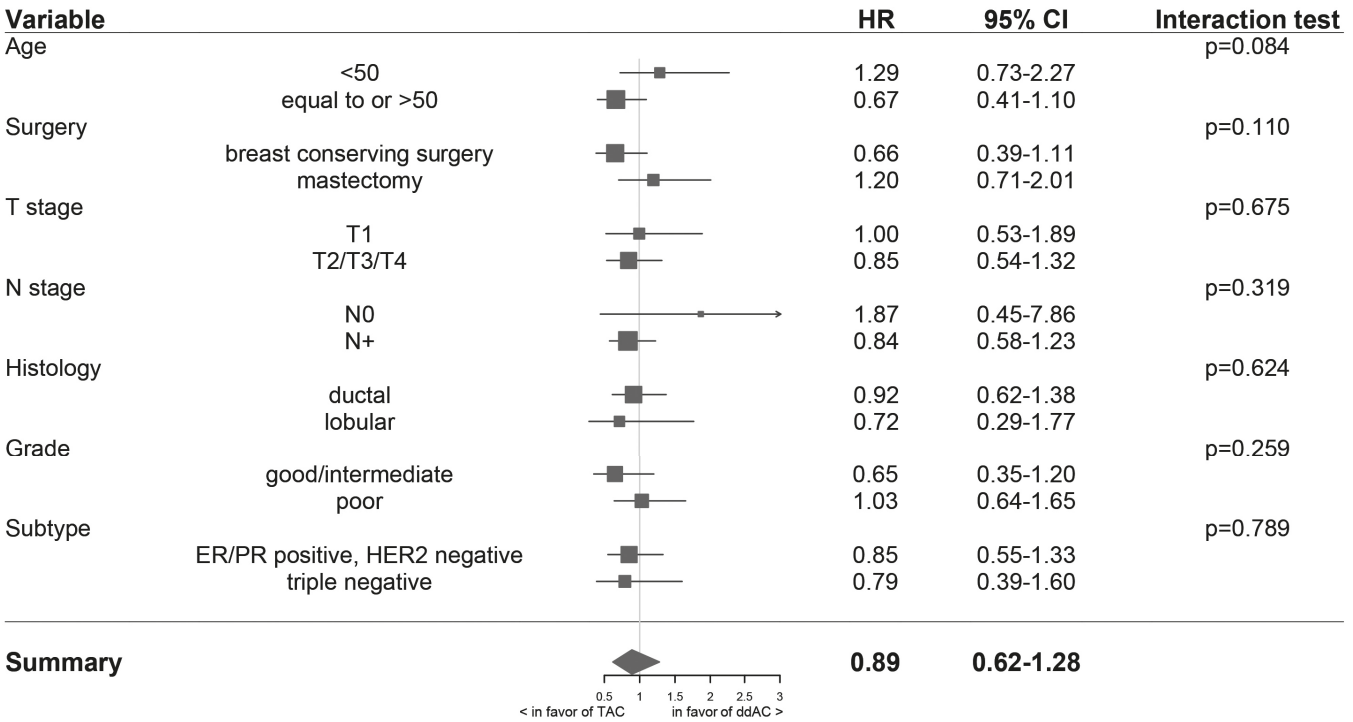
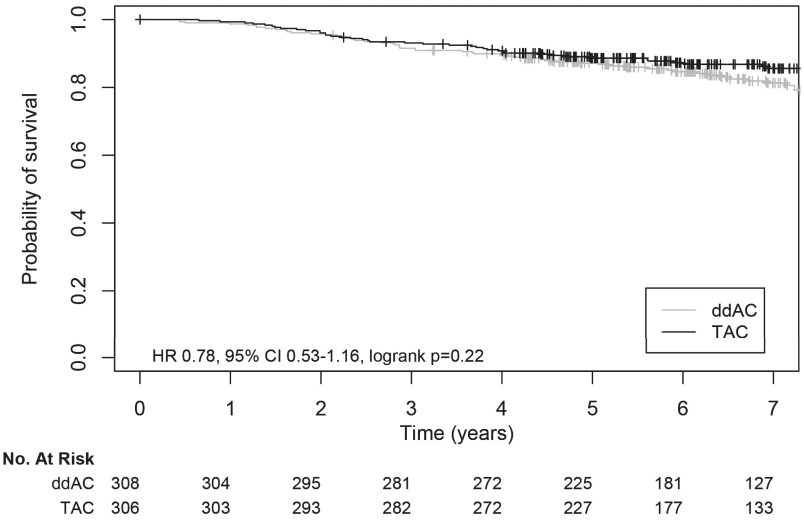


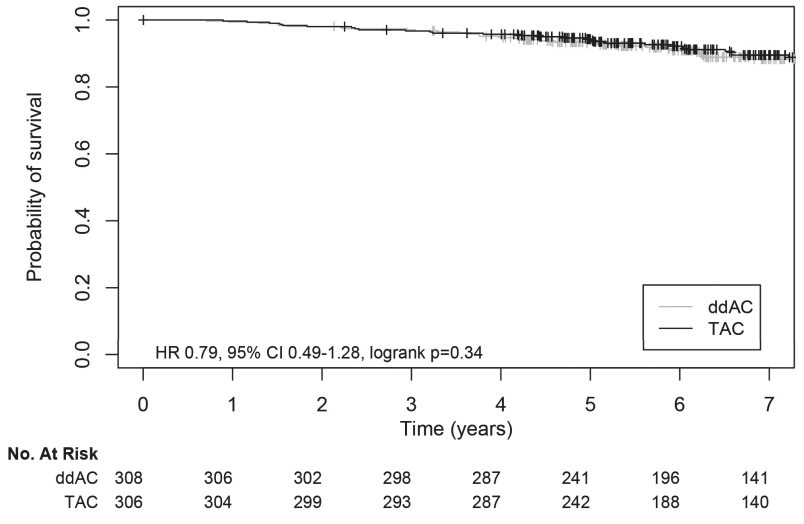
Figure S4. Recurrence free survival (a) and overall survival (b) of per-protocol treated patients. A=doxorubicin; C=cyclophosphamide; T=docetaxel; dd=dose-dense; HR= hazard ratio; 95% CI=95% confidence interval

a

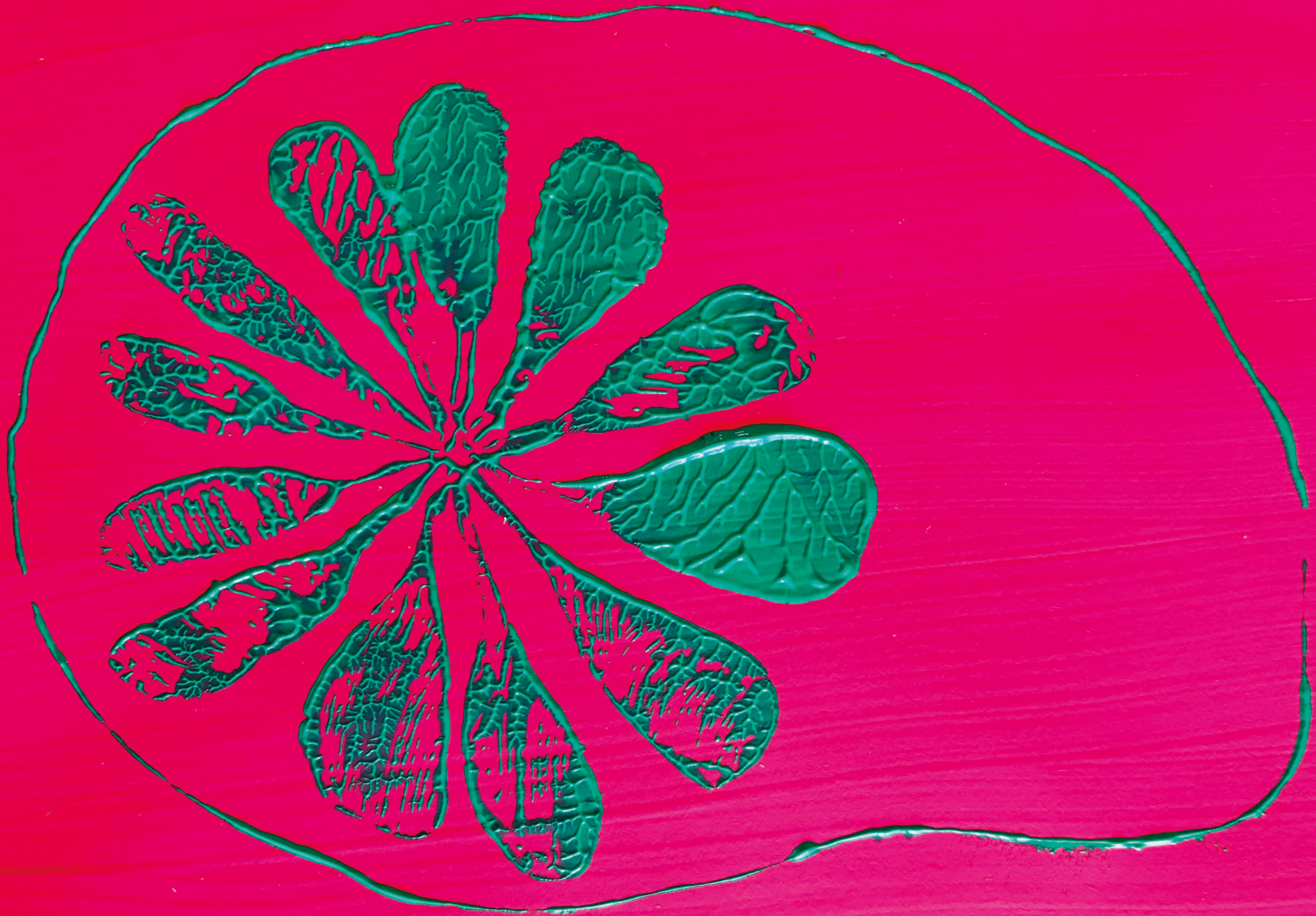


No. At Risk								
ddAC	308	304	295	281	272	225	181	127
TAC	306	303	293	282	272	227	177	133

b



No. At Risk								
ddAC	308	306	302	298	287	241	196	141
TAC	306	304	299	293	287	242	188	140



3

Tumor-infiltrating lymphocytes predict benefit from adjuvant taxane-based chemotherapy in triple negative breast cancer in the randomized MATADOR trial (BOOG 2004-04)

A.G.J. van Rossum, M. Hoogstraat, G. Bounova, H.M. Horlings, E. van Werkhoven, M. Opdam, I.A.M. Mandjes, A.E. van Leeuwen – Stok, S. Canisius, H. van Tinteren, A.L.T. Imholz, J.E.A. Portielje, M.E.M.M. Bos, A. van Bochove, J. Wesseling, E.J. Rutgers, L.F.A. Wessels, H.M. Oosterkamp*, M. Kok*, S.C. Linn*; and the MATADOR trialists' group.

*contributed equally to this work

Submitted.

ABSTRACT

Background: Gene expression profiles to assess the prognosis of breast cancer patients are well-known and used in the clinic. However, biomarkers that can predict sensitivity to a specific treatment schedule are lacking. In the MATADOR trial, we aimed to find a predictive gene expression profile for recurrence free survival (RFS) benefit of either dose-dense or taxane-containing chemotherapy.

Methods: 664 patients were randomized between 6 cycles adjuvant docetaxel-doxorubicin-cyclophosphamide ($T_{75}A_{50}C_{500}$) and 6 cycles dose-dense AC ($ddA_{60}C_{600}$). We employed RNA-sequencing data of pretreatment tumor samples to investigate the association between expression levels and RFS via a data-driven and a knowledge-driven approach using Molecular Signatures Database (MSigDB) hallmark gene sets.

Results: With a median follow up of 7 years, we observed 102 RFS events. Analyses revealed a profile with prognostic value (adjusted P=0.001), but limited predictive utility. Interestingly, hallmark gene set analyses showed significant association between enrichment in immune-related gene expression and favorable outcome after TAC, particularly in the basal subgroup. We evaluated the clinical applicability of this association by testing the predictive capacity of tumor-infiltrating lymphocytes (TILs assessed using H&E) in the triple negative breast cancer (TNBC) patients. In patients with TILs ≥20% (median) RFS after TAC was numerically better compared with ddAC, while ddAC was associated with longer RFS in patients with TILs <20% (adjusted $P_{interaction}$=0.03).

Conclusions: The gene expression profile could not predict RFS benefit of ddAC or TAC. However, high TILs is associated with longer RFS after adjuvant TAC and worse survival after ddAC in TNBC.

Trial registration ID: ISRCTN61893718

INTRODUCTION

The addition of taxanes and dose-dense scheduling of adjuvant chemotherapy markedly reduced the risk of early breast cancer relapse and death.[1,2] However, it is still not known whether an individual patient will benefit most from adding a taxane, from increasing the dose-density of the chemotherapy, or from both.

Most randomized clinical trials are designed with treatment efficacy as primary endpoint. Predictive biomarkers are generally, if incorporated at all, a secondary objective. A clinical trial with the primary objective to develop a predictive biomarker for a specific treatment will have a higher likelihood to result in a clinically useful test.[3]

Several attempts have been undertaken to identity gene expression profiles that might predict sensitivity or resistance to taxanes or dose-dense chemotherapy.[4-9] Most of these investigations comprised single-arm studies resulting in a profile predictive of response to that particular treatment or a profile simply reflecting the natural course of the disease. Others were well designed, but lacked power to assess the biomarker-by-treatment interaction.[10]

In the randomized, phase 3 MATADOR trial, the primary objective was to find a gene expression profile predictive of recurrence free survival (RFS) benefit of either dose-dense, anthracycline-based chemotherapy or a taxane-and-anthracycline-based regimen without dose-densification and to assess its predictive performance. To our knowledge, this is the first trial designed to develop a gene expression profile that could be used to estimate the treatment benefit of one chemotherapy regimen over the other. Such a profile would enable us to predict which treatment will result in the largest survival benefit for an individual patient.

METHODS

Patients

The MATADOR (Microarray Analysis in breast cancer to Tailor Adjuvant Drugs Or Regimens, ISRCTN61893718) study is an open-label, multicenter trial conducted in 29 centers in the Netherlands. Six hundred sixty-four female patients with pT1-3, N0-3, M0 breast cancer were recruited onto the trial. The inclusion criteria were described in detail elsewhere.[11,12] At the start of the trial, trastuzumab was not part of standard adjuvant treatment for patients with HER2-positive breast cancer yet. Therefore, these patients were initially enrolled in the MATADOR study. With emerging evidence that trastuzumab improved survival in HER2-positive breast cancer patients, these patients became ineligible to participate in the trial.

The study protocol and amendments were approved by the ethical committee of the Netherlands Cancer Institute. The study was conducted in agreement with Good Clinical Practice guidelines and with the Declaration of Helsinki. All patients provided written informed consent to participate in the trial and to use the tumor tissue removed at surgery for translational research. The REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria were used to report this study.[13]

Treatment

Patients were randomly assigned (1:1) to 6 cycles of doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 every 2 weeks (dose dense [dd] AC) or docetaxel 75 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2 every 3 weeks (TAC) by means of the automated ALEA system (FormsVision BV, Abcoude, the Netherlands) using Pocock's minimization technique[14]. Randomization was performed centrally at the Netherlands Cancer Institute. Granulocyte-colony stimulating factor (pegfilgrastim) was given to all patients. Radiation therapy and endocrine therapy were given according to the contemporary Dutch guidelines.[15]

Objectives and endpoints

The primary objective of the trial was to identify a gene expression profile for RFS benefit of either dose-dense or taxane-containing chemotherapy and to assess its predictive performance. RFS is defined as the time from randomization to locoregional recurrence, distant metastasis or death by any cause, whichever occurred first.

The clinical risk of recurrence was assessed using the modified Adjuvant!Online classification in line with the classification used in the MINDACT trial.[16] Patients with a clinically low risk of recurrence would not receive adjuvant chemotherapy nowadays according to current guidelines. For these patients, there is no clinical need for a predictive test guiding the decision which chemotherapy regimen will be most effective. Therefore, these patients were excluded for the analysis of the primary objective, as defined in the statistical analysis plan. The secondary objective was to directly compare RFS, overall survival (OS) and toxicity of the two treatment arms.[11,12]

RNA isolation and sequencing

RNA was isolated from formalin-fixed, paraffin-embedded tissue with a tumor cell percentage of at least 40% using the AllPrep DNA/RNA mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Quantification and purity were measured using the NanoDrop 2000 spectrophotometer (Thermofisher Scientific, Waltham, Massachussets, USA) and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Libraries of cDNA were constructed with the TruSeq RNA Access Library Prep Kit (Illumina, San Diego, California, USA) and single-end sequenced using the HiSeq 2500 (Illumina). Reads were aligned to the reference genome (hg38) using TopHat[17]. The number of uniquely assigned reads per gene was calculated with HTSeq[18]. Gene expression data were normalized and log2 transformed using DESeq2[19].

Molecular subtypes

Patients were grouped in five molecular subtypes using the PAM50 gene expression-based classifier.[20]

Predictive gene expression profile

A predictive score was constructed as follows. Applying leave-one-out cross-validation (LOOCV), a penalized Cox proportional hazards regression model was fitted which included treatment, the main effect of each gene and all pairwise treatment-gene interactions as explanatory variables for each patient. A LASSO penalty was used on the main effect of the genes and the treatment-gene interaction effects. The LASSO is a penalty on the sum of the absolute values of the regression coefficients, which means that they depend on the unit of the predictors. With the usual coding of the treatment by zeros and ones (known as treatment contrast), the resulting model would depend on the arbitrary choice of the reference treatment. Therefore, treatment was coded

instead as 0.5 and the other treatment as -0.5. This has the advantage that the resulting model does not depend on the arbitrary choice of the reference treatment. For every leave-one-out iteration, the penalty parameter lambda was obtained by maximizing the partial-likelihood of the Cox model via a 10-fold cross-validation on all patients but the left-out patient. The resulting model was used to calculate a profile score for each left-out patient. The profile score is the inner product of the optimally penalized, non-zero LASSO regression coefficients and the expression values of the corresponding genes.

Patients were classified into two groups based on the median split of the profile scores, which were compared regarding RFS using the log-rank test and entered into a multivariable Cox regression model to correct for the main effects of tumor size, lymph node status, histologic grade, age and type of surgery. For the high and low profile score subgroups, the association between treatment and RFS was tested using the Kaplan-Meier method and compared using the log-rank test. This procedure was repeated within the PAM50 subgroups. For the basal subgroup, double LOOCV was performed instead of 10-fold to optimize lambda, as the number of samples was low. The same procedure as described above was used to develop a model without the interaction effects. The concordance index (C-index) was calculated for the two resulting cross-validated profile scores (with and without interaction) and compared with a paired t-test.

The analyses were done in R version 3.4.3 with package glmnet version 2.0-16[21] and survcomp version 1.28.5[22].

Hallmark gene sets

As an exploratory analysis, we assessed the association between the activity of defined biological processes and RFS using the R package globaltest.[23] As gene sets we employed the Molecular Signatures Database (MSigDB) hallmark gene sets (Broad Institute, Cambridge, MA, USA)[24]. Each gene set consists of a number of genes involved in a certain biological process. The normalized expression of the genes in these gene sets was used as input for the test. We tested the association between the MSigDB gene sets and RFS in all patients and in the PAM50 subgroups.

Tumor histology and immunohistochemistry (IHC)

Histologic grade was assessed according to the modified Bloom-Richardson classification[25]. Tumors were scored centrally for expression of the estrogen receptor

(ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) as previously described.[11] ER and PR were considered positive if 10% or more cells showed nucleic staining. HER2 score of 3+ and, if confirmed by in situ hybridization, 2+ were classified as positive. IHC-based breast cancer subtype was defined as 1. ER and/or PR-positive, HER2-negative; 2. HER2-positive, regardless of ER and PR status or 3. triple negative.

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) were scored by a pathologist (HH) for the patients with triple negative breast cancer (TNBC). Scoring of TILs was performed on hematoxylin and eosin stained whole slide according to previously published recommendations[26] with high inter-observer concordance[27]. The association between TILs and RFS was assessed using the Kaplan-Meier method and compared using the log-rank test. The interaction between TILs and treatment was tested in a multivariable Cox regression model while correcting for the main effect of tumor size, lymph node status, histologic grade, age and type of surgery.

Statistics

The primary objective was to identify a gene expression profile that can predict RFS benefit of either dose-dense chemotherapy or a taxane-containing regimen and to assess its predictive performance. Subsequently, the gain in RFS attributed to the predictive profile could be calculated. This gain was defined as the improvement in RFS at 5 years with the treatment strategy using the profile, over the strategy in which all patients would get the same treatment (either ddAC or TAC), whichever would appear better from the direct comparison. It was calculated that if the profile would be developed using data from 400 patients, the standard error (SE) of the estimate of the gain would be less than 2.5%. The SE was calculated by propagation of error (delta-method). In this calculation, the variance resulting from the fact that it would be random which arm serves as the reference in the calculation of the gain, was considered negligible. The sample size of the study was set at 660 so that 1/3 of the data could be used as a validation cohort, allowing for 10% early dropout. During the course of the study, it became clear that the event rate was lower than expected. Therefore, an amendment was made to the protocol to use a cross-validation method instead of a separation in a training and a validation cohort.

RESULTS

Between August 2004 and November 2012, 664 patients were enrolled. For 604 (90.9%) patients, tumor tissue was available for gene expression analysis (Figure S1). Library preparation failed in 7 patients. Six out of 597 sequenced samples did not meet the quality checks. Another 62 patients had a clinically low risk of recurrence according to AdjuvantOnline![16] and would not receive adjuvant chemotherapy according to current guidelines. In this group no events occurred (Table S1). Therefore, these patients were excluded from the analyses to develop a predictive gene expression profile. Finally, for one patient survival data were unknown. This patient was excluded from the survival analyses. A total of 528 patients, 270 ddAC treated patients and 258 TAC treated patients, were used for the analysis of the primary objective.

Treatment groups were not significantly different regarding clinicopathologic characteristics (Table 1). With a median follow up of 7 years for the whole cohort (including patients who developed an event), we observed 102 RFS events.

Table 1. Characteristics of patients for whom gene expression data was available. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense. * Pearson Chi-square test, missing values excluded; † According to AJCC staging 6th edition; ‡ Grading according to the modified Bloom-Richardson grading system; § ER and PR nucleic staining of 10% staining or more was scored as positive, HER2-score of 3+ was considered positive, in case of a 2+ HER2-score, an in situ hybridization assay was performed; Subtypes were defined as 1. estrogen receptor (ER) and/or progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-negative; 2. HER2-positive, regardless of ER or PR status; 3. Triple (ER, PR, HER2) negative

		6x ddAC n=270		6x TAC n=258		P-value*
Age groups (%)	< 50 years	115	(42.6)	122	(47.3)	0.319
	≥ 50 years	155	(57.4)	136	(52.7)	
Surgery (%)	breast conserving surgery	148	(54.8)	132	(51.2)	0.475
	mastectomy	122	(45.2)	126	(48.8)	
T stage† (%)	T1	121	(44.8)	110	(42.6)	0.706
	T2-T3-T4	149	(55.2)	147	(57.0)	
	missing	0	(0.0)	1	(0.4)	
N stage† (%)	N0	42	(15.6)	40	(15.5)	1.000
	N+	228	(84.4)	218	(84.5)	
Grade‡ (%)	good	12	(4.4)	14	(5.4)	0.918
	intermediate	126	(46.7)	109	(42.2)	
	poor	125	(46.3)	119	(46.1)	
	missing	7	(2.6)	16	(6.2)	
Histology	ductal	220	(81.5)	208	(80.6)	0.719
	lobular	41	(15.2)	37	(14.3)	
	other	8	(3.0)	9	(3.5)	
	missing	1	(0.4)	4	(1.6)	
Subtype§ (%)	ER and/or PR-positive, HER2-negative	214	(79.3)	207	(80.2)	0.920
	HER2 positive	10	(3.7)	8	(3.1)	
	triple negative	46	(17.0)	43	(16.7)	

Gene expression profile

Unsupervised hierarchical clustering grouped patients according to the PAM50 classification (Figure S2). The association between the PAM50 classification and RFS is shown in Figure S3. As expected, most RFS events in the basal subgroup and the HER2-enriched subgroup occurred in the first years after diagnosis, while the events in the luminal A subgroup and the luminal B subgroup were observed during the entire follow up time.

Using Cox regression with a LASSO penalty, we computed the leave-one-out cross-validated profile score (linear predictor) for each patient. We then set out to determine the association of these scores with outcome. To this end we binarized the profile score by employing the median value as cut-off. This binary profile score had a significant association with RFS (adjusted hazard ratio [HR] 2.06, 95% confidence interval [CI] 1.33-3.19, P=0.001), with a significantly longer RFS for patients with a low profile score compared with those with a high profile score (Figure 1a). However, when considering the low and high profile score groups per treatment arm, no significant association with RFS was observed, indicating that the profile score had limited predictive value (Figure 1b and 1c). Moreover, there was no significant difference between the C-index of the model with and the model without interaction term in any of the subtypes. The genes that were selected with this approach are listed in Table S2.

In the basal subgroup, the binary profile score did not associate with treatment and RFS (Figures S4a and S4b, Table S3).

Similarly, in the luminal B and luminal A subgroup the binary profile score had limited predictive value (Figure S4c-S4f, Table S3).

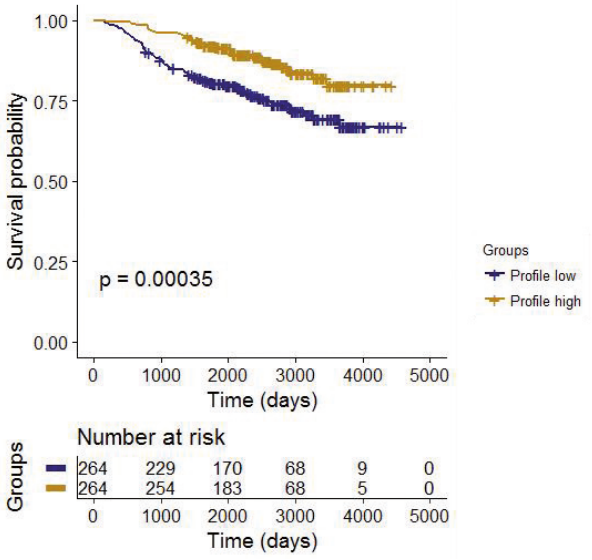
Hallmark gene sets

In an exploratory analysis, we tested the association between well-described biological processes represented in the MSigDB hallmark gene sets[24] and survival in the treatment subgroups using the globaltest[23]. In Figure 2, the associations between the gene sets and RFS are listed according to the globaltest statistic. Whereas 11 gene sets had a significant association with RFS in the ddAC treated patients (Figure 2a and Table S4), 34 gene sets were significantly associated with RFS in the TAC treated subgroup. However, none were significant after correction for multiple testing. In the basal subgroup, we observed a striking difference in the associations with RFS between the treatment arms. Whereas no gene sets were significantly associated with RFS in the ddAC treated patients with a basal tumor, 31 gene sets had a significant association with RFS in TAC treated subgroup (Figure 2b and Table S4). Interestingly, high expression of immune-related gene sets (top hits in Figure 2b) was associated with favorable outcome in the TAC treated subgroup, while this was not observed in ddAC treated patients.

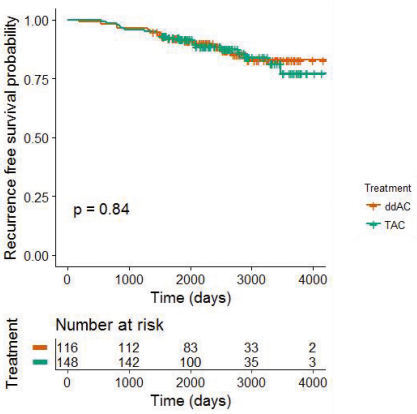
The association between the individual genes of the top 3 immune gene sets (interferon gamma response, allograft rejection and interferon alpha response) and RFS split by treatment subgroup are displayed in Figure S5. Significantly associated genes are listed

Figure 1. Association between validation profile score as obtained using a model with LASSO penalty[21] and recurrence free survival (RFS) in all patients (**a**). Association between treatment and RFS, split by a low profile score (**b**) or a high profile score (**c**). Log rank p-values are reported. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

a. All patients



b. Low profile score



c. High profile score

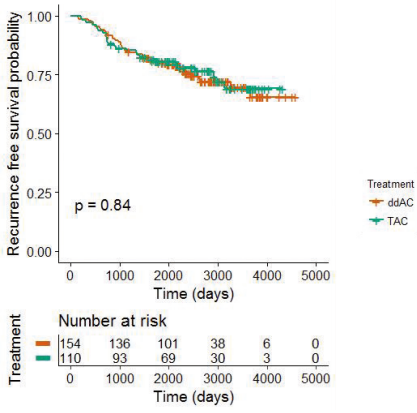
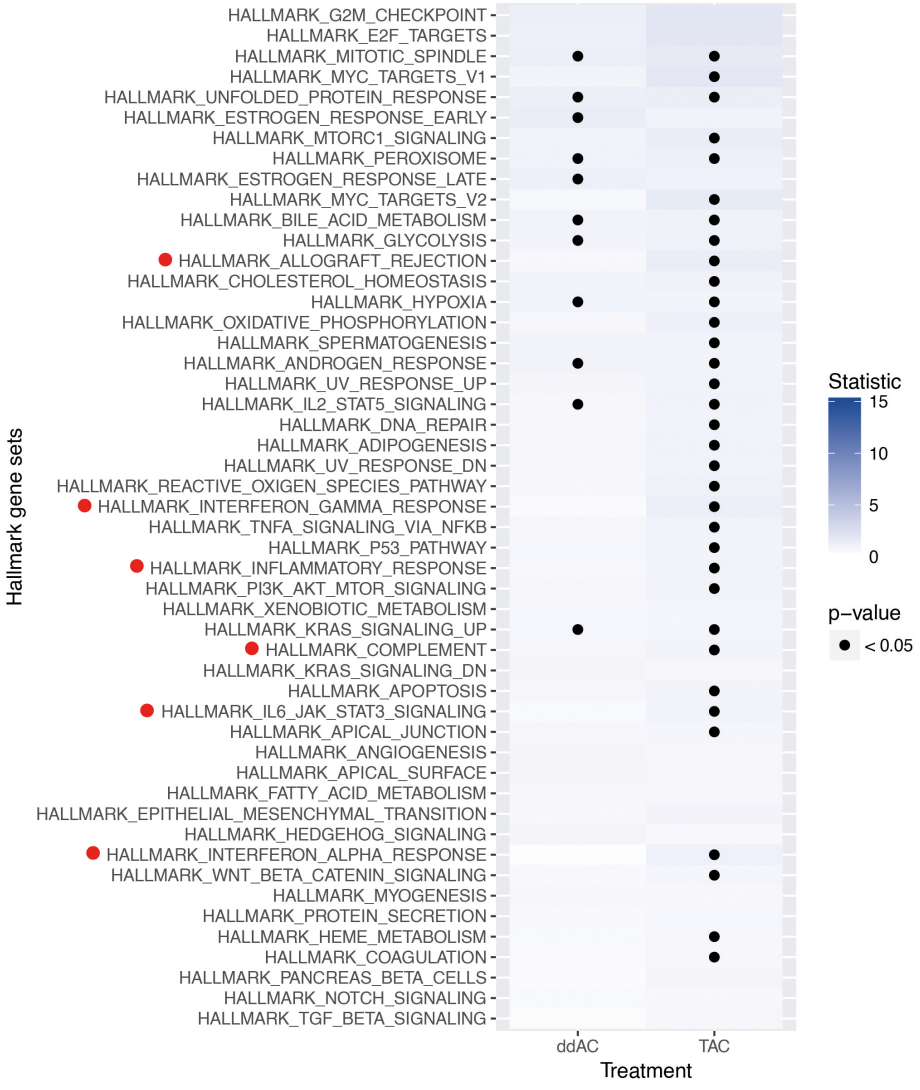
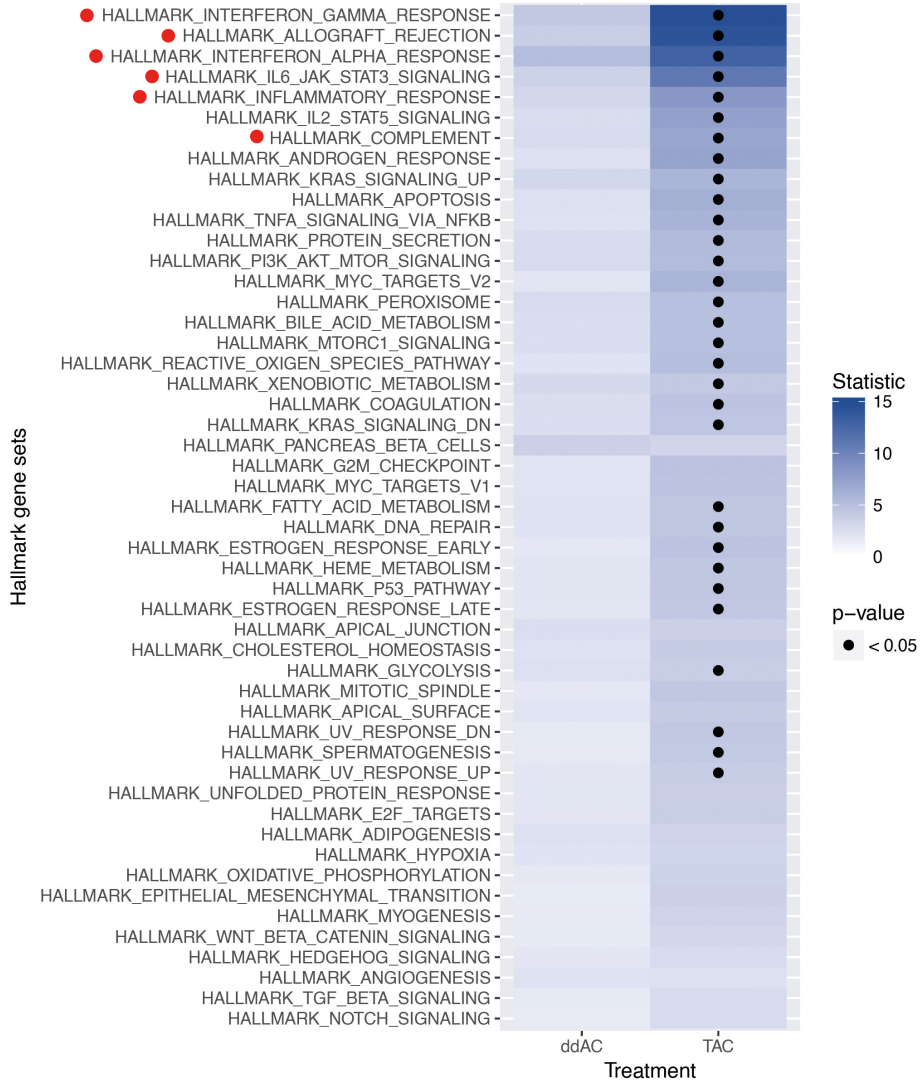


Figure 2. Strength of associations of hallmark gene sets[24] with recurrence free survival (RFS) measured by Goeman's *globaltest* statistic and its p-value (R package *globaltest*[23]) split by treatment arm in all patients (a), and in the basal subgroup(b). The gene sets are ordered according to the *globaltest* statistic. Immune-related processes are depicted by a red dot. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

a. All patients



b. Basal subgroup



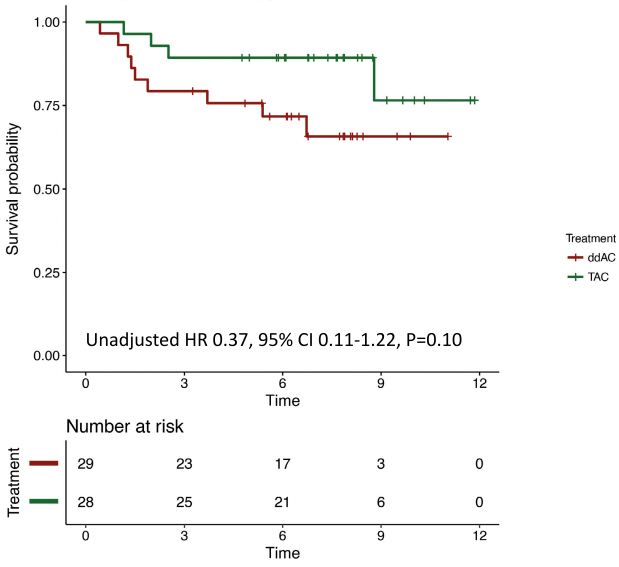
in Table S5. Interestingly, *CD74*, involved in formation and transport of MHC class II molecules, was positively associated with survival in all three selected gene sets in the TAC treated subgroup, but not in the ddAC treated patients. Another 6 genes (*HLA-DMA, HLA-G, JAK2, PSMB9, ST8SIA4 and IFNAR2*) related to both innate as well as adaptive immune responses were among the top hits in two gene sets in the TAC treated patients, but not in the ddAC treated group.

TILs

To assess the clinical applicability of the association between immune-related gene sets and survival as outlined above, we tested the predictive value of H&E-based tumor-infiltrating lymphocytes (TILs). Scoring of the TILs was done according to the international guideline[26], which results in high concordance among pathologists[27]. In addition, this biomarker is close to clinical application[28] in TNBC, making it an ideal candidate biomarker to assess endogenous immune responses in breast cancer. For 101 (93.5%) of 108 IHC-based triple negative breast cancer patients, tumors were scored for the abundance of TILs. The median value of TILs was 20% (IQR 10-50). Patients were divided in two groups according to the median: low TILs (<20%) and high TILs (≥20%). Abundance of TILs was not significantly associated with survival in TNBC patients (Figure S6) in our dataset. However, high TILs was predictive of numerically longer RFS after TAC, while low TILs was linked to better outcome after ddAC. The interaction between TILs and treatment was significant (adjusted $P_{interaction}$=0.03; Figure 3a and 3b, Table 2). Specifically, patients with high TILs had a significantly better survival than patients with low TILs in the TAC treated group (unadjusted HR 0.23, 95% CI 0.07-0.76, P=0.02; Figure S7a). This effect was not observed in the ddAC treated patients (Figure S7b).

Figure 3. Association between treatment and recurrence free survival split by tumor-infiltrating lymphocytes (TILs) high (a) and low (b) in triple negative breast cancer patients. TILs were scored as high (≥20%) and low (<20%). A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense; HR= hazard ratio; 95% CI=95% confidence interval.

a. High TILs



b. Low TILs

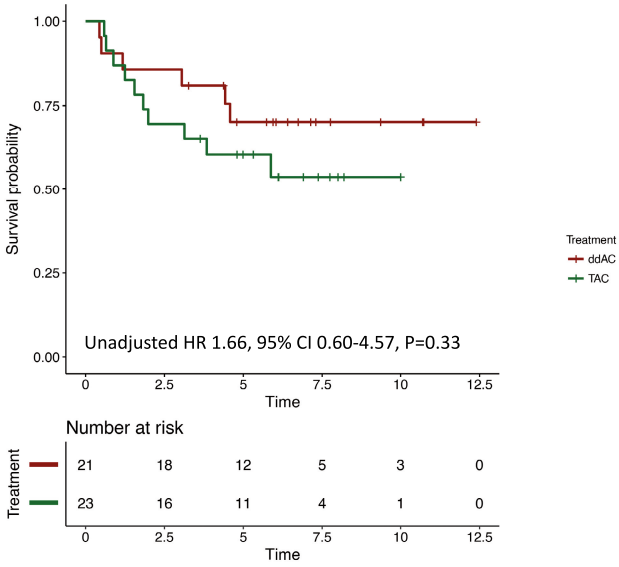


Table 2. Multivariable Cox regression model of the association between tumor-infiltrating lymphocytes (TILs) in an interaction with treatment and recurrence free survival (RFS) in TNBC patients. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense; TIL = tumor-infiltrating lymphocytes; HR = hazard ratio; CI = confidence interval

Variable		HR	95% CI	P-value
Treatment	ddAC	reference		
	TAC	1.67	0.58-4.85	0.34
Age	< 50 years	reference		
	≥ 50 years	1.02	0.47-2.19	0.96
T stage	T1	reference		
	T2-4	1.27	0.58-2.77	0.54
N stage	N0	reference		
	N+	15.06	2.03-111.81	**0.008**
Histologic grade	good/intermediate	reference		
	poor	1.26	0.28-5.58	0.76
Type of surgery	breast conserving surgery	reference		
	mastectomy	1.09	0.50-2.36	0.83
TILs	< 20%	reference		
	≥ 20%	1.19	0.40-3.55	0.76
TILs*treatment		0.18	0.04-0.87	**0.03**

DISCUSSION

The randomized MATADOR trial aimed to find a gene expression profile predictive of RFS benefit of either dose-dense chemotherapy (ddAC) or taxane-containing chemotherapy (TAC). Applying Cox regression with a LASSO penalty, we identified a gene expression profile which was prognostic for RFS, but unfortunately had limited predictive value for survival benefit of either ddAC or TAC.

Several challenges hampered defining a predictive gene expression profile. First, although in line with the high-risk patients of the MINDACT study[16], the survival of our patients was better than anticipated at the start of the trial. The statistical assumptions for this study were based on trials with a larger proportion of HER2 enriched breast cancer patients, who had a poor prognosis in the pre-anti-HER2 treatment era.[29,30] Also, stage migration might have played a role in the case mix of patients within these trials.[31] Secondly, adjuvant radiotherapy and endocrine therapy may have influenced survival, which might have interfered with finding a gene expression profile predictive

solely of adjuvant chemotherapy benefit in the total study population. In an exploratory analysis we therefore focused on the TNBC subgroup, to avoid the confounding effect of endocrine therapy.

Although previous groups could define a putative predictive profile[4-9], reports on the validation of these classifications are lacking, indicating the difficulty of generating a robust predictive gene expression-based classification. The variety of resistance mechanisms in the dataset of tumors in these studies likely plays a role.[32] If a resistance mechanism is not shared by a large fraction of the tumors, finding a predictive gene expression profile will be complicated. Also, the heterogeneity of the subclones within a tumor might influence the process. By analyzing bulk RNA derived from the tumor, it will only reflect the most prevalent type of tumor cells. Indeed, by disentangling the bulk signal into contributions of individual cellular components seems to have predictive value (Seinstra et al. Submitted for publication).

Besides genome-based expression profiling, we assessed the association between various biological processes and outcome using the hallmark gene sets. Since the addition of docetaxel is an absolute difference between TAC and ddAC, we hypothesized that mitotic spindle related gene sets and cell cycle related gene sets would form the top hits based on the conventional ideas of a genetic mechanism of action by stabilizing microtubules.[33] However, immune related gene sets appeared to have the strongest association with survival in the TAC treated patients with a basal tumor. These associations were less pronounced in the ddAC treated patients, suggesting that tumors with a stronger endogenous immune response derive more benefit from the addition of a taxane (or the higher dose of steroids accompanied with docetaxel) or a regimen without dose intensification. The literature on the direct effect of docetaxel on the anti-cancer immune response is limited. In a mouse model for TNBC docetaxel was able to deplete myeloid-derived immune suppressive cells in a specific manner[34] and in blood of metastatic breast cancer patients docetaxel resulted in an increased ratio between effector T cells and regulator T cells[35]. Also, high expression of immune-related genes has been linked to high likelihood of pCR in women with TNBC treated with TAC.[5,36] Further functional studies are needed to dissect the differential effects of TAC and ddAC on various components of the immune system.

Importantly, a simple H&E-based score of the immune infiltrate confirmed our observation that patients with a tumor with upregulation of immune-related genes have a better outcome after TAC. In TNBC patients, high abundance of TILs was associated with numerically longer RFS after TAC, while better outcome after ddAC

was observed in the low TIL group, with a significant interaction between abundance of TILs and treatment. These results were in line with previous reports on TILs and pCR after docetaxel-containing chemotherapy.[37,38] However, in another study no significant interaction was observed between TILs and adjuvant anthracycline only or anthracycline/docetaxel-containing chemotherapy for disease free survival in ER-negative, HER2-negative breast cancer patients[39]. This may be explained by a substantial difference in chemotherapy schedules (A or AC followed by CMF [cyclophosphamide – methotrexate – 5-fluorouracil] vs A-T or AT followed by CMF), the definition of positive ER and PR status (>1% instead of ≥10%), and differences in cut-off levels for lymphocytic infiltration. If validation in other cohorts confirms our finding, TILs could be a cheap and simple biomarker to select TNBC patients for a taxane-based chemotherapy regimen.

In conclusion, we identified a gene expression profile with limited value in predicting RFS benefit of either adjuvant dose-dense chemotherapy or a taxane-based regimen. However, analyses using well-established gene sets revealed immune-related processes as important predictors of RFS in the treatment subgroups of patients with a basal tumor, suggesting that grouping genes based on biological processes might be more useful than algorithms that use the expression of all measured genes independently in order to find a predictive biomarker for chemotherapy sensitivity. Furthermore, high abundance of TILs appeared to be a significant predictor of RFS benefit from docetaxel-based adjuvant chemotherapy in TNBC. The predictive value of TILs requires validation in an independent cohort. If found to be valid, the abundance of TILs in the primary tumor will help us to further personalize adjuvant chemotherapy in patients with triple negative breast cancer.

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SUPPLEMENTARY DATA

Figure S1. CONSORT diagram of the MATADOR patients. The clinical risk of recurrence was assessed using the modified Adjuvant!Online classification. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

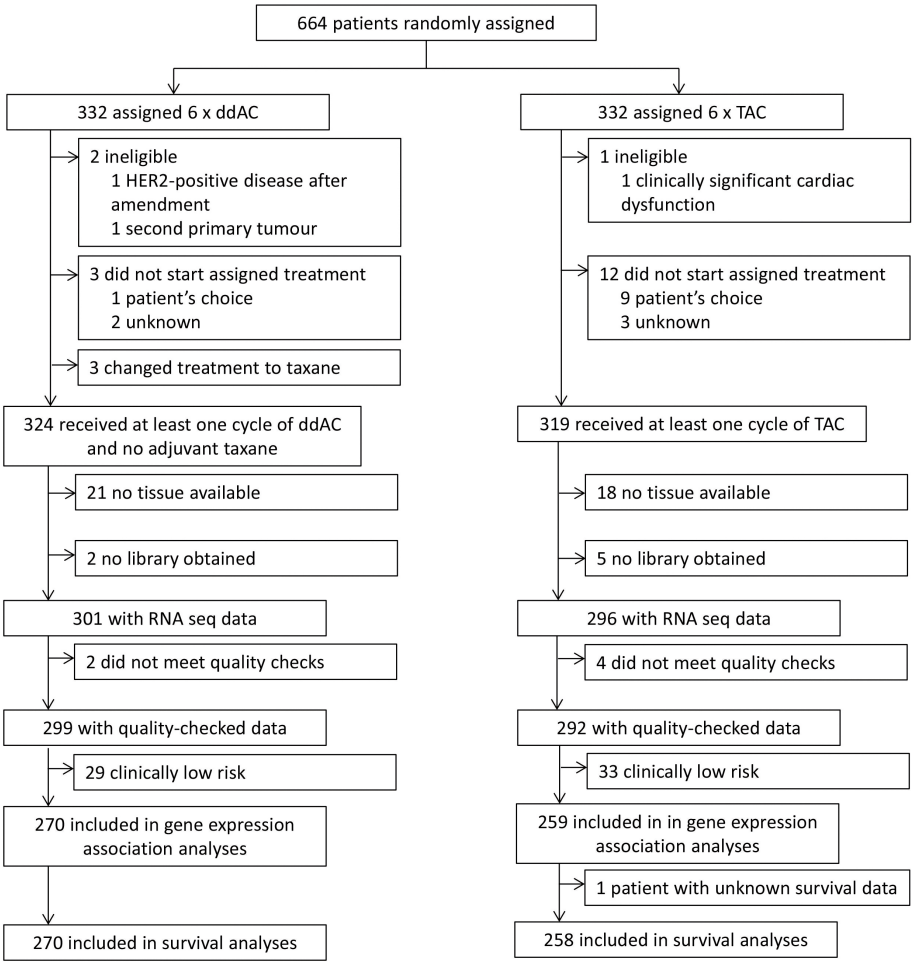


Figure S2. Unsupervised hierarchical clustering on all patients. RFS = recurrence free survival. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

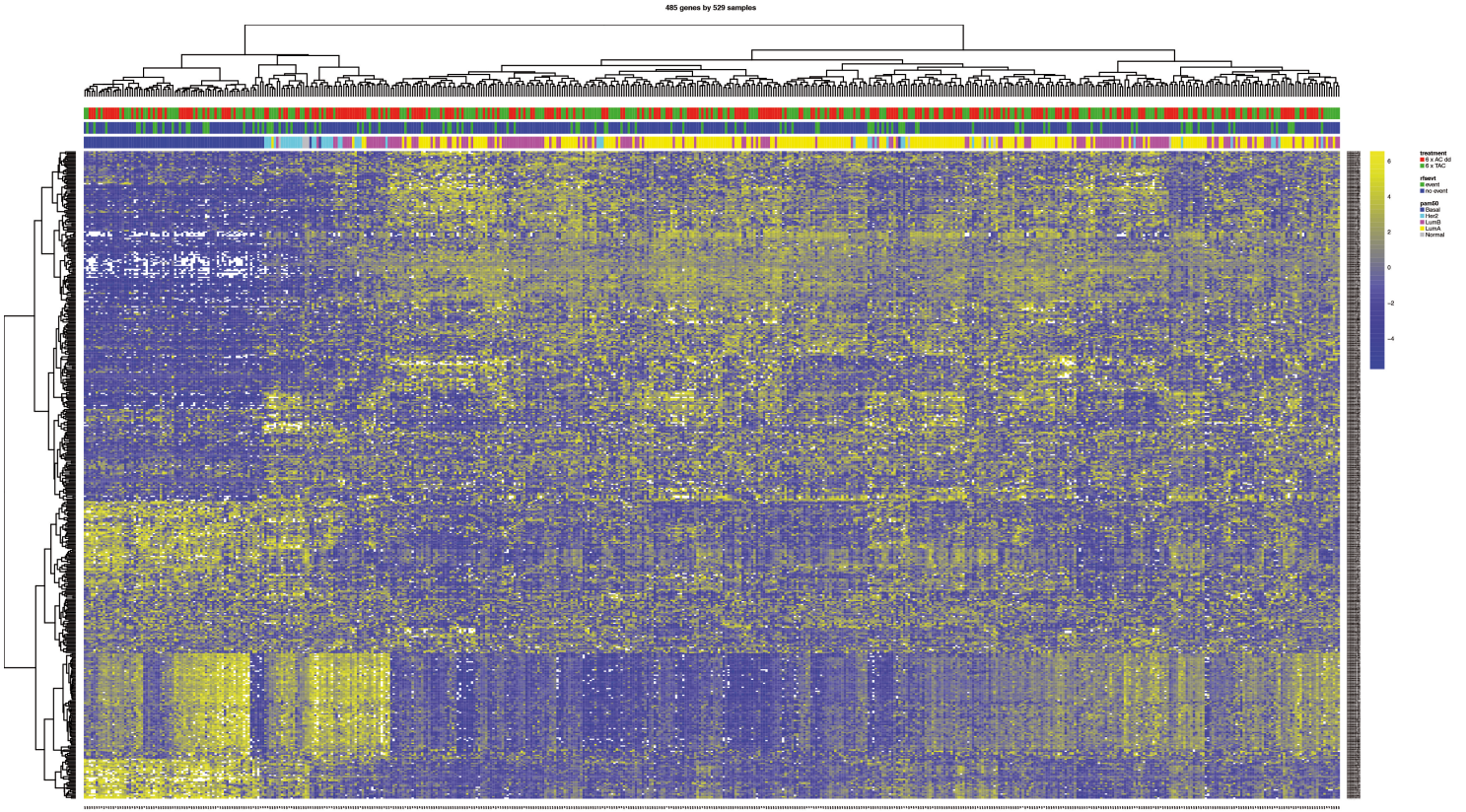


Figure S3. Association between PAM50 classification and recurrence free survival (RFS). LumA = luminal A; LumB = luminal B; HER2 = human epidermal growth factor receptor 2-enriched

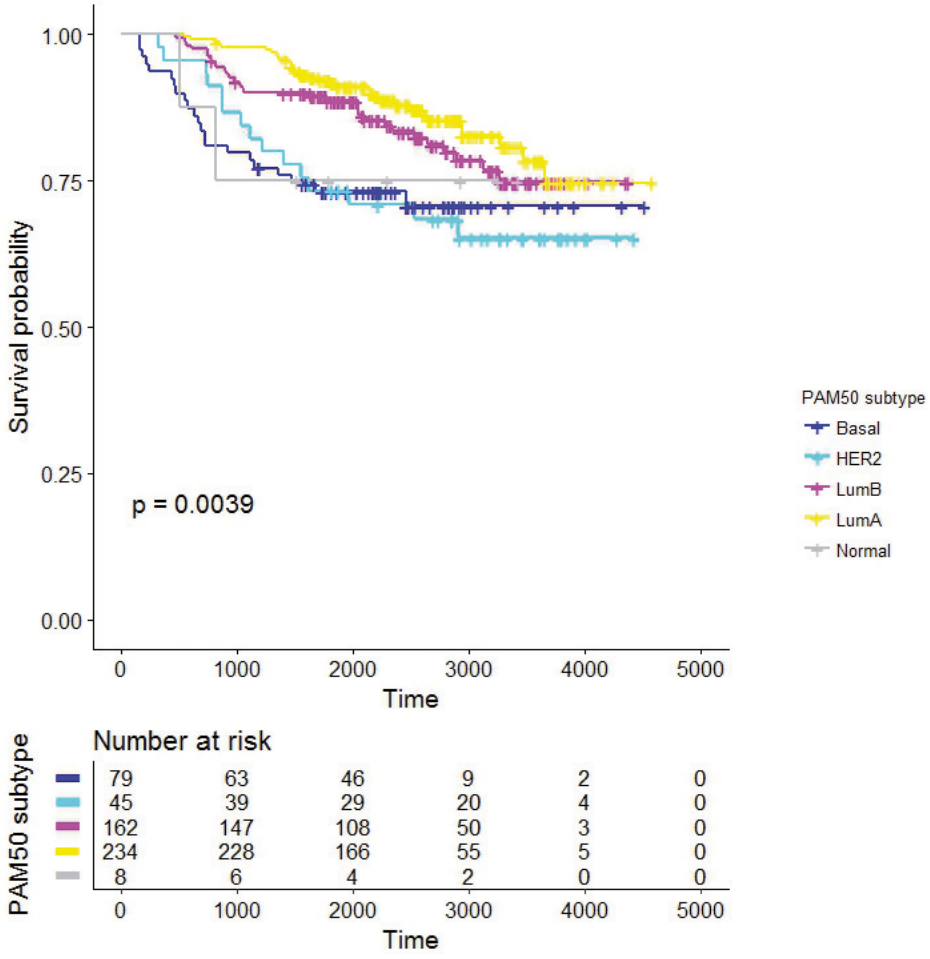


Figure S4. Association between treatment and recurrence free survival (RFS) in the basal subgroup (a, b), the luminal B subgroup (c, d), and the luminal A subgroup (e, f), split by a low profile score (a, c, e) or a high profile score (b, d, f). Log rank p-values are reported. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

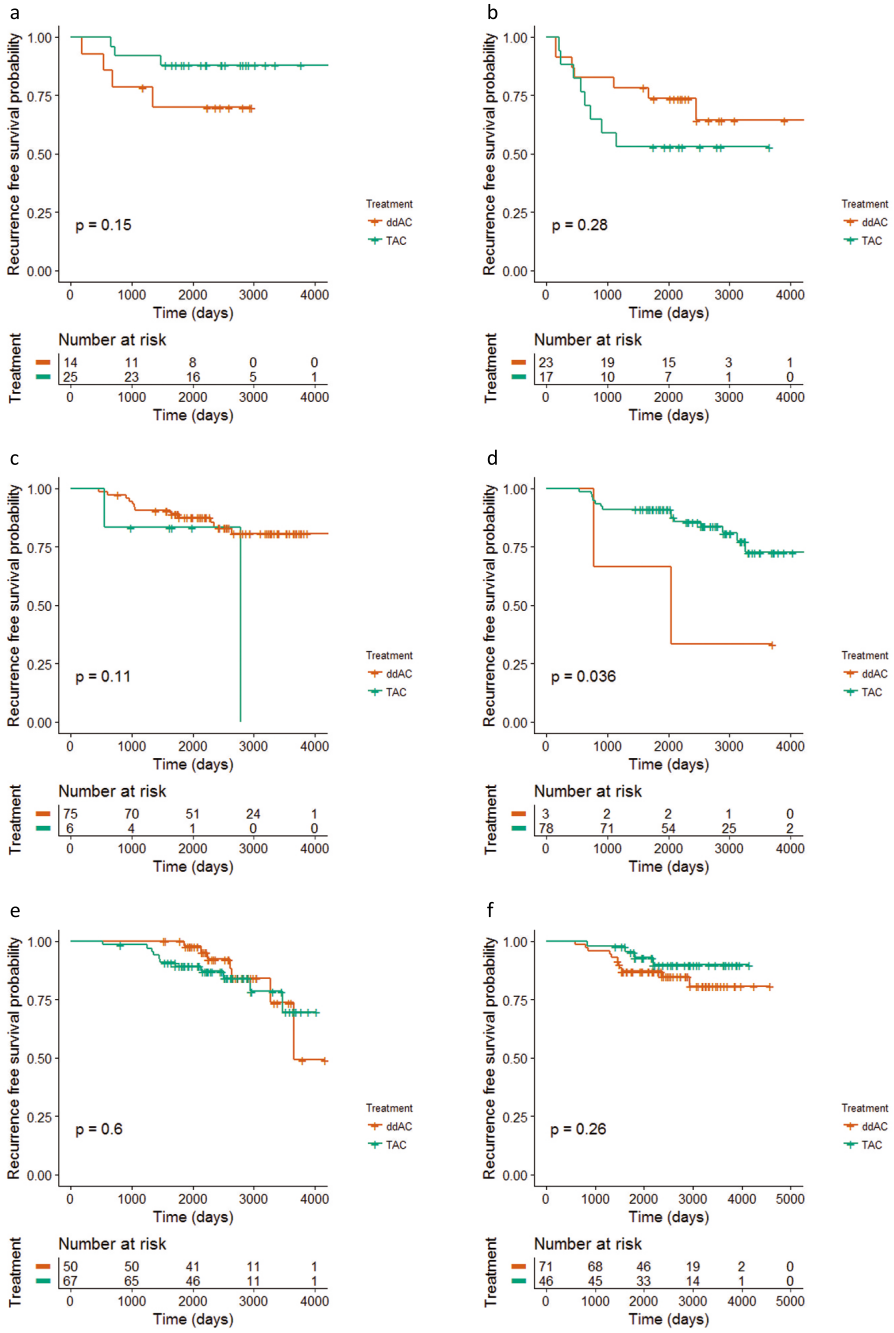
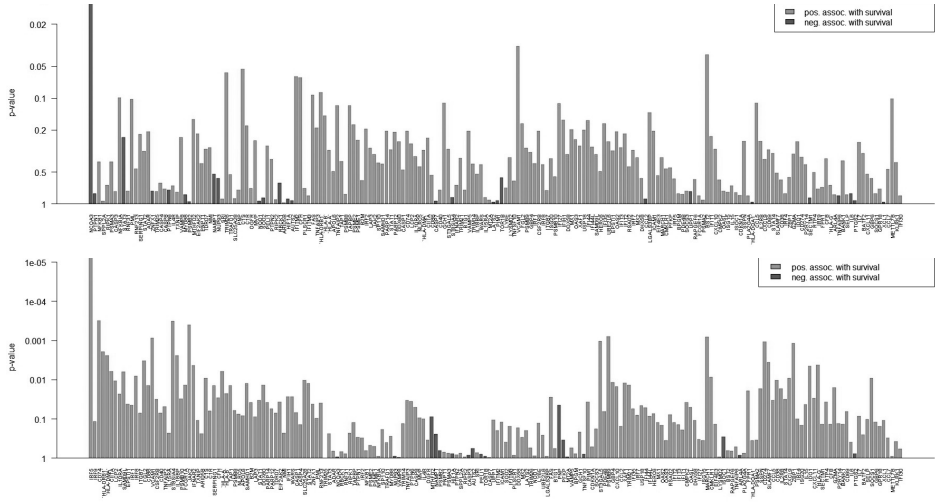


Figure S5. Association between the genes of the hallmark gene sets (a = interferon gamma response; b=allograft rejection; c=interferon alpha response) and recurrence free survival split by treatment subgroup. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

a



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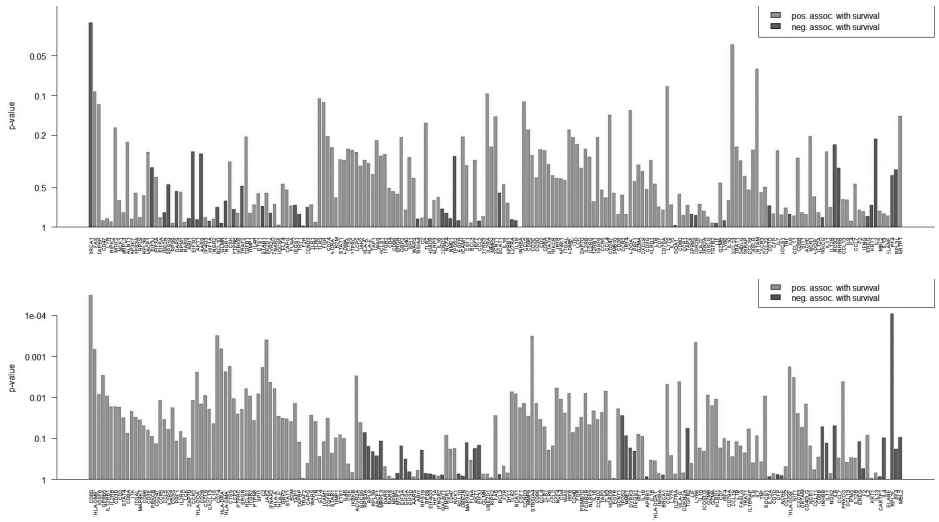

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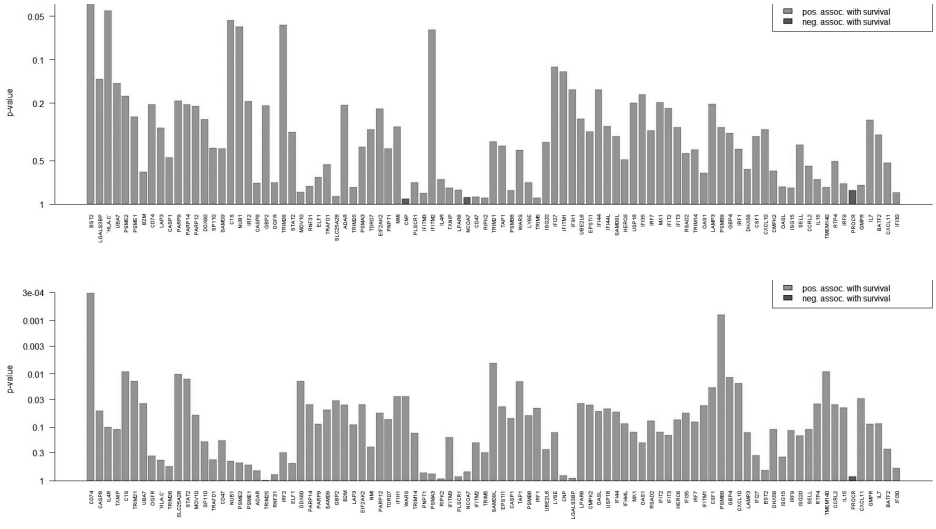


Figure S6. Association between tumor-infiltrating lymphocytes (TILs) and recurrence free survival (RFS) in patients with triple negative breast cancer. TILs were classified as low (<20%) and high (≥20%). HR = hazard ratio; CI = confidence interval

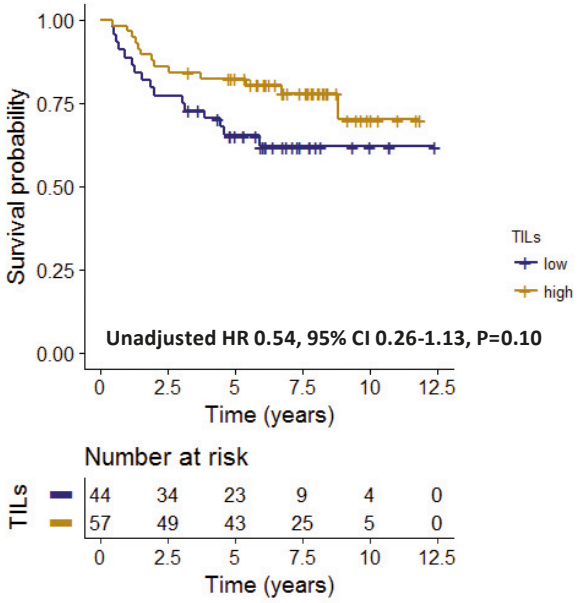
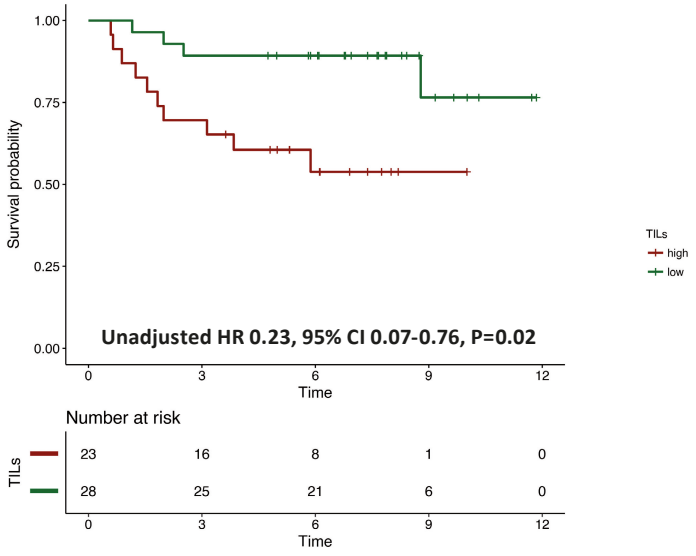


Figure S7. Association between tumor-infiltrating lymphocytes and recurrence free survival (RFS), split by treatment (TAC in a and ddAC in b), in patients with triple negative breast cancer. TILs were classified as low (<20%) and high (≥20%). HR = hazard ratio; CI = confidence interval

a



b

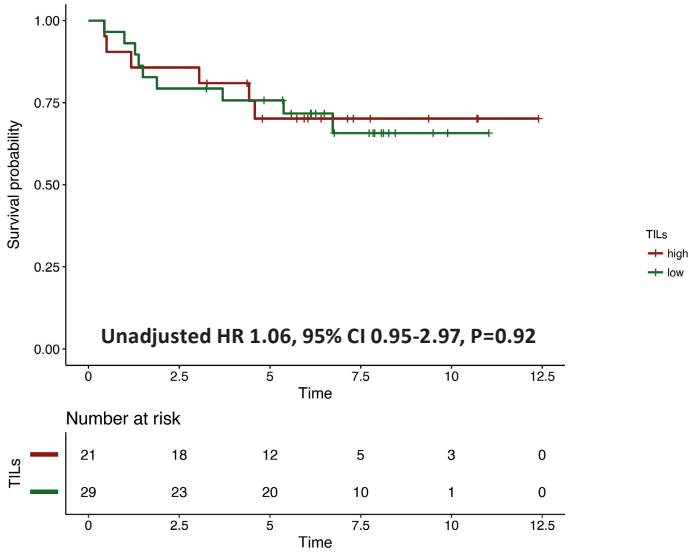


Table S1. Patient characteristics split by patients who were included in the analyses for the primary objective, patients who had a clinically low risk using the modified Adjuvant!Online classification and patients who were excluded for other reasons (ineligibility, no follow up data, no gene expression data available). RFS = recurrence free survival. † According to AJCC staging 6th edition; ‡ Grading according to the modified Bloom-Richardson grading system; § ER and PR nucleic staining of 10% staining or more was scored as positive, HER2-score of 3+ was considered positive, in case of a 2+ HER2-score, an in situ hybridization assay was performed; Subtypes were defined as 1. estrogen receptor (ER) and/or progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-negative; 2. HER2-positive, regardless of ER or PR status; 3. Triple (ER, PR, HER2) negative

		Analysed for primary endpoint n=528		**Clinically low risk n=62**		**Others n=74**	
Age groups (%)	< 50 years	237	(44.9)	27	(43.5)	33	(44.6)
	≥ 50 years	291	(55.1)	35	(56.5)	41	(55.4)
Surgery (%)	breast conserving surgery	280	(53.0)	38	(61.3)	32	(43.2)
	mastectomy	248	(47.0)	24	(38.7)	37	(50.0)
	missing	0	(0.0)	0	(0.0)	5	(6.8)
T stage† (%)	T1	231	(43.8)	55	(88.7)	27	(36.5)
	T2-T3-T4	296	(56.1)	7	(11.3)	42	(56.8)
	missing	1	(0.2)	0	(0.0)	5	(6.8)
N stage† (%)	N0	82	(15.5)	31	(50.0)	15	(20.3)
	N+	446	(84.5)	31	(50.0)	54	(73.0)
	missing	0	(0.0)	0	(0.0)	5	(6.8)
Grade‡ (%)	good	26	(4.9)	37	(59.7)	4	(5.4)
	intermediate	235	(44.5)	22	(35.5)	32	(43.2)
	poor	244	(46.2)	3	(4.8)	29	(39.2)
	missing	23	(4.4)	0	(0.0)	9	(12.2)
Histology	ductal	428	(81.1)	50	(80.6)	49	(66.2)
	lobular	78	(14.8)	5	(8.1)	10	(13.5)
	other	17	(3.2)	7	(11.3)	8	(10.8)
	missing	5	(0.9)	0	(0.0)	7	(9.5)
Subtype§ (%)	ER and/or PR-positive, HER2-negative	421	(79.7)	60	(96.8)	54	(73.0)
	HER2 positive	18	(3.4)	0	(0.0)	3	(4.1)
	triple negative	89	(16.9)	2	(3.2)	17	(23.0)
RFS event (%)	no	426	(80.7)	62	(100.0)	59	(79.7)
	yes	102	(19.3)	0	(0.0)	15	(20.3)

Table S2. Genes included in the predictive score according to the optimal lambda across the bootstraps of the Cox regression model with lasso penalty in all patients.

Gene	**Ensemble gene ID**
CYP4B1	ENSG00000142973
RGS7	ENSG00000182901
MKX	ENSG00000150051
DMBT1	ENSG00000187908
PGR	ENSG00000082175
CLEC4E	ENSG00000166523
KLRC2	ENSG00000205809
SERPINA6	ENSG00000170099
GP2	ENSG00000169347
RPL13P12	ENSG00000215030
CXCL17	ENSG00000189377
LRP2	ENSG00000081479
CLIC6	ENSG00000159212
ATP13A5	ENSG00000187527
GZMK	ENSG00000113088
RIMS1	ENSG00000079841
RIMS2	ENSG00000176406
SH2D1A	ENSG00000183918

Table S3. Genes included in the predictive score according to the optimal lambda across the bootstraps of the Cox regression model with lasso penalty in all patients according to molecular subtype.

	Gene	**Ensemble gene ID**
BASAL	*PIGR*	ENSG00000162896
	DMBT1	ENSG00000187908
	ANO5	ENSG00000171714
	SPDYC	ENSG00000204710
	LGR5	ENSG00000139292
	OLFM4	ENSG00000102837
	GPR12	ENSG00000132975
	GOLGA6L3	ENSG00000188388
	EEF1A2	ENSG00000101210
	CXCL13	ENSG00000156234
	PRSS12	ENSG00000164099
	FDCSP	ENSG00000181617
	RNF182	ENSG00000180537
	OFCC1	ENSG00000181355
	CTAGE4	ENSG00000225932
	IDO1	ENSG00000131203
	SDR16C5	ENSG00000170786
LUMINAL B	-	
LUMINAL A	*MUCL1*	ENSG00000172551
	CXCL17	ENSG00000189377
	RIMS1	ENSG00000079841

Table S4. Significance of the association between the average expression of the hallmark gene sets and recurrence free survival (RFS) in all patients and in the basal subgroup. If high expression of a gene set is associated with better outcome, the p-value is depicted in black. If high expression of a gene set is associated with worse outcome, the p-value is depicted in gray. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

Gene sets	all patients		basals	
	ddAC	TAC	ddAC	TAC
	P-value	P-value	P-value	P-value
HALLMARK_ADIPOGENESIS	0.14	0.02	**0.81**	0.09
HALLMARK_ALLOGRAFT_REJECTION	**0.19**	**0.00**	**0.35**	**0.005**
HALLMARK_ANDROGEN_RESPONSE	**0.05**	0.05	0.75	0.002
HALLMARK_ANGIOGENESIS	0.11	0.18	**0.68**	**0.53**
HALLMARK_APICAL_JUNCTION	0.09	**0.03**	**0.63**	**0.09**
HALLMARK_APICAL_SURFACE	**0.10**	**0.18**	0.82	**0.08**
HALLMARK_APOPTOSIS	**0.08**	**0.01**	**0.66**	**0.01**
HALLMARK_BILE_ACID_METABOLISM	**0.03**	**0.02**	**0.66**	**0.007**
HALLMARK_CHOLESTEROL_HOMEOSTASIS	**0.07**	0.01	0.75	0.06
HALLMARK_COAGULATION	**0.34**	**0.04**	**0.63**	**0.007**
HALLMARK_COMPLEMENT	**0.08**	**0.002**	**0.50**	**0.01**
HALLMARK_DNA_REPAIR	0.08	0.03	0.85	0.02
HALLMARK_E2F_TARGETS	0.13	0.08	0.92	0.19
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	**0.19**	**0.09**	**0.97**	**0.15**
HALLMARK_ESTROGEN_RESPONSE_EARLY	**0.04**	**0.12**	**0.98**	0.008
HALLMARK_ESTROGEN_RESPONSE_LATE	**0.05**	**0.07**	**0.95**	0.01
HALLMARK_FATTY_ACID_METABOLISM	0.08	0.07	**0.87**	0.002
HALLMARK_G2M_CHECKPOINT	0.11	0.07	0.81	0.07
HALLMARK_GLYCOLYSIS	0.05	0.02	0.69	0.03
HALLMARK_HEDGEHOG_SIGNALING	0.07	**0.20**	0.82	**0.37**
HALLMARK_HEME_METABOLISM	**0.20**	0.04	**0.86**	0.01
HALLMARK_HYPOXIA	0.02	**0.04**	0.80	**0.14**
HALLMARK_IL2_STAT5_SIGNALING	0.03	**0.006**	**0.53**	**0.01**
HALLMARK_IL6_JAK_STAT3_SIGNALING	**0.22**	**0.001**	**0.34**	**0.006**
HALLMARK_INFLAMMATORY_RESPONSE	**0.07**	**0.003**	**0.40**	**0.004**
HALLMARK_INTERFERON_ALPHA_RESPONSE	**0.85**	**0.01**	**0.29**	**0.01**
HALLMARK_INTERFERON_GAMMA_RESPONSE	**0.55**	**0.003**	**0.32**	**0.003**
HALLMARK_KRAS_SIGNALING_DN	0.05	**0.05**	0.64	**0.002**
HALLMARK_KRAS_SIGNALING_UP	**0.02**	**0.01**	**0.36**	**0.005**
HALLMARK_MITOTIC_SPINDLE	0.04	0.04	**0.98**	0.06
HALLMARK_MTORC1_SIGNALING	0.08	0.02	0.61	0.01
HALLMARK_MYC_TARGETS_V1	0.14	0.04	**0.88**	0.08
HALLMARK_MYC_TARGETS_V2	0.18	0.05	**0.82**	0.03
HALLMARK_MYOGENESIS	**0.06**	**0.21**	0.97	**0.08**
HALLMARK_NOTCH_SIGNALING	**0.33**	0.21	**0.90**	**0.36**
HALLMARK_OXIDATIVE_PHOSPHORYLATION	0.09	0.02	0.95	0.16
HALLMARK_P53_PATHWAY	**0.05**	**0.03**	**0.85**	**0.02**
HALLMARK_PANCREAS_BETA_CELLS	**0.52**	**0.08**	**0.20**	**0.16**

Gene sets	all patients		basals	
	ddAC	TAC	ddAC	TAC
	P-value	P-value	P-value	P-value
HALLMARK_PEROXISOME	**0.04**	**0.02**	**0.49**	**0.01**
HALLMARK_PI3K_AKT_MTOR_SIGNALING	0.09	0.009	0.50	**0.03**
HALLMARK_PROTEIN_SECRETION	0.22	0.06	0.55	0.004
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	0.21	0.03	0.76	0.03
HALLMARK_SPERMATOGENESIS	0.06	0.04	0.99	**0.02**
HALLMARK_TGF_BETA_SIGNALING	**0.60**	**0.06**	**0.94**	**0.35**
HALLMARK_TNFA_SIGNALING_VIA_NFKB	**0.11**	**0.002**	**0.69**	**0.01**
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.04	0.03	**0.85**	0.08
HALLMARK_UV_RESPONSE_DN	**0.10**	**0.02**	**0.98**	**0.03**
HALLMARK_UV_RESPONSE_UP	0.06	0.02	0.86	0.03
HALLMARK_WNT_BETA_CATENIN_SIGNALING	**0.34**	**0.02**	0.85	**0.26**
HALLMARK_XENOBIOTIC_METABOLISM	0.06	**0.05**	**0.37**	**0.04**

Table S5. Genes from the hallmark gene sets 'interferon gamma response', 'allograft rejection' and 'interferon alpha response' with a significant association (P<0.05) with recurrence free survival (RFS) split by treatment subgroup. Genes for which high expression is associated with longer RFS are depicted in black, genes for which low expression is associated with longer RFS are depicted in gray. A=doxorubicine; C=cyclophosphamide; T=docetaxel; dd=dose-dense

Interferon gamma response		Allograft rejection		Interferon alpha response	
ddAC	TAC	ddAC	TAC	ddAC	TAC
BST2	**CD74**	**CXCL13**	**CD74**	**BST2**	**CD74**
VCAM1	**VCAM1**	BRCA1	**HLA-DMA**	**HLA-C**	**PSMB9**
NCOA3	**HLA-DMA**		**HLA-G**		**SAMD9L**
	HLA-G		**JAK2**		
	JAK2		**ST8SIA4**		
	PSMB9		**IFNAR2**		
	ST8SIA4		**CD80**		
	IFNAR2		**CTSS**		
	IL18BP		**C2**		
	HLA-DRB1		**GPR65**		
	ST3GAL5		**HLA-DRA**		
	IFR5		**ACVR2A**		
	MARCH1		**HLA-DOA**		
	CD274		**CCR1**		
	SLAMF7		**HLA-DMB**		
	CXCL9		**LY86**		
	IDO1		**HLA-DOB**		
	FGL2		**SIT1**		
			PRKCG		
			RPL3L		

4

Independent replication of polymorphisms predicting toxicity in breast cancer patients randomized between dose-dense and docetaxel-containing adjuvant chemotherapy

A.G.J. van Rossum, M. Kok, D. McCool, M. Opdam, N.C. Miltenburg, I.A.M. Mandjes, A.E. van Leeuwen - Stok, A.L.T. Imholz, J.E.A. Portielje, M.M.E.M. Bos, A. van Bochove, E. van Werkhoven, M. Schmidt, H.M. Oosterkamp* and S.C. Linn*

* contributed equally to this work

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ABSTRACT

Introduction: Although pharmacogenomics has evolved substantially, a predictive test for chemotherapy toxicity is still lacking. We compared the toxicity of adjuvant dose-dense doxorubicin-cyclophosphamide (ddAC) and docetaxel-doxorubicin-cyclophosphamide (TAC) in a randomized multicenter phase III trial and replicated previously reported associations between genotypes and toxicity.

Methods: Patients with pT1-3, pN0-3 breast cancer were randomized between six cycles $A_{60}C_{600}$ every 2 weeks or $T_{75}A_{50}C_{500}$ every 3 weeks. Associations of 13 previously reported single nucleotide polymorphisms (SNPs) with the most frequent toxicities: anemia (AN), febrile neutropenia (FN) and peripheral neuropathy (PNP) were analyzed using logistic regression models.

Results: 646 patients (97%) were evaluable for toxicity (grade 2 and higher). Whereas AN was more frequent after ddAC ($P < 0.001$), TAC treated patients more often had PNP ($P < 0.001$). We could replicate 2 previously reported associations: *TECTA* (rs1829; OR 4.18, 95% CI 1.84-9.51, P = 0.001) with PNP, and *GSTP1* (rs1138272; OR 2.04, 95% CI 1.13-3.68, P = 0.018) with PNP.

Conclusions: In this independent replication, we could replicate an association between 2 out of 13 SNPs and chemotherapy toxicities. These results warrant further validation in order to enable tailored treatment for breast cancer patients.

INTRODUCTION

Adjuvant chemotherapy for early breast cancer has improved substantially over the past decades.[1] The introduction of two classes of drugs has been particularly important: anthracyclines and taxanes. However, treatment with these very effective drugs causes significant toxicities[2].

Anthracyclines are associated with an increased risk of nausea, vomiting, bone marrow suppression, myelodysplastic syndrome, leukemia and congestive heart failure[3,4]. Taxanes on the other hand are associated with peripheral neuropathy, febrile neutropenia and diarrhea[5]. These toxicities may put patients at risk of unfavorable outcome[2], decrease health-related quality of life and raise health-care costs due to hospital admissions. Hence, there is a great clinical need for tests that can predict which patients will encounter significant toxicity[6].

The ultimate goal is to develop a clinical test with a short lead-time that predicts treatment-specific toxicity with high accuracy. Patients with a test result indicating substantial toxicity may be spared from these side effects when an alternative systemic treatment would be prescribed. To date, numerous associations between toxicity of anthracyclines and taxanes and single nucleotide polymorphisms (SNPs) have been described[7–29]. These SNPs usually reside in genes that encode for the enzymes involved in the pharmacokinetics of these drugs. Despite plausible biological rationales, none of these associations were validated in independent studies and incorporated into clinical practice. Proper validation could have been hampered due to the methodological limitations of these studies[30]. Studies were often retrospective series instead of randomized trials with relatively small sample sizes. Moreover, these studies evaluated multiple associations, thereby increasing the risk of type I errors (false positive findings).

Here we present the toxicity of a multicenter randomized phase III trial of six cycles of dose-dense doxorubicin/cyclophosphamide (ddAC) and docetaxel/doxorubicin/cyclophosphamide (TAC). Additionally, we aim to replicate previously reported associations between side effects and clinical variables or SNPs. To our knowledge, this is the first trial that investigates 6 cycles of ddAC instead of 4. Moreover, it is the first replication of reported associations between genotype and chemotherapy toxicity in a large independent dataset including a randomization between two adjuvant regimens for breast cancer treatment.

METHODS

Study design

The MATADOR trial (Microarray Analysis in breast cancer to Tailor Adjuvant Drugs Or Regimens, ISRCTN61893718) is a prospective, multicenter, non- blinded randomized phase III trial conducted in the Netherlands during 2004-2012. Twenty-nine centers participated in this study. The primary objective of this study was to discover a gene expression profile that can predict recurrence free survival (RFS) benefit of either dose-dense or docetaxel-containing, anthracycline-based adjuvant therapy. Here we present SNP and toxicity data of this study. Female patients with a stage pT1-3, pN0-3, M0 invasive adenocarcinoma of the breast were eligible (Supplementary Figure 1). A WHO performance status of 0 or 1 and adequate bone marrow, liver and renal function were required. Patients with pre-existing motor or sensory neuropathy of grade 2 or more were ineligible, as well as patients who received previous systemic anticancer therapy. At the start of the trial, trastuzumab was not part of daily clinical practice and patients with HER2-positive disease were therefore included in this study. In February 2006 however, the protocol was amended to allow trastuzumab treatment for HER2-positive disease after completion of study treatment. In view of the accumulating evidence of improved disease free survival after concurrent chemotherapy and trastuzumab, patients with HER2-positive disease became ineligible in September 2007.

Patients were stratified according to menopausal status, type of surgery, tumor size, nodal status, hormone receptor status (estrogen receptor and progesterone receptor), HER2 status and treatment center. Subsequently, patients were allocated to receive either six cycles of doxorubicin 60 mg/m² plus cyclophosphamide 600 mg/m² every 2 weeks (ddAC), or six cycles of docetaxel 75 mg/m², doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m² every 3 weeks (TAC). All patients received granulocyte-colony stimulating factor (pegfilgrastim). Prophylactic antibiotic therapy was not recommended. Anti-emetic treatment was given according to the local standards. Patients received adjuvant radiotherapy and/or endocrine therapy according to the Dutch guidelines.

Toxicities were reported in the clinical record form according to common toxicity criteria for adverse events (AEs; CTCAE version 3.0). All adverse events (AE) of grade 2 or higher were recorded. Anemia was defined as a baseline hemoglobin concentration 6.2 mmol/L or less, febrile neutropenia was described as a body temperature of ≥ 38.5°C and an absolute neutrophil count of $< 1.0 \times 10^9$/L, and peripheral neuropathy was defined as

sensory alterations, paresthesia or weakness interfering with function. Any event that was fatal, life threatening, required hospitalization, led to prolonged hospitalization or resulted in significant disability was described as a serious adverse event (SAE).

The study protocol was approved by the medical ethical committee of the Netherlands Cancer Institute (approval 24 March 2004) and the research was conducted in accordance with the Declaration of Helsinki (version 17C, 1964). All patients had given written informed consent to participate in the study, including side studies meant to improve breast cancer diagnostics or therapy.

Tumor histology and immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) tumor tissue was assessed for morphology, histological grade according to the modified Bloom-Richardson classification[43], expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) by the pathologists of the participating centers according to established local procedures. The Dutch guidelines specified ER and PR nucleic staining of 10% staining or more as positive. HER2 score of 3+ was considered positive. In case of a 2+ HER2 score, an in situ hybridization assay was performed. Breast cancer subtype was defined as 1. ER and/or PR positive, HER2 negative; 2. HER2 positive, regardless of ER and PR status; or 3. triple negative.

DNA isolation

Fresh frozen (FF) and FFPE tumor tissue as well as normal tissue was requested from all patients. FFPE tumor tissue was available for the majority of the cases (75%). If unavailable, FF tumor tissue (18%) or FFPE normal tissue (7%) was used. For FFPE tissue, DNA was isolated as previously described using 10 slides of 10 µm, the QIAamp DNA extraction kit and protocol (QIAgen)[44]. For FF tissue, 15 slides of 30 µm were used. DNA was isolated using the DNeasy Blood & Tissue Kit (QIAgen). DNA was available for 642 patients.

Single nucleotide polymorphisms (SNPs)

To reduce the risk of multiple testing, three toxicity categories were selected for SNP analyses based on a combination of most frequent, largest clinical impact (hospital admission) and potentially long-term disability. These three categories were anemia (A), febrile neutropenia (FN) and peripheral neuropathy (PNP).

The SNP selection procedure is illustrated in Figure 2. First, a PubMed search was performed to select SNPs based on previously reported associations between toxicity of either doxorubicin, cyclophosphamide or docetaxel and a SNP. The literature search contained three elements: 1. one of the three toxicity categories, 2. the study drugs, and 3. single nucleotide polymorphism. SNPs associated with toxicity reported until September 2015 were selected. An update of the search was performed in June 2016. The search resulted in 24 SNPs with a possible association with toxicity. Secondly, we selected 105 SNPs that could be involved in the metabolism of one of the study drugs from the PharmaADME database (http://www. pharmaadme.org/). These two strategies resulted in a total of 129 SNPs. A SNP was excluded from further analyses if the assay failed due to technical reasons (n = 7), if the minor allele frequency (MAF) was below 5% (n = 105), or if the genotype frequencies of a SNP deviated from Hardy-Weinberg equilibrium (HWE, $P < 0.001$, n = 4, Supplementary Table 4). A total of 13 different SNPs were included in the final analyses. The previously reported associations between these SNPs and the toxicities were summarized in Supplementary Table 5.

A customized, mass-spectrometry based genotyping assay (Sequenom MassARRAY platform, Sequenom Inc, CA, USA) was designed to analyze these SNPs. Genotypes were determined using Sequenom's TyperAnalyzer software.

Statistics

Differences in clinicopathological characteristics, AEs and SAEs between treatment groups were compared with a chi-square test. When the count in any of the groups was less than 5, a Fisher's exact test was applied.

To accurately replicate previously reported associations between clinical parameters (if a cut off was reported) or SNPs and one of the three toxicities, univariable binary logistic regression models were constructed using previously reported genotype categories. All variables that were significantly associated with toxicity in the univariable models were included in a multivariable binary logistic regression model.

Secondly, tests for interaction were performed to evaluate whether the risk of a genotype-based patient group for a particular toxicity was different per given treatment (ddAC or TAC). The association of the allocated treatment with toxicity was investigated using a logistic regression model in subgroups of patients. Interactions between clinical parameters or SNPs and treatment were tested using logistic regression model with an interaction term.

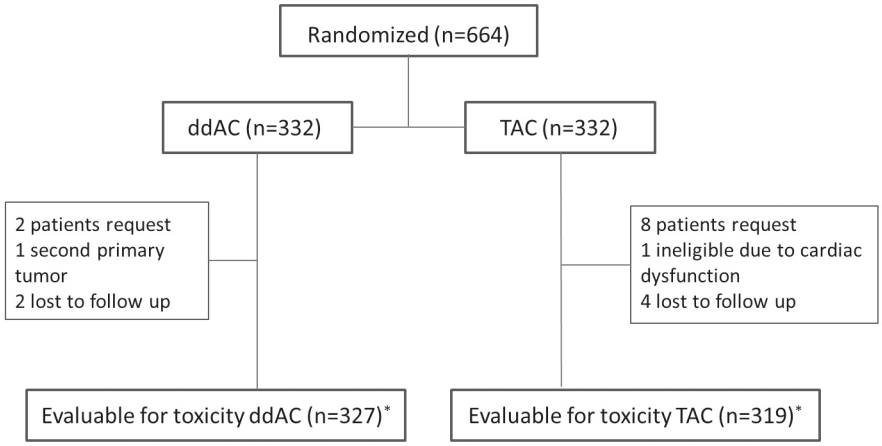
The association analyses were exploratory and were not pre-specified in the analysis plan of the MATADOR trial. Since our objective was to replicate previously described associations between SNPs and toxicity, we did not correct for multiple testing. For all analyses, a p-value of less than 0.05 was considered significant. Analyses were performed using SPSS software version 22 (IBM Corporation, Armonk, NY).

RESULTS

Clinicopathological characteristics

Between August 2004 and November 2012, 664 patients were randomized (Figure 1). Sixteen patients were excluded after randomization on their own request or lost to follow up. Two patients were considered ineligible for other reasons: one patient had a second primary tumor and one patient had significant cardiac dysfunction at baseline. In total, 646 patients were evaluable for toxicity.

Figure 1. Flow chart of patients evaluable for toxicity * received at least one cycle of allocated treatment; ddAC = dose-dense doxorubicin, cyclophosphamide; TAC = docetaxel, doxorubicin, cyclophosphamide



The two treatment groups were not significantly different according to clinicopathological characteristics (Table 1). After the introduction of trastuzumab in routine clinical practice, patients with a HER2-positive tumor were no longer eligible and consequently only a small proportion of the patients included in this trial had HER2-positive disease.

Table 1. Patient characteristics. A = doxorubicin; C = cyclophosphamide; T=docetaxel ¶ Subtypes were defined as 1. estrogen receptor (ER) and/or progesterone receptor (PR) positive, human epidermal growth factor receptor 2 (HER2) negative; 2. HER2 positive, regardless of ER or PR status; 3. Triple (ER, PR, HER2) negative; * Pearson Chi-square test or Fisher's exact test (2-sided), missing values excluded; † Menopausal status was based on patients' history; ‡ According to AJCC staging 6th edition § Grading according to the modified Bloom-Richardson grading system[33]

	dose dense AC n (%)	TAC n (%)	Total n (%)	p-value*
Age (yrs)				0.667
≤29	2 (0.6)	1 (0.3)	3 (0.5)	
30-39	23 (7.0)	25 (7.8)	48 (7.4)	
40-49	115 (35.2)	123 (38.6)	238 (36.8)	
50-59	125 (38.2)	115 (36.1)	240 (37.2)	
60-69	62 (19.0)	53 (16.6)	115 (17.8)	
≥70	0	2 (0.6)	2 (0.3)	
Menopausal status†				0.323
premenopausal	168 (51.4)	175 (54.9)	343 (53.1)	
postmenopausal	154 (47.1)	137 (42.9)	291 (45.0)	
missing	5 (1.5)	7 (2.2)	12 (1.9)	
Surgery				0.490
breast conserving surgery	178 (54.4)	165 (51.7)	343 (53.1)	
mastectomy	148 (45.3)	153 (48.0)	301 (46.6)	
missing	1 (0.3)	1 (0.3)	2 (0.3)	
Endocrine therapy				0.934
none	55 (16.8)	57 (17.9)	102 (15.8)	
tamoxifen	76 (23.2)	69 (21.6)	145 (22.4)	
aromatase inhibitor	26 (8.0)	28 (8.8)	54 (8.4)	
sequential tamoxifen-aromatase inhibitor	170 (52.0)	164 (51.4)	334 (51.7)	
missing	0 (0.0)	1 (0.3)	1 (0.2)	
T Stage‡				0.691
T1	157 (48.0)	151 (47.3)	308 (47.7)	
T2	152 (46.5)	148 (46.4)	300 (46.4)	
T3	16 (4.9)	18 (5.6)	34 (5.3)	
T4	2 (0.6)	0	2 (0.3)	
Tx	0	1 (0.3)	1 (0.2)	
missing	0	1 (0.3)	1 (0.2)	
N Stage‡				0.918
N0	61 (18.7)	61 (19.1)	122 (18.9)	
N1	207 (63.3)	195 (61.1)	402 (62.2)	
N2	44 (13.5)	44 (13.8)	88 (13.6)	
N3	15 (4.6)	18 (5.6)	33 (5.1)	
missing	0	1 (0.3)	1 (0.2)	
Histology				0.310
ductal	269 (82.3)	254 (79.6)	523 (81.0)	
lobular	46 (14.1)	45 (14.1)	91 (14.1)	

Table 1. (continued)

	dose dense AC n (%)	TAC n (%)	Total n (%)	p-value*
other	12 (3.7)	20 (6.3)	32 (5.0)	
Grade§				0.480
good	32 (9.8)	40 (12.5)	72 (11.1)	
intermediate	155 (47.4)	141 (44.2)	296 (45.8)	
poor	140 (42.8)	138 (43.3)	278 (43.0)	
Subtype¶				0.666
ER and/or PR positive, HER2 negative	267 (81.6)	258 (80.9)	525 (81.3)	
HER2 positive	12 (3.7)	11 (3.4)	23 (3.5)	
Triple negative	48 (14.7)	50 (15.7)	98 (15.2)	

Dose reductions and delays

A total of 280 out of 327 patients randomized to ddAC (85.6%) and 271 out of 319 patients randomized to TAC (85.0%) received 6 full-dosed cycles of treatment (P = 0.809). For the patients who prematurely stopped treatment, ddAC was discontinued due to toxicity in 22 out of 327 patients (6.7%) and TAC in 26 out of 319 patients (8.2%, P = 0.491; Supplementary Table 1). Dose reductions of more than 10% occurred more frequently for TAC (39 out of 1817 cycles, 2.1%) than for ddAC (13 out of 1914 cycles, 0.7%, P < 0.001).

Adverse events (AEs)

Supplementary Table 2 shows all AEs (grade 2 or higher) per treatment arm per CTCAE category.

Table 2 shows the toxicities that were significantly different between the treatment groups. Anemia was observed more often in the ddAC group than in the TAC group: 62 out of 327 patients (19.0%) versus 15 out of 319 patients (4.7%) respectively (P < 0.001). Also, hand-foot syndrome (4.3% vs 0.6%, P = 0.004), cough (5.8% vs 2.2%, P = 0.019) and phlebitis (4.3% vs 1.3%, P = 0.029) were observed more often in the ddAC treated patients.

Table 2. Toxicities (grade 2 or higher) with significantly different frequencies in the treatment groups.
* Pearson Chi-square test 2-sided; † Fisher's exact test 2-sided; A = doxorubicin; C = cyclophosphamide; T=docetaxel

	Total n=646 (%)	dose dense AC n=327 (%)	TAC n=319 (%)	p-value*
Anemia	77 (11.9)	62 (19.0)	15 (4.7)	<0.001
Hand-foot syndrome	16 (2.5)	14 (4.3)	2 (0.6)	0.004†
Diarrhea	74 (11.5)	21 (6.4)	53 (16.6)	<0.001
Edema limb	16 (2.5)	1 (0.3)	15 (4.7)	<0.001†
Peripheral neuropathy	61 (9.4)	15 (4.6)	46 (14.4)	<0.001
Cough	26 (4.0)	19 (5.8)	7 (2.2)	0.019
Phlebitis	18 (2.8)	14 (4.3)	4 (1.3)	0.029†

Peripheral neuropathy was seen in 46 out of 319 patients (14.4%) in the TAC treatment group and in 15 out of 327 ddAC treated patients (4.6%; P < 0.001). In addition, diarrhea was observed more often in patients treated with TAC (16.6%) than in patients treated with ddAC (6.4%; P < 0.001), as was edema of the limbs (4.7% vs 0.3%; P < 0.001). Of note, febrile neutropenia was observed in 36 out of 327 patients treated with ddAC (11.0%) and 40 out of 319 patients treated with TAC (12.5%) which was not significantly different (P = 0.546).

Serious adverse events (SAEs)

Two patients were diagnosed with acute myeloid leukemia during follow up, one in the ddAC group and one in the TAC group (Supplementary Table 3). One ddAC treated patient developed myelodysplasia. Two TAC treated patients and one ddAC treated patient, all without known cardiovascular history, developed grade 3 or 4 symptoms of heart failure.

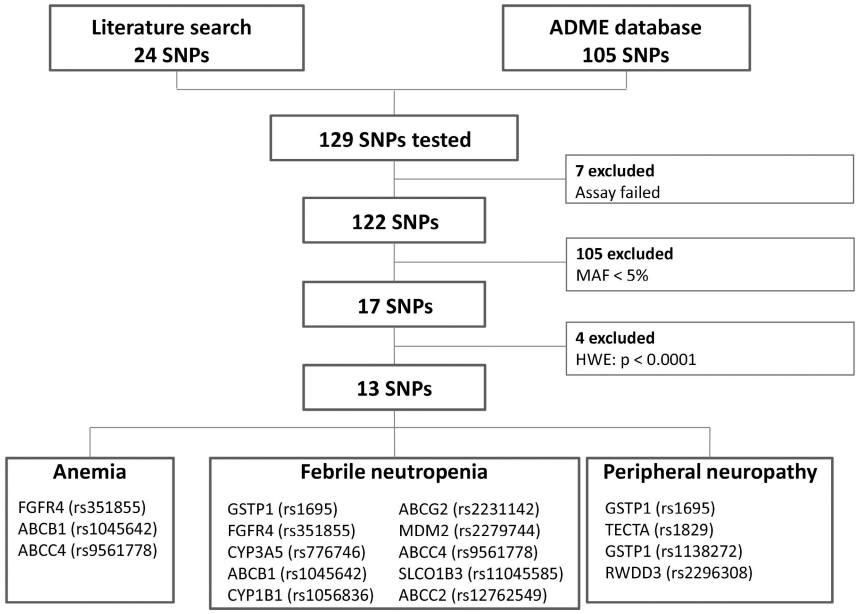
In total, 130 out of 646 patients (20.1%) experienced at least one SAE: 60 out of 327 patients (18.3%) in the ddAC treated group and 70 out of 319 patients (21.9%) in the TAC treated group (P = 0.255). Admission to the hospital due to a SAE was needed at least once in 121 patients: 55 of 327 ddAC treated patients (16.8%) and 66 of 319 TAC treated patients (20.7%; P = 0.207). Although there was no difference in the frequency of febrile neutropenia between the ddAC group and the TAC group, the first episode was on average after 3.7 cycles of ddAC and 1.4 cycles of TAC (P < 0.001).

SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

Replication of associations between clinicopathologic variables, SNPs and toxicity

We aimed to replicate previously reported associations between clinicopathologic variables or SNPs and toxicity. SNPs were selected if they were associated previously with toxicity of one of the treatment agents or if they were involved in the metabolism of one of the treatment agents (Figure 2). The results are listed in Supplementary Table 5, the significant findings are listed in Table 3.

Figure 2. Flow chart of single nucleotide polymorphisms (SNPs) that were selected for association analyses. MAF = minor allele frequency, HWE= Hardy-Weinberg Equilibrium



Anemia (AN)[7–10]

The odds of anemia in patients who were 65 years or older was 3.45 times the odds in the younger patients (30% vs 11%, P = 0.003) (Table 3). Baseline platelet count of 200 × 10^9 cells/L or less was also associated with higher risk of anemia (25.5% vs 10.8%, P = 0.002). Previously reported genotypes for *FGFR4* (CC vs CT/TT)[8], *ABCB1* (TT/TC vs

CC)[9] and *ABCC4* (GG vs GT/TT)[10] were not significantly associated with anemia in our dataset (Supplementary Table 6). The associations of age and baseline platelet count with anemia remained stable in a multivariable model.

Febrile neutropenia (FN)[9, 11–25]

Baseline absolute neutrophil count (ANC ≤ 3.1 × 10^9 cells/L)[15] and the following previously reported genotypes did not have a significant association with FN: *GSTP1* (AG rs1695 and CC rs1138272 vs other; rs1695 AA vs AG/GG)[18,19], *ABCB1* (TT vs TC/CC)[9,17], *ABCG2* (CC vs CA/AA)[22], *MDM2* (TT/TG vs GG)[23], *ABCC4* (GG vs GT/TT)[24], *SLCO1B3* (AA vs AG/ GG)[25] and *ABCC2* (CC/CG vs GG)[25] and a haplotype of *ABCB1* and *CYP1B1* (rs1045642*rs1056836)[21] (Supplementary Table 6).

Peripheral neuropathy (PNP)[26–29]

The odds of PNP in homozygous variant carriers of *TECTA* (TT, rs1829) was 4.18 times increased compared with the odds in homozygous wildtype or heterozygous variant carriers (CC/CT) in our cohort (28.1% vs 8.6%, P = 0.001) (Table 3). In addition, heterozygous and homozygous variant carriers of *GSTP1* (CT/TT, rs1138272) had 2.04 times increased odds of PNP (15.4% vs 8.2%, P = 0.018). In our dataset, a history of diabetes as previously described by Bhatnagar et al[27], was not related with PNP (Supplementary Table 6). Also, previously reported genotype subgroups for *GSTP1* (AA vs AG/GG)[28] and *RWDD3* (GG/GT vs TT)[26] were not significantly associated with PNP.

SNPs and differential toxicity of ddAC or TAC

Next, we evaluated whether the associations between the SNPs and toxicities of interest were different in the two treatment arms. The significant tests for interaction of the treatment effect are included in Table 3.

Anemia

We found no significant interaction between a clinical variable or SNP and treatment (ddAC vs TAC) for the risk of developing anemia (Supplementary Table 7).

Table 3. Significant associations between toxicities and clinical variables or SNPs. ddAC = dose-dense doxorubicin, cyclophosphamide; TAC = docetaxel, doxorubicin, cyclophosphamide; OR = odds ratio; CI = confidence interval

Toxicity	Variable	Groups	All patients			ddAC	TAC	TAC vs ddAC		Test for interaction
			No. of patients with toxicity (%)	OR (95% CI)	p-value	No. of patients with toxicity (%)	No. of patients with toxicity (%)	OR (95% CI)	p-value	p-value
AN	Age	< 65 years vs ≥ 65 years	68/616 (11.0) 9/30 (30.0)	**3.45 (1.52-7.85)**	**0.003**	56/310 (18.1) 6/17 (35.3)	12/306 (3.9) 3/13 (23.1)	**0.19 (0.10-0.35)** 0.55 (0.11-2.81)	**< 0.001** 0.472	0.223
AN	Baseline platelet count	> 200x10^9 cells/L vs ≤ 200x10^9 cells/L	63/585 (10.8) 14/55 (25.5)	**2.83 (1.46-5.48)**	**0.002**	52/294 (17.7) 10/28 (35.7)	11/291 (3.8) 4/27 (14.8)	**0.18 (0.09-0.36)** 0.31 (0.08-1.16)	**< 0.001** 0.083	0.475
FN	FGFR4 (rs351855)	CC/CT *vs* TT	66/579 (11.4) 8/59 (13.6)	1.22 (0.55-2.68)	0.622	28/293 (9.6) 7/30 (23.3)	38/286 (13.3) 1/29 (3.4)	1.45 (0.86-2.43) 0.12 (0.01-1.02)	0.160 0.053	**0.027**
PNP	*TECTA* (rs1829)	CC/CT vs TT	52/608 (8.6) 9/32 (28.1)	**4.18 (1.84-9.51)**	**0.001**	14/315 (4.4) 1/9 (11.1)	38/293 (13.0) 8/23 (34.8)	**3.20 (1.70-6.05)** 4.27 (0.45-40.44)	**< 0.001** 0.206	0.810
PNP	*GSTP1* (rs1138272)	CC vs CT/TT	43/525 (8.2) 18/117 (15.4)	**2.04 (1.13-3.68)**	**0.018**	11/275 (4.0) 4/50 (8.0)	32/250 (12.8) 14/67 (20.9)	**3.52 (1.74-7.15)** 3.04 (0.93-9.88)	**< 0.001** 0.065	0.833

Febrile neutropenia

Although treatment was not significantly associated with toxicity in the *FGFR4* (rs351855) genotype subgroups, we did observe a significant interaction between treatment and this SNP (P = 0.027, Table 3 and Supplementary Figure 2). Interaction analyses of other clinical variables or SNPs with FN were not significant. Of note, AG carriers of rs1695 and CC carriers of rs1138272 in *GSTP1* had a significantly higher risk of FN when treated with TAC (OR 2.14, 95% CI 1.08–4.23, P = 0.029), which was not observed in the ddAC treated group (OR 0.98, 95% CI 0.47–2.05, P = 0.959).

Peripheral neuropathy

None of the investigated factors had a significant interaction with treatment on the risk of PNP.

DISCUSSION

The main objective of the research presented here was to replicate previously described associations between certain clinical parameters or genetic polymorphisms and three frequently observed and clinically important chemotherapy-induced toxicities. Regarding the clinical parameters, we were able to replicate the associations of age and baseline platelet count with risk of anemia as previously described by Dranitsaris et al[7]. Of the 13 SNPs tested, the variant genotypes of rs1829 in *TECTA* and rs1138272 in *GSTP1* were related to peripheral neuropathy. However, the test for interaction between use of docetaxel, these variant genotypes and PNP was not significant. Given the relatively low sample size of our study, validation is required to determine the clinical value of our findings.

Most previously described associations could not be replicated in our study. This might be due to the fact that these associations were often described in patients treated with a different regimen than the agents used in our study. Also, previously described associations could have been incidental findings in inadequately designed studies. Instead of taking an agnostic approach in evaluating the predictive value of numerous SNPs, we focused on already described associations between genotype and frequently occurring side effects. With this starting point we reduce the type I error (false positive findings). The randomized nature of our dataset allowed us to evaluate whether these associations are treatment-specific and could therefore be of use in tailoring adjuvant chemotherapy for breast cancer patients. None of the SNPs in the different toxicity models were in linkage disequilibrium, except for a minor linkage between both *GSTP1* SNPs (rs1695 and rs1138272; r2 0.162), indicating that we investigated independent SNPs.

The largest difference in risk of toxicity was observed for *TECTA*. Homozygous variant carriers of *TECTA* (rs1829) had an increased risk of PNP. The mechanistic explanation regarding the link between *TECTA* and PNP is elusive. Tectorin Alpha (TECTA) is a major component of the tectorial membrane in the inner ear, which is important for transducing sound into electrical signals for our nervous system. Mutations in the *TECTA* gene are therefore often linked to deafness[31]. Our findings are in line with the preliminary findings of Schneider et al[26], who reported an association between *TECTA* polymorphism and taxane-induced-PNP. However, in the final report of Schneider et al[32] and two other genome wide association studies[33,34] the association could not be replicated. In addition, in our study the association between treatment and PNP was not significantly different in the *TECTA* genotype subgroups as tested by the interaction

analysis. This might be due to the relatively small sample size and an imbalance in the distribution of *TECTA* genotypes between the treatment arms. Alternatively, *TECTA* homozygous variant alleles may be associated with higher vulnerability of nerve tissues to cytotoxic damage in general. In the latter case, *TECTA* genotype analysis might only appear valuable when balancing risks and benefits of adjuvant chemotherapy in an equivocal case where PNP might be detrimental (e.g. a professional violin player). However, before introducing *TECTA* genotype analysis in daily clinical practice, these data require validation in an independent, large, preferably prospective cohort using the exact same subgrouping of patients according to their genotype.

To explore potential tailored chemotherapy based on SNP analysis, we tested the effect of treatment on the risk of toxicity in the genotype-based patient subgroups by performing an interaction analysis. The risk of FN according to *FGFR4* genotype was significantly different in the ddAC subgroup compared to the TAC subgroup as determined by the test for interaction. However, the absolute number of patients in the investigated subgroups is very small and an explanation for the opposite effect in the TAC arm versus the ddAC arm is lacking. Moreover, the mechanism by which a polymorphism of fibroblast growth factor receptor 4 (*FGFR4*) can lead to an increased risk of FN is unknown. Therefore, the observed interaction between this *FGFR4* variant, treatment and FN should be considered hypothesis-generating.

To our knowledge, this is the first report describing toxicity data of 6 cycles of adjuvant ddAC in high risk breast cancer patients in the context of a multicenter phase III randomized trial. In the ddAC treated subgroup as well as the TAC treated subgroup, 85% of the patients received 6 full-dosed cycles of treatment. Compared with 4 cycles of ddAC as described by Jones et al[35], anemia was more frequently observed in our ddAC treated cohort (19% vs 7%, resp.) suggesting that this might be related to the two additional cycles of ddAC. Indeed, 32 out of 62 occurrences of anemia (52%) were observed in cycles 5 and 6. In addition, the prevalence of anemia after 6 cycles of AC in the Cancer and Leukemia Group B 40101 trial (6%) was also less than in our cohort[36], indicating that the combination of the dose dense schedule and two additional cycles cause an increased frequency of anemia. In line with the observations by Jones[35], we observed febrile neutropenia in 11% of the patients during six cycles of ddAC, despite the use of G-CSF. In the CALGB 40101 cohort[36], febrile neutropenia was seen in only 6% of the patients. Although these comparisons are indirect, it suggests that the dose dense schedule has a considerable effect on the incidence of FN. As observed rarely in the CALGB 40101 trial (AC, <1%)[36] and during a single institution trial evaluating FAC (10%)[37], also ddAC treated patients encountered PNP, which might be related

to cyclophosphamide. For our TAC treated subgroup, we compared our results with adverse events in patients receiving equally dosed TAC in the GeparTrio trial and the Breast Cancer International Research Group (BCIRG) trial 001[38,39]. Whereas 1.3% of the GeparTrio trial patients had grade 3–4 neuropathy and up to 47.1% had any grade of neuropathy, PNP grade 2 or higher was observed in 14.4% of our TAC treated patients. In the BCIRG 001, 3.6% of the patients treated with TAC had neurosensory effects grade 2 or higher and 25.5% had neurosensory effects of any grade. The incidence of heart failure (ddAC 0.3%, TAC 0.6%) and leukemia or myelodysplastic syndrome (ddAC 0.6%, TAC 0.6%) was low in our study.

This study has some limitations. Most GWAS and SNP association studies use germline DNA from normal tissue, often peripheral blood cells. In our cohort, normal tissue was available in only 25% of the patients, the remainder 75% was based on FFPE tumor tissue. In line with a previous report on genotype classifications in tumor tissue and normal tissue[40], concordance of 19 SNP genotypes, including the 13 selected SNPs, on 15 pairs of tumor tissue and normal tissue of our cohort was 93-100%. Likewise, concordance on 20 pairs of fresh frozen tumor tissue and FFPE tumor tissue was 94–100%. Although similarity is high, we cannot exclude that we had some misclassification of genotypes, especially for those assays that were excluded due to violation of the Hardy-Weinberg equilibrium and whose genotype distribution deviated from what was reported in the Database of Single Nucleotide Polymorphisms (dbSNP; http://www.ncbi.nlm.nih.gov/snp/). However, importantly, MAFs of the 13 SNPs were in line with those reported in dbSNP. In addition, all 13 SNPs were in Hardy-Weinberg equilibrium and as expected there was no correlation between type of tissue (normal vs tumor) used for analyses and AN, FN and PNP respectively (data not shown). These observations support the idea that the type of tissue does not seem to have a significant influence on the genotype calls of the 13 SNPs included in our analyses.

Secondly, frequencies of genetic variants, including ADME genes, are related to ethnic origin[32,41]. Therefore, many association studies take ethnicity into account. Unfortunately, we did not have data on ethnic origin. However, the study was conducted across the Netherlands, in a probably mainly Caucasian population. Moreover, since the European population has relatively low diversity in functionally important ADME genes[41], it is unlikely that ethnic background has influenced these findings to a relevant extent.

Thirdly, the sample size of our cohort is limited. The original randomized trial was powered to define a gene expression profile predictive of recurrence free survival

benefit of either of the two treatments. Because of limited power, we selected only three commonly observed toxicities to test for associations with SNPs. However, when split by treatment and subsequently by genotype subgroup, the numbers of patients who encountered any of these toxicities are low. Our data should therefore be assessed as contributing to existing evidence and hypothesis-generating.

Finally, methods used in this study may deviate from the methods of the previously reported association studies. Treatments might differ with regard to the combination of agents, the number of cycles and the schedule of administration. Besides, grades of the reported toxicity or endpoints might vary between studies. These distinct methods hamper replication of the associations for some SNPs. However, an association between a SNP and toxicity that is of potential clinical relevance should be found in a variety of studies regardless of applied methods.

The strength of our study is that we analyzed a prospective randomized dataset. However, our SNP analyses were exploratory and not prespecified in primary or secondary objectives. Since our patients were not stratified for the investigated genotypes, the distribution of these variables over the treatment arms was occasionally imbalanced (e.g. genotypes of *TECTA*). However, by replicating previously reported associations instead of identifying new ones, this study contributes to expanding evidence on these associations and provides information on what the potential role is of these SNPs in clinical practice.

This randomized study allowed us to directly compare the toxicity profile of 6 cycles of ddAC and TAC and replicate previously reported associations between toxicities and specific genotypes. The majority of these associations were not found in our cohort. This is in line with a study on radiation toxicity and SNPs in which none of the previously reported relations could be detected in a large independent dataset[42]. However, we were able to replicate some of the associations despite the relatively limited cohort size and the unplanned nature of the analyses. Also, SNP selection was limited by the time frame of the literature search, excluding more recently published, promising associations. Validation of high priority candidate SNPs in an independent cohort or a meta-analysis is desirable and will create a solid basis for biomarker driven prospective trials. These trials are needed to facilitate the entry of robust, simple and cost- effective methods to predict chemotherapy-induced toxicities into the clinic.

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SUPPLEMENTARY MATERIAL

Supplemental table 1. Number of treatment cycles, dose reductions of at least 10%, dose delays and discontinuation of therapy due to toxicity per treatment arm. A = doxorubicin; C = cyclophosphamide; T=docetaxel

	dose dense AC				TAC			
	No. of cycles	No. of dose reductions (%)	Stop due to toxicity after cycle no. (%)	Delays due to toxicity	No. of cycles	No. of dose reductions (%)	Stop due to toxicity after cycle no. (%)	Delays due to toxicity
Cycle 1	327	0	0	0	319	0	5 (1.6)	0
Cycle 2	326	1 (0.3)	0	9 (2.8)	310	9 (2.9)	4 (1.3)	7 (2.3)
Cycle 3	326	4 (1.2)	4 (1.2)	13 (4.0)	306	2 (0.7)	4 (1.3)	10 (3.3)
Cycle 4	321	4 (1.2)	4 (1.2)	12 (3.7)	302	8 (2.6)	5 (1.7)	6 (2.0)
Cycle 5	317	1 (0.3)	14 (4.4)	19 (6.0)	295	8 (2.7)	8 (2.7)	16 (5.4)
Cycle 6	297	3 (1.0)	0	22 (7.4)	285	12 (4.2)	0	5 (1.8)
Total	1914	13 (0.7)	22 (6.7)	75 (3.9)	1817	39 (2.1)	26 (8.2)	44 (2.4)

Supplemental table 2. Number of adverse events (grade 2 or higher) for each CTCAE category. For the CTCAE categories, the numbers reflect the number of patients that had at least one side effect in that CTCAE category. For the individual side effects (blanc rows), the observed toxicity is counted once per patient. * Pearson's chi square test (2-sided); † Fisher's exact test was applied

	dose dense AC n=327	**TAC n=319**	**Total n=646**	**p-value***
Allergy/Immunology	2	7	9	0.103†
Blood/Bone marrow	78	41	119	**<0.001**
Anemia	62 (18.9)	15 (4.7)	77 (11.9)	**<0.001**
Leukocytopenia	30 (9.2)	20 (6.3)	50 (7.7)	0.167
Neutropenia	9 (2.8)	8 (2.5)	17 (2.6)	0.846
Thrombopenia	7 (2.1)	3 (0.9)	10 (1.5)	0.340†
Cardiac Arrhythmia	7	3	10	0.340†
Cardiac general	0	4 (1.3)	4 (0.6)	0.059†
Constitutional symptoms	130	118	248	0.470
Fatigue	117 (35.8)	109 (34.2)	226 (35.0)	0.668
Fever (without neutropenia)	14 (4.3)	10 (3.1)	24 (3.7)	0.441
Dermatology/Skin	118	106	224	0.446
Endocrine	4	9	13	0.170†
Gastrointestinal	125	133	258	0.368
Anorexia	19 (5.8)	9 (2.8)	28 (4.3)	0.062
Constipation	16 (5.0)	25 (7.8)	41 (6.3)	0.125

Supplemental table 2. (Continued)

	dose dense AC n=327	TAC n=319	Total n=646	p-value*
Diarrhea	21 (6.4)	53 (16.6)	74 (11.5)	**<0.001**
Mucositis	15 (4.6)	11 (3.4)	26 (4.0)	0.462
Nausea	65 (20.0)	52 (16.3)	117 (18.1)	0.238
Vomiting	35 (10.7)	21 (6.6)	56 (8.7)	0.063
Hemorrhage/Bleeding	1	0	1	1.000†
Hepatobilliary/Pancreas	1	0	1	1.000†
Infection	94	94	188	0.840
Febrile neutropenia	36 (11.0)	40 (12.5)	76 (11.8)	0.546
Edema limb	1	17	18	**<0.001**
Metabolic/Laboratory	11	8	19	0.235
Musculoskeletal/Soft tissue	2	2	4	1.000†
Neurology	32	62	94	**0.001**
Peripheral neuropathy	15 (4.6)	46 (14.4)	61 (9.4)	**<0.001**
Ocular/Visual	14	11	25	0.583
Pain	42	49	91	0.358
Bone	8 (2.4)	13 (4.1)	21 (3.3)	0.243
Head	17 (5.2)	8 (2.5)	25 (3.9)	0.076
Pulmonary/Upper respiratory	48	28	76	**0.020**
Cough	19 (5.8)	7 (2.2)	26 (4.0)	**0.019**
Dyspnea	19 (5.8)	20 (6.3)	39 (6.0)	0.806
Renal/Genitourinary	3	0	3	0.249†
Sexual/Reproductive system	3	2	5	1.000†
Syndromes	3	4	7	0.722†
Vascular	21	10	31	0.051

Supplemental table 3. Toxicities of special interest.

	dose dense AC n=327	TAC n=319	Total n=646
Acute myeloid leukemia	1	1	2
Myeolodysplastic syndrome	1		1
Heart failure grade 3-4	1	2	3

Supplemental table 4. Distribution of genotypes and Hardy Weinberg Equilibrium test for selected genetic variants. * Pearson chi-square test (2-sided), missing values excluded.

		Genotype	No.	HWE*	
				χ2	p-value
GSTP1	rs1695	G	83	0.041	0.840
		AG	297		
		A	275		
		NA	4		
TECTA	rs1829	C	398	0.003	0.960
		CT	227		
		T	32		
		NA	2		
FGFR4	rs351855	C	312	<0.001	0.988
		CT	280		
		T	63		
		NA	4		
CYP3A5	rs776746	G	546	6.110	0.0134
		AG	100		
		A	11		
		NA	2		
ABCB1	rs1045642	C	133	0.028	0.867
		TC	317		
		T	194		
		NA	15		
CYP1B1	rs1056836	G	157	1.817	0.178
		GC	310		
		C	189		
		NA	3		
CYP2D6	rs1065852	C	408	27.826	**1.328E-07**
		CT	218		
		T	0		
GSTP1	rs1138272	C	537	4.897	0.027
		TC	110		
		T	12		
		NA	0		
ABCG2	rs2231142	C	522	0.074	0.785
		CA	128		
		A	7		
		NA	2		
CYP2B6	rs2279343	G	52	457.206	**1.949E-101**
		GA	586		
		A	1		
MDM2	rs2279744	G	94	1.547	0.214
		GT	287		
		T	270		
		NA	8		

Supplemental table 4. (Continued)

		Genotype	No.	HWE*	
				χ2	p-value
RWDD3	rs2296308	G	496	0.177	0.674
		GT	150		
		T	13		
		NA	0		
ABCC4	rs9561778	G	415	0.030	0.863
		GT	215		
		T	29		
		NA	0		
SLCO1B3	rs11045585	G	12	0.255	0.614
		GA	165		
		A	480		
		NA	2		
ABCC2	rs12762549	C	163	7.567	0.006
		CG	364		
		G	132		
		NA	0		

Supplemental table 5. Summary of the original association studies for anemia (A), febrile neutropenia (B) and peripheral neuropathy (C).

A

Variable	Groups	Genetic source	Method	Treatment	Endpoint	Reference	Highest risk
Age at randomization	≥ 65 years *vs* < 65 years			CEF, CAF, CMF, AC, FAC, FEC, AC-paclitaxel	Hemoglobin concentration < 100 g/L	Dranitsaris et al. Lancet Oncol. 2005	Age ≥ 65 years
Baseline hemoglobin level	Continuous			CEF, CAF, CMF, AC, FAC, FEC, AC-paclitaxel	Hemoglobin concentration < 100 g/L	Dranitsaris et al. Lancet Oncol. 2005	Low baseline hemoglobin level (no cut off)
Baseline platelet count	≤ $200x10^9$ cells/L vs > $200x10^9$ cells/L			CEF, CAF, CMF, AC, FAC, FEC, AC-paclitaxel	Hemoglobin concentration < 100 g/L	Dranitsaris et al. Lancet Oncol. 2005	Baseline platelet count ≤ 200x10(9) cells/L
FGFR4 (rs351855)	CC *vs* CT/TT	Germline DNA	Sequenom MassARRAY	3-6 cycles of FEC or FEC+docetaxel	Grade 3-4 anemia (CTC 3.0)	Vulsteke et al. Ann Oncol. 2013	CC genotype
ABCB1 (MDR1; rs1045642)	TT *vs* TC/CC	Whole blood DNA	ABI Prism 3730 genetic analyzer	docetaxel-containing chemotherapy	Any grade anemia	Choi et al. Cancer Res Treat. 2015	TT genotype
ABCC4 (rs9561778)	GG *vs* GT/TT	Whole blood DNA	PCR-RFLP	cyclophosphamide-containing chemotherapy	Grade 3-4 anemia	Islam et al. Tumour Biol. 2015	GT or TT genotypes

B

Variable	Groups	Genetic source	Method	Treatment	Endpoint	Reference	Highest risk
Age at randomization	≥ 65 years *vs* < 65 years			Multiple regimens, containing doxorubicin and/or cyclophosphamide and/or docetaxel	FN: oral temperature ≥ 38.3 °C and neutrophil count of <500 cells/ mm^2	Aapro et al. Eur J Cancer. 2006	Age ≥ 65 years
Baseline hemoglobin level	Continuous			3-6 cycles of FEC or FEC+docetaxel	FN: ANC < $0.5x10^9$/L and body temperature ≥ 38°C	Pfeil et al. BMC Cancer. 2014	Low hemoglobin level (no cut off)
	Continuous			Dose-dense AC-paclitaxel	Grade 3-4 hematologic toxicity	Zauderer et al. Breast Cancer Res Treat. 2009	Low hemoglobin level (no cut off)
Absolut neutrophil count	≤ $3.1x10^9$ cell/L *vs* > $3.1x10^9$ cell/L			6 cycles FEC	FN (undefined)	Jenkins et al. Ann Oncol. 2009	Low neutrophil count (≤ $3.1x10^9$ cell/L)
Baseline platelet count	Continuous			3-6 cycles of FEC or FEC+docetaxel	FN: ANC < $0.5x10^9$/L and body temperature ≥ 38°C	Pfeil et al. BMC Cancer. 2014	Low platelet count (no cut off)
	Continuous			6 cycles FEC	FN	Jenkins et al. Ann Oncol. 2009	Low platelet count was significant risk factor for FN in BC patients
Creatinine	Continuous			cyclophosphamide, methotrexate and fluorouracil (CMF) or anthracycline-based regimen	Grade 3-4 hematologic toxicity	Hurria et al. Drugs Aging. 2005	Increased creatinine
GSTP1 (rs1695)	AG (rs1695) and CC (rs1138272) *vs* other genotypes	Whole blood DNA	PCR-RFLP	docetaxel	FN: temperature ≥ 38.5 °C twice and neutrophil count <$1.0x10^9$/L	Tran et al. Clin Pharmacol Ther. 2006	AG genotype of rs1695 and CC genotype of rs1138272

B (Continued)

Variable	Groups	Genetic source	Method	Treatment	Endpoint	Reference	Highest risk
	AA *vs* AG/GG	FFPE tissue of normal lymph node	Sequenom MALDI-TOF mass spectrometry	6 cycles of CAF or CMF	Grade 3-4 neutropenia (<1,000/mm3	Yao et al. Clin Cancer Res. 2010	AA genotype
		Whole blood DNA	TaqMan SNP Genotyping Assays	4 or 6 cycles of FEC100 or 4 cycles of EC	FN: temperature > 38 °C and ANC < 1000/µl	Sugishita et al. Breast Cancer. 2016	AA genotype
FGFR4 (rs351855)	CC/CT *vs* TT	Germline DNA	Sequenom MassARRAY	3-6 cycles of FEC or FEC+docetaxel	FN: ANC < $0.5x10^9$/L and body temperature ≥ 38°C	Pfeil et al. BMC Cancer. 2014	TT lower risk of FN than TC or CC in BC patients
	CC/CT *vs* TT	Normal and tumor tissue	TaqMan OpenArray technology	6 cycles of TAC	FN according to NCI-CTCAE version 4.0	Charehbili et al. Pharmacogenomics. 2015	TT genotype
CYP3A5 (rs7767746)	GG vs GA/AA	Whole blood DNA	LightCycler®480 Real-Time PCR system	4 cycles of AC	Grade 4 neutropenia (neutrophil count <500/µl)	Tang et al. J Cancer Res Clin Oncol. 2013	AA and GA genotypes
ABCB1 (MDR1; rs1045642)	TT *vs* TC/CC	Whole blood DNA	ABI Prism 3730 genetic analyzer	docetaxel-containing chemotherapy	Any grade neutropenia	Choi et al. Cancer Res Treat. 2015	TT genotype
		Whole blood DNA	PCR-RFLP	docetaxel	Grade 3 neutropenia	Tran et al. Clin Pharmacol Ther. 2006	TT genotype
CYP1B1 (rs1056836)	CC *vs* CG/GG	Whole blood DNA	PCR-RFLP	4 cycles 5-FU, doxorubicin/epirubicin and cyclophosphamide followed by paclitaxel or docetaxel	Dose reduction/ delay due to neutropenia	Tulsyan et al. Gene. 2014	Haplotype with ABCB1 (rs1045642)

B (Continued)

Variable	Groups	Genetic source	Method	Treatment	Endpoint	Reference	Highest risk
ABCG2 (rs2231142)	CC *vs* CA/AA	Whole blood DNA	METPlus arrays Affimetrix	AC or FAC followed by 3-6 cycles of docetaxel with or without trastuzumab	FN	Awada et al. OMICS. 2013	CC genotype
MDM2 (rs2279744)	TT/TG *vs* GG	Genomic DNA from peripheral monocytes	TaqMan SNP Genotyping Assays	6 cycles of FEC	Severe neutropenia: neutrophil count <100/mm^3	Okishiro et al. Breast Cancer Res Treat. 2012	TT and TG genotypes
ABCC4 (rs9561778)	GG *vs* GT/TT	Genomic DNA	multiplex PCR-invader assay or direct sequencing	Cyclophosphamide-containing chemotherapy	Grade 3-4 neutropenia	Low et al. J Hum Genet. 2009	TT genotype
SLCO1B3 (rs11045585)	AA *vs* AG/GG	Genomic DNA	multiplex PCR-invader assay or direct sequencing	Docetaxel-containing chemotherapy	Grade 3-4 neutropenia	Kiyotani et al. Cancer Sci. 2008	AG and GG genotypes
ABCC2 (rs12762549)	CC vs CG/GG	Genomic DNA	multiplex PCR-invader assay or direct sequencing	docetaxel	Grade 3-4 neutropenia	Kiyotani et al. Cancer Sci. 2008	CG and GG genotypes

C

Variable	Groups	Genetic source	Method	Treatment	Endpoint	Reference	Highest risk
Age at randomization	Continuous			AC-paclitaxel, AC+bevacizumab followed by paclitaxel+ bevacizumab, AC+ bevacizumab followed by paclitaxel+ bevacizumab followed by bevacizumab	Grade 2-4 neuropathy	Schneider et al. J Clin Oncol. 2011	Higher age (no cut off)
Diabetes	Yes *vs* No			Docetaxel or paclitaxel single agent or in combination with other agents	Dose reductions	Bhatnagar et al. Springerplus. 2014	Pre-existence of diabetes
GSTP1 (rs1695)	AA vs AG/GG	Genomic DNA from peripheral lymphocytes	PCR-RFLP	Docetaxel	Grade 2-4 docetaxel-induced peripheral neuropathy	Mir et al. Ann Oncol. 2009	AA genotype
TECTA (rs1829)	CC/CT vs TT	Germline DNA	Infinium Human Omni1 array	AC + placebo or bevacizumab followed by paclitaxel and, in some cases, bevacizumab	Grade 2-4 neuropathy	Schneider et al. J Clin Oncol. 2011	TT genotype
GSTP1 (rs1138272)	CC vs CT/TT	Whole blood DNA	TaqMan SNP Genotyping Assays	3 cycles of EC followed by 3 cycles of docetaxel or 6 ccyles of cyclophosphamide/docetaxel	Grade 2-4 docetaxel-induced peripheral neuropathy	Eckhoff et al. Acta Oncol. 2015	TT and CT genotypes
RWDD3 (rs2296308)	GG/GT vs TT	Germline DNA	Infinium Human Omni1 array	AC + placebo or bevacizumab followed by paclitaxel and, in some cases, bevacizumab	Grade 2-4 neuropathy	Schneider et al. J Clin Oncol. 2011	TT genotype

Supplemental table 6. Validation of previously reported associations between anemia (A1), febrile neutropenia (B1) and peripheral neuropathy (C1) and SNPs using univariate binary logistic regression analyses. Multivariate binary logistic regression analyses (A2, C2) were made with only the significantly different factors. OR = odds ratio; CI = confidence interval

A1

			MATADOR study		
Factor	**Groups**	**Reference**	**No. of patients with toxicity (%)**	**All patients**	
				OR	**95% CI**
Age	< 65 years vs ≥ 65 years	Dranitsaris	68/616 (11.0) 9/30 (30.0)	**3.45**	**1.52-7.85**
Baseline platelet count	> 200x10^9 cells/L vs ≤ 200x10^9 cells/L	Dranitsaris	63/585 (10.8) 14/55 (25.5)	**2.83**	**1.46-5.48**
FGFR4 (rs351855)	CC *vs* CT/TT	Vulsteke	32/304 (10.5) 42/334 (12.6)	1.22	0.75-1.99
ABCB1 (rs1045642)	TT/TC *vs* CC	Choi	58/497 (11.7) 17/130 (13.1)	1.14	0.64-2.03
ABCC4 (rs9561778)	GG *vs* GT/TT	Islam	54/403 (13.4) 22/239 (9.2)	0.66	0.39-1.11

A2

			MATADOR study		
Factor	**Groups**	**Reference**	**No. of patients with toxicity (%)**	**All patients**	
				OR	**95% CI**
Age	< 65 years vs ≥ 65 years	Dranitsaris	68/616 (11.0) 9/30 (30.0)	**2.99**	**1.28-7.02**
Baseline platelet count	> 200x10^9 cells/L vs ≤ 200x10^9 cells/L	Dranitsaris	63/585 (10.8) 14/55 (25.5)	**2.48**	**1.25-4.89**

B1

Factor	Groups	Reference	MATADOR study		
			No. of patients with toxicity (%)	All patients	
				OR	95% CI
Baseline ANC ANC	>3.1 x 10^9 cells/L vs ≤ 3.1 x 10^9 cells/L	Jenkins	56/514 (10.9) 19/126 (15.1)	1.45	0.83-2.55
GSTP1 (rs1695)	other genotypes vs AG (rs1695) and CC (rs1138272)	Tran	44/429 (10.3) 30/209 (14.4)	1.47	0.89-2.41
	AA *vs* AG/GG	Sugishita Yao	27/268 (10.1) 47/370 (12.7)	1.30	0.79-2.15
FGFR4 (rs351855)	CC/CT *vs* TT	Pfeil Charehbili	66/579 (11.4) 8/59 (13.6)	1.22	0.55-2.68
CYP3A5 (rs776746)	GG vs GA/AA	Tang	57/532 (10.7) 18/108 (16.7)	1.67	0.94-2.96
ABCB1 (rs1045642)	TT *vs* TC/CC	Choi Tran	23/190 (12.1) 50/437 (11.4)	0.94	0.55-1.60
ABCB1 CYP1B1 (rs1045642* rs1056836)		Tulsyan		0.64	0.38-1.06
ABCG2 (rs2231142)	CC *vs* CA/AA	Awada	56/509 (11.0) 19/131 (14.5)	1.37	0.78-2.40
MDM2 (rs2279744)	TT/TG *vs* GG	Okishiro	64/543 (11.8) 8/91 (8.8)	0.72	0.33-1.56
ABCC4 (rs9561778)	GG *vs* GT/TT	Low	54/403 (13.4) 21/239 (8.8)	0.62	0.37-1.06
SLCO1B3 (rs11045585)	AA *vs* AG/GG	Kiyotani	55/465 (11.8) 20/175 (11.4)	0.96	0.56-1.66
ABCC2 (rs12762549)	CC/CG vs GG	Kiyotani	59/515 (11.5) 16/127 (12.6)	1.11	0.62-2.01

C1

Factor	Groups	Reference	MATADOR study		
			No. of patients with toxicity (%)	All patients	
				OR	95% CI
diabetes	no vs yes	Bhatnagar	58/643 (9.1) 2/11 (18.2)	2.21	0.47-10.46
GSTP1 (rs1695)	AA vs AG/GG	Mir	20/268 (7.5) 40/370 (10.8)	1.50	0.86-2.64
TECTA (rs1829)	CC/CT vs TT	Schneider	52/608 (8.6) 9/32 (28.1)	**4.18**	**1.84-9.51**
GSTP1 (rs1138272)	CC vs CT/TT	Eckhoff	43/525 (8.2) 18/117 (15.4)	**2.04**	**1.13-3.68**
RWDD3 (rs2296308)	GG/GT vs TT	Schneider	61/630 (9.7) 0/12 (0.0)	0.00	0.00

C2

Factor	Groups	Reference	MATADOR study		
			No. of patients with toxicity (%)	All patients	
				OR	95% CI
TECTA (rs1829)	CC/CT vs TT	Schneider	52/608 (8.6) 9/32 (28.1)	**4.51**	**1.96-10.37**
GSTP1 (rs1138272)	CC vs CT/TT	Eckhoff	43/525 (8.2) 18/117 (15.4)	**2.19**	**1.20-3.99**

Supplemental table 7. Risk of anemia (A), febrile neutropenia (B) and peripheral neuropathy (C) per treatment arm in previously reported clinical or genotype subgroups. OR = odds ratio; CI = confidence interval

A

Factor	Groups	Reference	ddAC	TAC	TAC vs ddAC	Test for interaction
			No. of patients with toxicity (%)	No. of patients with toxicity (%)	OR (95% CI)	p-value
Age	< 65 years vs ≥ 65 years	Dranitsaris	56/310 (18.1) 6/17 (35.3)	12/306 (3.9) 3/13 (23.1)	**0.19 (0.10-0.35)** 0.55 (0.11-2.81)	0.223
Baseline platelet count	> 200x10^9 cells/L vs ≤ 200x10^9 cells/L	Dranitsaris	52/294 (17.7) 10/28 (35.7)	11/291 (3.8) 4/27 (14.8)	**0.18 (0.09-0.36)** 0.31 (0.08-1.16)	0.475
FGFR4 (rs351855)	CC *vs* CT/TT	Vulsteke	25/145 (17.2) 34/178 (19.1)	7/159 (4.4) 8/156 (5.1)	**0.22 (0.09-0.53)** **0.23 (0.10-0.51)**	0.954
ABCB1 (rs1045642)	TT/TC *vs* CC	Choi	47/256 (18.4) 13/62 (21.0)	11/241 (4.6) 4/68 (5.9)	**0.21 (0.11-0.42)** **0.24 (0.07-0.77)**	0.883
ABCC4 (rs9561778)	GG *vs* GT/TT	Islam	42/203 (20.7) 19/122 (15.6)	12/200 (6.0) 3/117 (2.6)	**0.25 (0.13-0.48)** **0.14 (0.04-0.50)**	0.456

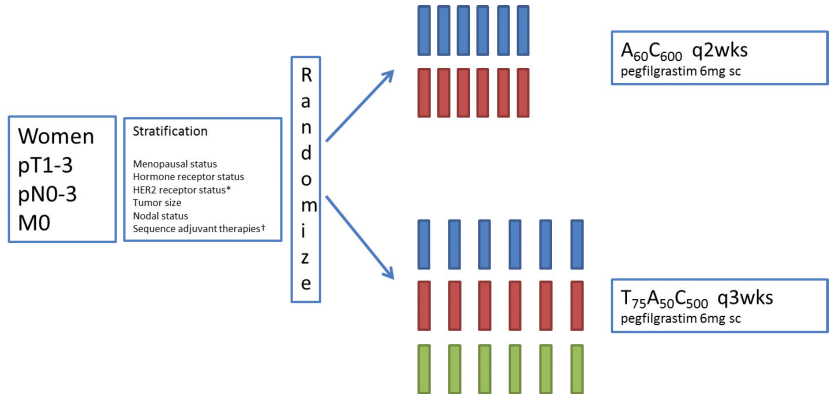
B

Factor	Groups	Reference	ddAC No. of patients with toxicity (%)	TAC No. of patients with toxicity (%)	TAC vs ddAC OR (95% CI)	Test for interaction p-value
Baseline ANC ANC	>3.1 x 10^9 cells/L vs ≤ 3.1 x 10^9 cells/L	Jenkins	24/256 (9.4) 11/66 (16.7)	32/258 (12.4) 8/60 (13.3)	1.37 (0.78-2.40) 0.77 (0.29-2.06)	0.319
GSTP1 (rs1695)	other genotypes vs AG (rs1695) and CC (rs1138272)	Tran	23/211 (10.9) 12/112 (10.7)	21/218 (9.6) 18/97 (18.6)	0.87 (0.47-1.63) 1.90 (0.86-4.17)	0.129
	AA *vs* AG/GG	Sugishita Yao	15/138 (10.9) 20/185 (10.8)	12/130 (9.2) 27/185 (14.6)	0.83 (0.38-1.86) 1.41 (0.76-2.62)	0.309
FGFR4 (rs351855)	CC/CT *vs* TT	Pfeil Charehbili	28/293 (9.6) 7/30 (23.3)	38/286 (13.3) 1/29 (3.4)	1.45 (0.86-2.43) 0.12 (0.01-1.02)	**0.027**
CYP3A5 (rs776746)	GG vs GA/AA	Tang	29/271 (10.7) 6/52 (11.5)	28/261 (10.7) 12/56 (21.4)	1.00 (0.58-1.74) 2.09 (0.72-6.06)	0.229
ABCB1 (rs1045642)	TT *vs* TC/CC	Choi Tran	10/95 (10.5) 23/223 (10.3)	13/95 (13.7) 27/214 (12.6)	1.35 (0.56-3.24) 1.26 (0.70-2.27)	0.896
ABCG2 (rs2231142)	CC *vs* CA/AA	Awada	28/264 (10.6) 7/59 (11.9)	28/245 (11.4) 12/72 (16.7)	1.09 (0.62-1.90) 1.49 (0.55-4.05)	0.594
MDM2 (rs2279744)	TT/TG *vs* GG	Okishiro	33/277 (11.9) 2/46 (4.3)	31/266 (11.7) 6/45 (13.3)	0.98 (0.58-1.64) 3.39 (0.65-17.75)	0.160
ABCC4 (rs9561778)	GG *vs* GT/TT	Low	24/203 (11.8) 11/122 (9.0)	30/200 (15.0) 10/117 (8.5)	1.32 (0.74-2.34) 0.94 (0.39-2.31)	0.540
SLCO1B3 (rs11045585)	AA *vs* AG/GG	Kiyotani	25/240 (10.4) 10/85 (11.8)	30/225 (13.3) 10/90 (11.1)	1.32 (0.75-2.33) 0.94 (0.37-2.38)	0.535
ABCC2 (rs12762549)	CC/CG vs GG	Kiyotani	26/267 (9.7) 9/58 (15.5)	33/248 (13.3) 7/69 (10.1)	1.42 (0.82-2.46) 0.62 (0.21-1.77)	0.167

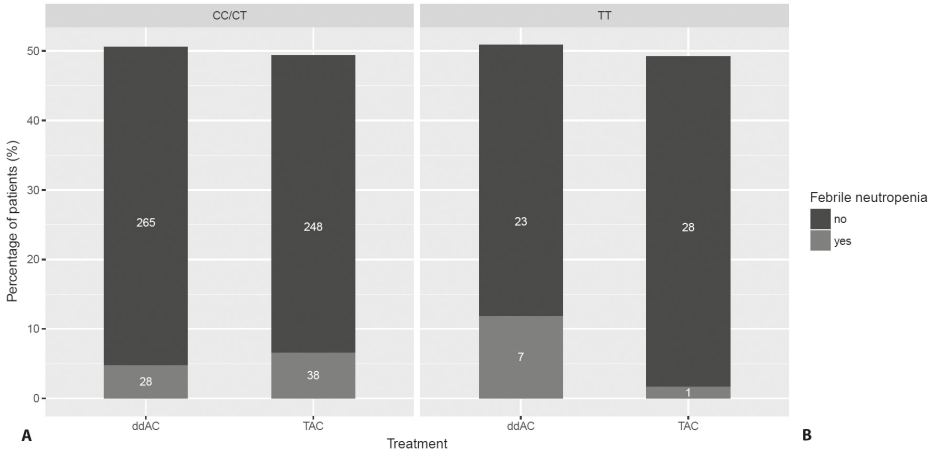
C

Factor	Groups	Reference	ddAC	TAC	TAC vs ddAC	Test for interaction
			No. of patients with toxicity (%)	No. of patients with toxicity (%)	OR (95% CI)	p-value
diabetes	no	Bhatnagar	15/323 (4.6)	43/311 (13.8)	**3.30 (1.79-6.06)**	0.999
	vs		0/4 (0)	2/7 (28.6)	-	
	yes					
GSTP1 (rs1695)	AA	Mir	4/138 (2.9)	16/130 (12.3)	**4.70 (1.53-14.46)**	0.492
	vs		11/185 (5.9)	29/185 (15.7)	**2.94 (1.42-6.08)**	
	AG/GG					
TECTA (rs1829)	CC/CT	Schneider	14/315 (4.4)	38/293 (13.0)	**3.20 (1.70-6.05)**	0.810
	vs		1/9 (11.1)	8/23 (34.8)	4.27 (0.45-40.44)	
	TT					
GSTP1 (rs1138272)	CC	Eckhoff	11/275 (4.0)	32/250 (12.8)	**3.52 (1.74-7.15)**	0.833
	vs		4/50 (8.0)	14/67 (20.9)	3.04 (0.93-9.88)	
	CT/TT					
RWDD3 (rs2296308)	GG/GT	Schneider	15/317 (4.7)	46/313 (14.7)	**3.47 (1.89-6.36)**	1.000
	vs		0/8 (0)	0/4 (0)	-	
	TT					

Supplementary Figure 1. Design of the Matador study: a multicenter, randomized phase III trial. *The Matador study included patients from 2004 to 2012; HER2 positive patients were included in the Matador study until August 2007, afterwards they were excluded due to perceived superiority of concurrent administration of trastuzumab with chemotherapy. †The sequence of adjuvant radiotherapy followed by chemotherapy or vice versa. HER2 = human epidermal growth factor receptor 2; A = doxorubicin; C = cyclophosphamide; T = docetaxel; wks=weeks; mg=milligram.



Supplementary Figure 2. Proportion of patients with febrile neutropenia per treatment arm in patients with a CC/CT genotype (A) or a TT genotype (B) for FGFR4. The numbers in the bars represent the number of patients.



5

BRCA1-like profile is not significantly associated with survival benefit of non-myeloablative intensified chemotherapy in the GAIN randomized controlled trial

A. G. J. van Rossum, P. C. Schouten, K. E. Weber, V. Nekljudova, C. Denkert, C. Solbach, C. H. Köhne, C. Thomssen, H. Forstbauer, G. Hoffmann, A. Kohls, S. Schmatloch, C. Schem, G. von Minckwitz, T. Karn, V. J. Möbus, S. C. Linn, S. Loibl, F. Marme

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ABSTRACT

Background: The *BRCA1*-like profile identifies tumors with a defect in homologous recombination due to inactivation of BRCA1. This profile has been shown to predict which stage III breast cancer patients benefit from myeloablative, DNA double-strand-break-inducing chemotherapy. We tested the predictive potential of the *BRCA1*-like profile for adjuvant non-myeloablative, intensified dose-dense chemotherapy in the GAIN trial.

Methods: Lymph node positive breast cancer patients were randomized to 3 x 3 dose-dense cycles of intensified epirubicin, paclitaxel, and cyclophosphamide (ETC) or 4 cycles concurrent epirubicin and cyclophosphamide followed by 10 cycles of weekly paclitaxel combined with 4 cycles capecitabine (EC-TX). Only triple negative breast cancer patients (TNBC) for whom tissue was available were included in these planned analyses. *BRCA1*-like or non-*BRCA1*-like copy number profiles were derived from low coverage sequencing data.

Results: 119 out of 163 TNBC patients (73%) had a *BRCA1*-like profile. After median follow-up of 83 months, disease free survival (DFS) was not significantly different between *BRCA1*-like and non-*BRCA1*-like patients [adjusted hazard ratio (adj.HR) 1.02; 95% confidence interval (CI) 0.55–1.86], neither was overall survival (OS; adj.HR 1.26; 95% CI 0.58–2.71). When split by *BRCA1*-like status, DFS and OS were not significantly different between treatments. However, EC-TX seemed to result in a trend to an improvement in DFS in patients with a *BRCA1*-like tumor, while the reverse accounted for ETC treatment in patients with a non-*BRCA1*-like tumor (p for interaction = 0.094).

Conclusions: The *BRCA1*-like profile is not associated with survival benefit for a non-myeloablative, intensified regimens in this study population. Considering the limited cohort size, capecitabine might have additional benefit for TNBC patients.

INTRODUCTION

Carriers of inactivating germline *BRCA1* (g*BRCA1*) mutations are known to have an increased incidence of breast cancer with a life time risk of 45–60%[1–3]. g*BRCA1* mutations can result in inactivation of the BRCA1 protein. In an active state, this protein plays a pivotal role in the repair of DNA double strand breaks (DSBs) via the error- free process of homologous recombination (HR). In an inactive state however, the cell will use more error-prone mechanisms of DSB repair, such as non-homologous end joining (NHEJ). This results in genetic instability, which in turn, when abundant enough, impairs cell viability[4].

Inactivation of the BRCA1 protein can originate from germline mutations as well as from somatic mutations, hypermethylation of the promotor, or from still unknown mechanisms[5]. The genetic instability that arises from an inactive BRCA1 protein leads to a characteristic copy number (CN) profile[6–8]. Breast tumors can be classified in tumors that display this characteristic CN profile (*BRCA1*-like) and tumors that do not (non-*BRCA1*-like)[9]. Identifying tumors with inactivated homologous recombination may allow targeting the defect with different classes of drugs, like bifunctional alkylators, platinum, or PARP1 inhibitors. The *BRCA1*-like classifier has shown its predictive value for benefit of high dose alkylating chemotherapy previously[10–12].

Vollebergh et al. showed that 41 patients with a *BRCA1*-like profile receiving adjuvant myeloablative, high dose, platinum-based chemotherapy with stem-cell transplantation had an eightfold lower risk of recurrence than patients who received conventional anthracycline-based chemotherapy (test for interaction p = 0.006)[10]. More- over, a disease free survival (DFS) and overall survival (OS) benefit was observed in 16 *BRCA1*-like patients when they were treated with a different myeloablative, high dose, alkylating chemotherapy regimen instead of conventionally dosed chemotherapy (hazard ratio 0.05, p = 0.003)[11]. Recently, the predictive capacity of the *BRCA1*-like profile was confirmed in 26 patients receiving tandem high dose chemotherapy with epirubicin, thiotepa, and cyclophosphamide[12]. Interestingly, all three studies have shown that *BRCA1*-like profile is associated with triple negative (TN) status. In the cohort of Vollebergh et al., up to 56% of the TN patients (34/60) had a *BRCA1*-like profile.

TN breast cancer (TNBC) has proven to be a difficult to treat subtype, partly due to its heterogeneity[13]. Taxanes, platinum compounds, alkylating agents, and several targeted agents (bevacizumab, cetuximab) have been investigated. Only taxanes provided a consistent survival benefit[14–17]. Although the value of capecitabine for TNBC patients

is still unsettled[18–20], there is evidence that capecitabine might be effective[21,22]. Clearly, predictive markers to optimize tailoring of treatment are war- ranted. Since the *BRCA1*-like profile is found in a substantial proportion of TNBC patients, this classifier might particularly be useful in this subgroup.

Although the survival benefit was striking, high dose chemotherapy treatment involved substantial toxicity. We therefore investigated the predictive value of the *BRCA1*-like classifier in patients treated with non-myeloablative intensified, dose-dense chemotherapy when compared to more conventional dose-dense chemotherapy in TNBC patients of the GAIN trial[23]. A previous study showed that the same intensified, dose-dense chemotherapy regimen improved survival compared to standard chemotherapy[24]. Our hypothesis was that *BRCA1*-like patients would derive a survival benefit when treated with the intensified chemotherapy regimen, since it contained high dose cyclophosphamide, a bifunctional alkylating agent. Since capecitabine was part of the conventional chemotherapy arm in the GAIN trial and not of the intensified chemotherapy treatment, we could also investigate what it would add in terms of efficacy.

PATIENTS AND METHODS

Patients

The German Adjuvant Intergroup Node-Positive (GAIN) study was an open label, phase III trial that was conducted between August 2004 and July 2008. Female patients biologically younger than 65 years of age with histologically confirmed invasive breast cancer, at least one positive axillary or internal mammary lymph node and no signs of distant metastases were considered eligible. Histologic complete resection (R0) of the primary tumor was required and patients needed to have an Eastern Cooperative Oncology Group (ECOG) performance score of < 2. Patient recruitment was described in detail previously[23]. The study protocol was approved by all involved ethical committees.

Treatment

The GAIN study (NCT00196872) had a 2 x 2 factorial design. First, patients were randomized between two chemotherapy regimens in a 1:1 ratio. The first arm consisted of three cycles of epirubicin 150 mg/m², three cycles of paclitaxel 225 mg/m², and three cycles of cyclophosphamide 2000 mg/m², sequentially given with a 2-week interval between cycles (ETC). The second treatment arm was four concurrent cycles

of epirubicin 112.5 mg/m² and cyclophosphamide 600 mg/m² given every 2 weeks fol- lowed by 10 weekly cycles of paclitaxel 67.5 mg/m² and capecitabine 2000 mg/m² administered on day 1–14, con- currently given in a three weekly schedule (EC-TX). During cyclophosphamide treatment, patients received prophylactic ciprofloxacine on day 5–12. Patients received growth factor support with pegfilgastrim, darbepoetin, or both for the complete duration of chemotherapy treatment. In a second randomization, patients were allocated to ibandronate (50 mg/day) for two years or observation in a 2:1 ratio.

Informed consent for study participation and biomaterial collection was obtained from all individual participants included in the study. The REMARK criteria were followed (see appendix)[25].

DNA extraction, low coverage whole genome sequencing and *BRCA1*-like classification

From 421 TNBC patients within the GAIN trial, tissue was available from 199 patients. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were revised and selected if they had a tumor cell percentage of 60% or more. Two unstained slides of 10 um thickness of tissue were prepared at the Institute of Pathology, Charité - Universitätsmedizin in Berlin and sent to the Netherlands Cancer Institute in Amsterdam. DNA was extracted using the QiaAmp DNA mini kit (Qiagen, Venlo, the Netherlands) as described elsewhere[26].

Low coverage whole genome sequencing was per- formed as described previously[27]. Input for the reactions was 20–1000 ng of DNA. Libraries were prepared according to the TruSeq protocol. Ten to fifteen cycles of enrichment PCR were required to obtain enough yield for sequencing. Ten uniquely indexed samples were pooled equimolarly and sequenced using an Illumina HiSeq2000 machine to a coverage of 0.5x. This was done in one lane of a single-end 50 bp run according to manufacturer's instructions.

Reads were aligned to the reference genome (hg19) using the BWA backtrack algorithm[28]. Reads were subsequently counted in 20 kb non-overlapping bins and corrected for GC bias with a loess fit, and for mappability, by multiplying the mappability of a bin with the loess-corrected read count of the bin[29]. The loess and mappability corrected read counts were converted to log2 read counts. Subsequently, the log2 read counts were mapped to the original BAC clone locations, which were extended to 1 MB to capture a sufficient number of reads for every BAC clone. These BAC mapped profiles were

subsequently used to classify samples as *BRCA1*-like or non-*BRCA1*-like. The *BRCA1*-like classification is a shrunken centroids classifier that assigns a probability that a new profile has similar amplifications and deletions to those found in *BRCA1*-mutated breast cancer. If a new profile shares many of these amplifications and deletions it is called *BRCA1*-like. If the profile better resembles amplifications and deletions found in cancers without *BRCA1* mutation it is called non-*BRCA1*-like. To classify a sample the algorithm uses 371 genomic locations. Samples with a probability of being *BRCA1*-like > 0.63 were called *BRCA1*-like. This threshold was obtained independently in previous work[10]. Details of the training of the classifier can be found in [6] and [10]. An R implementation of this classifier is available at http://ccb.nki.nl/software/nkibrca/. Classification of samples was done blinded to clinicopathological and outcome data.

Statistical analyses

We analyzed whether patients selected for these analyses have different characteristics compared to all TNBC patients. Relative total dose intensity (RTDI) is calculated as the ratio between the administered dose and the planned dose of the allocated treatment. Time to treatment (TTT) is the interval in days between surgery and the first cycle of the allocated chemotherapy. The categorical variables were compared using a Fisher's exact test or a Chi-square test; the continuous variables were compared using a Wilcoxon test.

Disease free survival (DFS) was defined as locoregional recurrence, distant recurrence, or death by any cause. Overall survival (OS) was defined as death by any cause. The Kaplan–Meier method was used to estimate survival in the *BRCA1*-like and the non-*BRCA1*-like subgroups. Survival was compared with log rank tests.

To ensure the robustness of multivariate Cox proportional hazards models we first tested all independent covariables in univariate models with respect to the end- point and subgroup. Only covariables with a Wald p value < 0.2 in their univariate model were included into the multivariate model. From these multivariate models adjusted hazard rates were derived. The predictive value of the *BRCA1*-like profile was evaluated by performing tests for interaction also based on Cox proportional hazards models.

All p values are two-sided, p values below 0.05 are considered significant. Confidence intervals (CI) are symmetric 95% confidence intervals. No corrections were made for multiple testing. All analyses were performed according to the statistical analysis plan using SAS Enterprise Guide V4.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

DNA extraction and library preparation was performed for 197 patients. A total of 34 samples were excluded: the quality of isolated DNA was insufficient, a library could not be constructed or data quality criteria were not met (Figure 1). The clinicopathologic characteristics of patients who were included in the analyses were not significantly different from those of the other TNBC patients of the GAIN cohort (Table S1).

BRCA1-like profile was found in 119/163 patients (73%). *BRCA1*-like tumors had a higher Bloom-Richardson grade than non-*BRCA1*-like tumors ($p < 0.001$). No other correlations with clinicopathologic characteristics were observed (Table 1).

Figure 1. Selection of TNBC patients for *BRCA1*-like analyses. TNBC=triple negative breast cancer

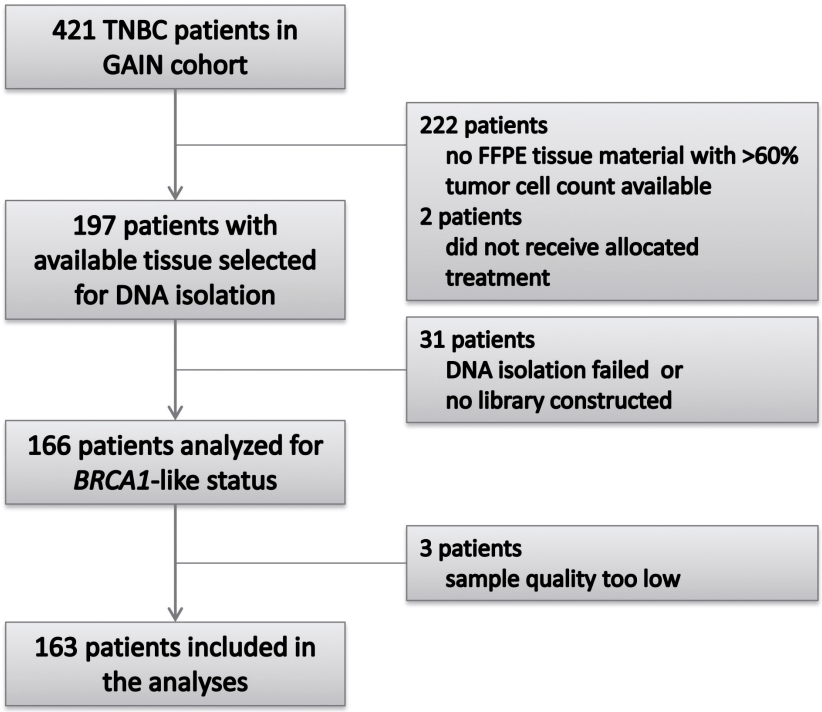


Table 1. Patient characteristics. Patient characteristics of all triple negative breast cancer patients in the current study, split in patients classified as *BRCA1*-like and non-*BRCA1*-like. *Fishers exact test for binary variables and Chi-square test for other variables (2-sided); TNBC = triple negative breast cancer; E = epirubicin; T = paclitaxel; C = cyclophosphamide; X = capecitabine; relative total dose intensity is the ratio between the administered dose and the planned dose of the allocated treatment. Time to treatment is the interval in days between surgery and the first cycle of the allocated chemotherapy.

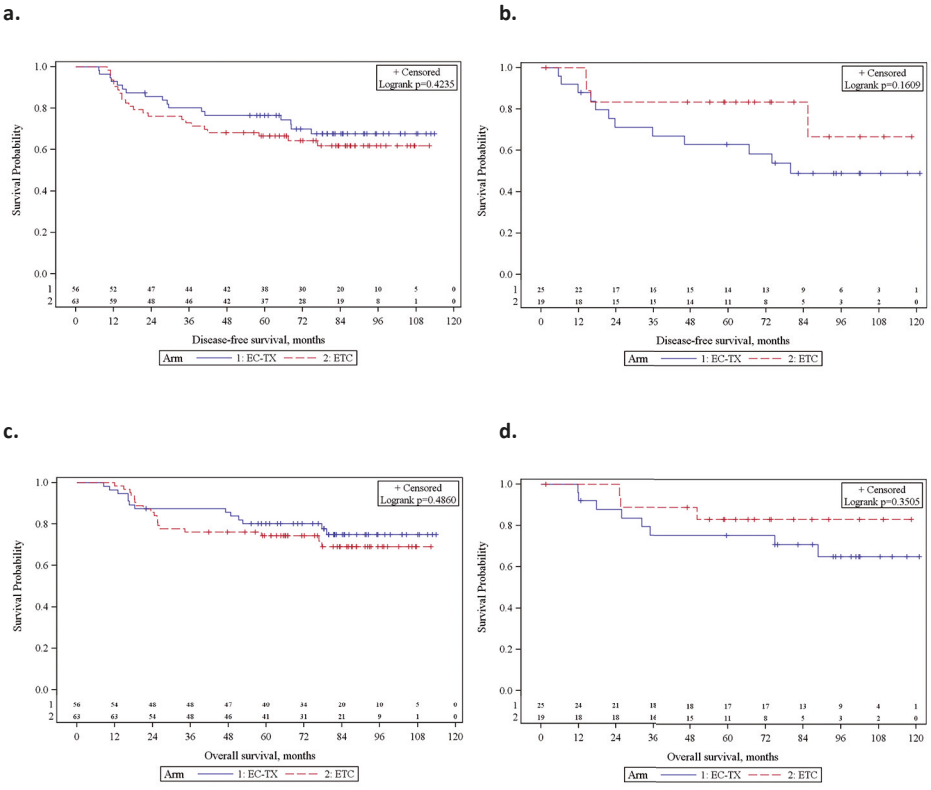
Parameter	Category	*BRCA1*-like patients (n=119)	Non-*BRCA1*-like patients (n=44)	All patients (n=163)	p-value*
Menopausal status (%)	pre- or perimenopausal	70 (58.8)	21 (48.8)	91 (56.2)	.285
	postmenopausal	49 (41.2)	22 (51.2)	71 (43.8)	
	missing	0	1	1	
Body mass index (%)	normal weight	51 (42.9)	22 (50.0)	73 (44.8)	.300
	underweight	0 (0.0)	1 (2.3)	1 (0.6)	
	overweight	38 (31.9)	11 (25.0)	49 (30.1)	
	obesity	30 (25.2)	10 (22.7)	40 (24.5)	
Surgery (%)	breast conserving surgery	80 (67.2)	26 (59.1)	106 (65.0)	.359
	mastectomy	39 (32.8)	18 (40.9)	57 (35.0)	
Tumor size (%)	pT1	30 (25.2)	13 (29.5)	43 (26.4)	.720
	pT2	76 (63.9)	24 (54.5)	100 (61.3)	
	pT3	11 (9.2)	6 (13.6)	17 (10.4)	
	pT4	2 (1.7)	1 (2.3)	3 (1.8)	
Nodal status (%)	pN0	0 (0.0)	0 (0.0)	0 (0.0)	.908
	pN1	53 (44.5)	18 (40.9)	71 (43.6)	
	pN2	37 (31.1)	15 (34.1)	52 (31.9)	
	pN3	29 (24.4)	11 (25.0)	40 (24.5)	
Histological type (%)	ductal invasive	103 (86.6)	35 (79.5)	138 (84.7)	.083
	lobular invasive	2 (1.7)	4 (9.1)	6 (3.7)	
	other	14 (11.8)	5 (11.4)	19 (11.7)	
Bloom Richardson grade (%)	I	0 (0.0)	0 (0.0)	0 (0.0)	<.001
	II	11 (9.3)	15 (34.1)	26 (16.0)	
	III	107 (90.7)	29 (65.9)	136 (84.0)	
	missing	1	0	1	
Treatment arm (%)	ETC	63 (52.9)	19 (43.2)	82 (50.3)	.294
	EC-TX	56 (47.1)	25 (56.8)	81 (49.7)	
Ibandronate (%)	no	43 (36.1)	15 (34.1)	58 (35.6)	.856
	yes	76 (63.9)	29 (65.9)	105 (64.4)	

Table 1. (Continued)

Parameter	Category	*BRCA1*-like patients (n=119)	Non-*BRCA1*-like patients (n=44)	All patients (n=163)	p-value*
Relative total dose intensity (%)	< 80%	8 (8.7)	5 (11.1)	13 (9.5)	0.745
	80-90%	11 (12.0)	8 (17.8)	19 (13.9)	
	90-100%	51 (55.4)	23 (51.1)	74 (54.0)	
	≥ 100%	22 (23.9)	9 (20.0)	31 (22.6)	
	missing	20	9	29	
Time to treatment (%)	≤ 21 days	23 (20.5)	8 (15.1)	31 (18.8)	0.307
	22-28 days	32 (28.6)	23 (43.4)	55 (33.3)	
	29-35 days	28 (25.0)	11 (20.8)	39 (23.6)	
	> 35 days	29 (25.9)	11 (20.8)	40 (24.2)	
	missing	1	0	1	

The median follow-up time of all included patients was 83.5 months. At the time of the analyses, 56 patients had a locoregional recurrence, distant recurrence, or died. In the total cohort, DFS was not significantly different between *BRCA1*-like patients and non-*BRCA1*-like patients [adjusted hazard ratio (adj. HR) 1.02; 95% confidence interval (CI) 0.55–1.86]. Similarly, there was no difference in OS (adj. HR 1.26; 95% CI 0.58–2.71). When split by *BRCA1*-like status (Figure 2a, b), DFS was not significantly different in *BRCA1*-like patients when they were treated with EC- TX or ETC (unadj. HR 0.78; 95% CI 0.41–1.45). Neither was DFS in non-*BRCA1*-like patients (unadj. HR 2.20; 95% CI 0.71-6.86). However, a trend for interaction between *BRCA1*-like status and treatment was observed (unadj. p = 0.094; Figure 3). Also in the multivariate model, EC-TX treatment seemed to result in a trend to an improvement in DFS in *BRCA1*-like patients (adj. HR 0.61; 95% CI 0.32–1.19, p = 0.147; data not shown), while ETC treatment showed an improvement for non-*BRCA1*-like patients (adj. HR 4.14; 95% CI 1.10–15.58, p = 0.036; data not shown). The same trends were observed for overall survival (unadj. HR 0.78; 95% CI 0.38–1.59 for *BRCA1*- like patients; unadj. HR 1.87; 95% CI 0.49–7.14 for non-*BRCA1*-like patients; Figure 2c, d).

Figure 2. Survival of *BRCA1*-like patients and non-*BRCA1*-like patients. Disease free survival in *BRCA1*-like patients (**a**) and non-*BRCA1*-like patients (**b**) when treated with ETC (red line) or EC-TX (blue line). Overall survival in *BRCA1*-like patients (**c**) and non-*BRCA1*-like patients (**d**) when treated with ETC (red line) or EC-TX (blue line). E=epirubicin; T=paclitaxel; C=cyclophosphamide; X=capecitabine



In a multivariate model, RTDI and TTT were significantly associated with DFS and lymph node status with DFS and OS (Tables 2, 3). When splitting the *BRCA1*-like subgroup according to lymph node (LN) status (Figure S2), patients with 10 or more positive LNs have a better DFS when they are treated with EC-TX compared to ETC (unadj. HR 0.33; 95% CI 0.11–0.94). However, OS was not significantly different between the treatment arms in these patients (unadj. HR 0.45; 95% CI 0.15–1.34). In non-*BRCA1*-like patients, neither DFS nor OS was significantly different between treatments in patients with 10 or more positive LNs (DFS: unadj. HR 0.86, 95% CI 0.10–7.52; OS: unadj. HR 0.93, 95% CI 0.11–8.09). However, sub- groups in non-*BRCA1*-like patients were very small.

Table 2. Multivariate cox model for disease free survival (DFS). Only covariates that had a univariate Wald p-value < 0.2 were included in this model.

Variable		Hazard ratio	Confidence interval		p-value
			Lower	Upper	
Surgery	mastectomy	1.39	0.63	3.09	0.421
	vs				
	breast conserving surgery				
Tumor size	pT3-4	2.48	0.95	6.43	0.063
	vs				
	pT1-2				
Nodal status	pN3	2.06	0.91	4.66	0.049
	vs				
	pN2	0.69	0.30	1.58	
	vs				
	pN1				
Treatment	ETC	1.11	0.56	2.21	0.770
	vs				
	EC-TX				
***BRCA1*-like status**	yes	0.92	0.45	1.87	0.813
	vs				
	no				
Relative total dose intensity (%)	≥ 100%	0.45	0.16	1.25	0.027
	vs				
	90-100%	0.30	0.12	0.74	
	vs				
	80-90%	0.17	0.05	0.63	
	vs				
	< 80%				
Time to treatment (%)	> 35 days	1.86	0.64	5.41	0.004
	vs				
	29-35 days	5.36	1.88	15.24	
	vs				
	22-28 days	1.30	0.47	3.60	
	vs				
	≤ 21 days				

Table 3. Multivariate cox model for overall survival (OS). Only covariates that had a univariate Wald p-value < 0.2 were included in this model.

Variable		Hazard ratio	Confidence interval		p-value
			Upper	Lower	
Surgery	mastectomy	1.61	0.78	3.31	0.200
	vs				
	breast conserving surgery				
Tumor size	pT3-4	1.85	0.79	4.36	0.157
	vs				
	pT1-2				
Nodal status	pN3	3.03	1.35	6.79	0.007
	vs				
	pN2	1.11	0.46	2.67	
	vs				
	pN1				
Histological type	non-lobular	0.90	0.24	3.42	0.883
	vs				
	lobular				
Treatment	ETC	1.48	0.77	2.85	0.246
	vs				
	EC-TX				
***BRCA1*-like status**	yes	1.26	0.58	2.71	0.559
	vs				
	no				

DISCUSSION

In this study, we investigated the predictive value of the *BRCA1*-like profile in non-myeloablative intensified, dose- dense chemotherapy and more conventional dose-dense chemotherapy with the addition of capecitabine. In a subset of 163 TNBC patients from the GAIN trial cohort, the *BRCA1*-like profile was not associated with treatment benefit of ETC or EC-TX.

Although both treatments were given in a dose-dense schedule, the differences between the treatments were sequential versus combination chemotherapy, the intensified doses of the ETC agents, and the addition of capecitabine in the EC-TX arm. While the cumulative dose of epirubicin and paclitaxel was the same for both regimens, the dose of the alkylating agent cyclophosphamide was 2.5 times higher in the ETC arm (6000 vs. 2400 mg/m²). Previous research has shown that *BRCA1*-mutated tumors and tumors with molecular features of *BRCA1*-mutated tumors—called *BRCA*ness—are sensitive to drugs that form interstrand DNA cross links or drugs that stall the replication fork[4]. Cyclophosphamide is an alkylating agent with the ability to generate DNA cross links. Also, there is evidence of an association between dose intensity and treatment effect[30]. Therefore, we hypothesized that the intensified regimen would improve survival in *BRCA1*-like patients when compared to treatment with a more conventional schedule. We could not confirm the hypothesis in this trial. Moreover, the *BRCA1*-like subgroup seemed to benefit from treatment with EC-TX, whereas this trend was observed for ETC treatment in non-*BRCA1*-like patients (p for interaction = 0.094). There are three possible explanations. First, sequential treatment might provide a window of opportunity for the tumor to regrow. While a standard dose of epirubicin induces DNA damage only to a certain extent, *BRCA1*-like tumors might not benefit from the subsequent taxane treatment due to their relative resistance[31]. The three cycles of cyclophosphamide might be insufficient to effectively treat the disease. Secondly, the dose-increase of cyclophosphamide to more than standard might not result in greater efficacy. Two previously conducted clinical trials showed that an intensification and dose-escalation of cyclophosphamide when combined with doxorubicin did not result in improved disease free survival or overall survival, while toxicity did increase with dose[32,33]. However, subgroup analyses were limited in these studies and it might be that a selected group of breast cancer patients would derive benefit from intensified and dose- increased cyclophosphamide. Thirdly, the addition of capecitabine to a combination regimen might have a greater effect than expected, especially in a subgroup of patients. In the recent 10 year survival update of the FinXX trial, Joensuu et al. showed that adding capecitabine to a taxane-anthracycline-based

chemotherapy regimen improved recurrence free survival and breast-cancer specific survival compared to a capecitabine-free treatment regimen in TNBC patients[34]. Also, O'Shaughnessy et al. concluded that capecitabine results in a better DFS and OS in TNBC patients with a low Ki67 score (≤ 65%)[35]. From our study, it seems that TNBC patients with deficient HR, i.e., *BRCA1*-like patients, also might have a better survival when treated with a capecitabine-containing regimens. In an exploratory analysis, DFS of *BRCA1*-like patients with 10 or more positive lymph nodes treated with EC-TX was even significantly better than patients with the same characteristics treated with ETC.

Being an oral prodrug of 5-fluorouracil (5-FU), capecitabine is metabolized via three enzymes into 5-FU of which the last step is done by thymidine phosphorylase (TP). Intracellularly, 5-FU is converted into its active metabolites 5-fluoro-deoxyuridine monophosphate (fdUMP) and 5-fluorouridine triphosphate (fdUTP). These metabolites hamper RNA synthesis and interfere with the function of thymidylate synthase (TS). Forming a complex with fdUMP, TS is unable to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This causes imbalances in the deoxynucleotide (dNTP) pool, leading to DNA damage[36]. If a tumor cell is incapable of repairing DNA damage in an error-free manner, this will result in abundancy of DNA lesions, which affects cell viability. Therefore, it seems valid that adding capecitabine will improve survival in *BRCA1*-like patients, although the exact mechanism remains elusive at present. Also, preclinical and clinical studies show that taxanes and capecitabine have a synergistic effect[37]. Tumor cells have a higher concentration of TP than normal cells. Moreover, taxanes cause an additional raise in TP levels in tumor cells, resulting in enhanced conversion of capecitabine into 5-FU and its subsequent active metabolites. This could clarify the seemingly enhanced efficacy of EC-TX in *BRCA1*-like patients, but not the moderate efficacy of this regimen in non-*BRCA1*-like patients. However, it is remarkable considering that tumors that harbor a *BRCA1* mutation or a *BRCA*ness signature are thought to be relatively resistant to taxanes or taxane-based combination regimens without capecitabine[31,38,39].

We investigated the predictive potential of the *BRCA1*-like classifier in a representative subset of TNBC patients of a randomized trial. The method that we used to classify patients as *BRCA1*-like or non-*BRCA1*-like is robust, as shown previously[27], and the investigators who performed the classification of samples were blinded for clinical outcome. However, the sample size of this predefined analysis is small. This might explain why we did not observe a significant treatment effect, despite the fact that the hazard rates for treatment in *BRCA1*-like patients and non-*BRCA1*-like patients are in opposite directions (HR 0.78 and HR 2.20 for DFS, resp.). Also, the univariate analysis

showed a trend for interaction (p = 0.094). When the cohort is further divided by LN status, numbers of patients are very low, especially in the non-*BRCA1*-like groups. The preferred design to confirm the predictive potential of a biomarker would be a prospective, randomized trial. Currently, these trials are ongoing (NCT01898117; NCT01057069; NCT01646034). Alternatively, a matched case–control set up could be used[40].

In conclusion, we found no significant difference between treatment with non-myeloablative intensified, dose-dense ETC, or dose-dense EC-TX using the *BRCA1*-like classifier as predictive marker. However, the investigated cohort was small. Despite these low numbers, our results indicate that adding capecitabine to dose-dense chemotherapy might improve survival in *BRCA1*-like patients. Further research is warranted.

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SUPPLEMENTARY MATERIAL

Table S1. Patient characteristics of all triple negative breast cancer patients in the GAIN cohort. Patient characteristics of all 421 triple negative breast cancer patients in the GAIN cohort, split in patients who were selected for *BRCA1*-like analyses and the remaining patients.* Fishers exact test for binary variables and Chi-square test for other variables (2-sided) TNBC = triple negative breast cancer; E = epirubicin; T = paclitaxel; C = cyclophosphamide; X = capecitabine

Parameter	Category	Patients selected for *BRCA1*-like analyses (n=163)	Patients not selected (n=258)	All TNBC patients in GAIN cohort (n=421)	p-value*
menopausal status (%)	pre- or perimenopausal	91 (56.2)	153 (59.3)	244 (58.1)	.543
	postmenopausal	71 (43.8)	105 (40.7)	176 (41.9)	
	missing	1	0	1	
body mass index (%)	normal weight	73 (44.8)	133 (51.6)	206 (48.9)	.421
	underweight	1 (0.6)	3 (1.2)	4 (1.0)	
	overweight	49 (30.1)	73 (28.3)	122 (29.0)	
	obesity	40 (24.5)	49 (19.0)	89 (21.1)	
surgery (%)	breast conserving surgery	106 (65.0)	161 (62.4)	267 (63.4)	.605
	mastectomy	57 (35.0)	97 (37.6)	154 (36.6)	
tumor size (%)	pT1	43 (26.4)	78 (30.2)	121 (28.7)	.805
	pT2	100 (61.3)	151 (58.5)	251 (59.6)	
	pT3	17 (10.4)	26 (10.1)	43 (10.2)	
	pT4	3 (1.8)	3 (1.2)	6 (1.4)	
nodal status (%)	pN0	0 (0.0)	0 (0.0)	0 (0.0)	.889
	pN1	71 (43.6)	110 (42.6)	181 (43.0)	
	pN2	52 (31.9)	88 (34.1)	140 (33.3)	
	pN3	40 (24.5)	60 (23.3)	100 (23.8)	
histological type (%)	ductal	138 (84.7)	214 (82.9)	352 (83.6)	.713
	lobular	6 (3.7)	14 (5.4)	20 (4.8)	
	other	19 (11.7)	30 (11.6)	49 (11.6)	
Bloom Richardson grade (%)	I	0 (0.0)	2 (0.8)	2 (0.5)	.529
	II	26 (16.0)	40 (15.5)	66 (15.7)	
	III	136 (84.0)	216 (83.7)	352 (83.8)	
	missing	1	0	1	
chemotherapy arm (%)	ETC	82 (50.3)	126 (48.8)	208 (49.4)	.841
	EC-TX	81 (49.7)	132 (51.2)	213 (50.6)	
Ibandronate (%)	yes	105 (64.4)	177 (68.6)	282 (67.0)	.396
	no	58 (35.6)	81 (31.4)	139 (33.0)	

Figure S1. Forest plot of hazard ratios (HR) for overall survival by patient subgroup. Whereas the HR of *BRCA1*-like patients is in favor of EC-TX, ETC seems better in non-*BRCA1*-like patients (not significant). Grade is according to the Bloom-Richardson grading system; BCS = breast conserving surgery; BMI = Body Mass Index; RTDI = relative total dose intensity; TTT = time to treatment

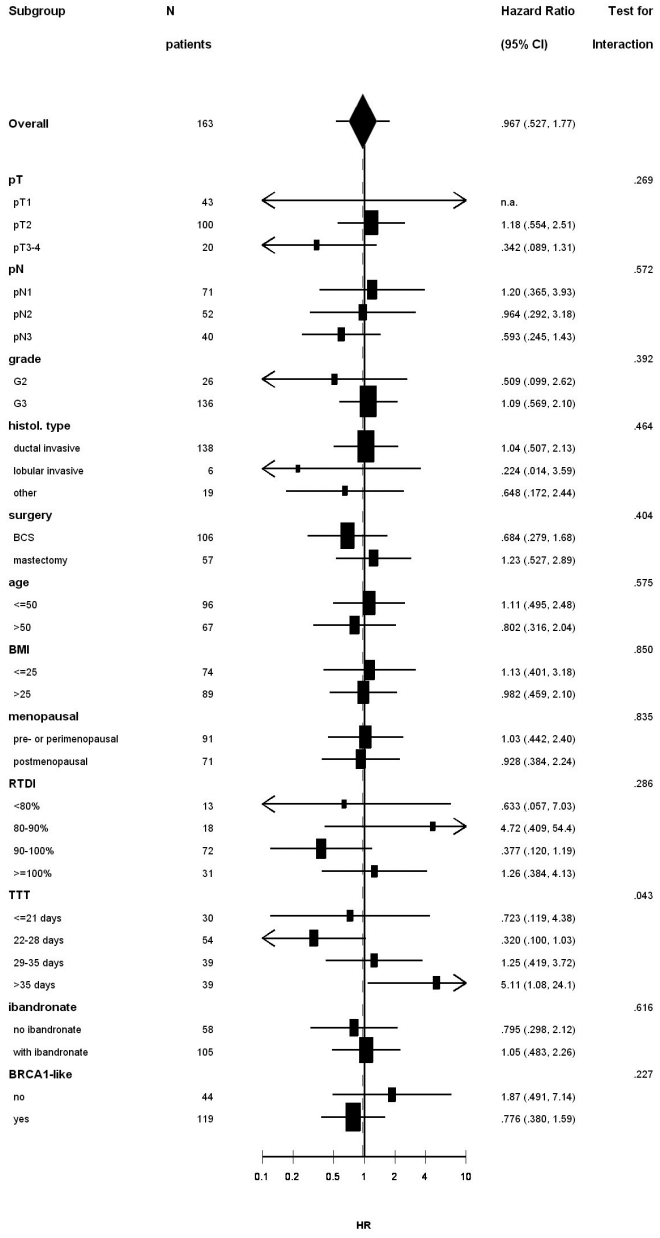
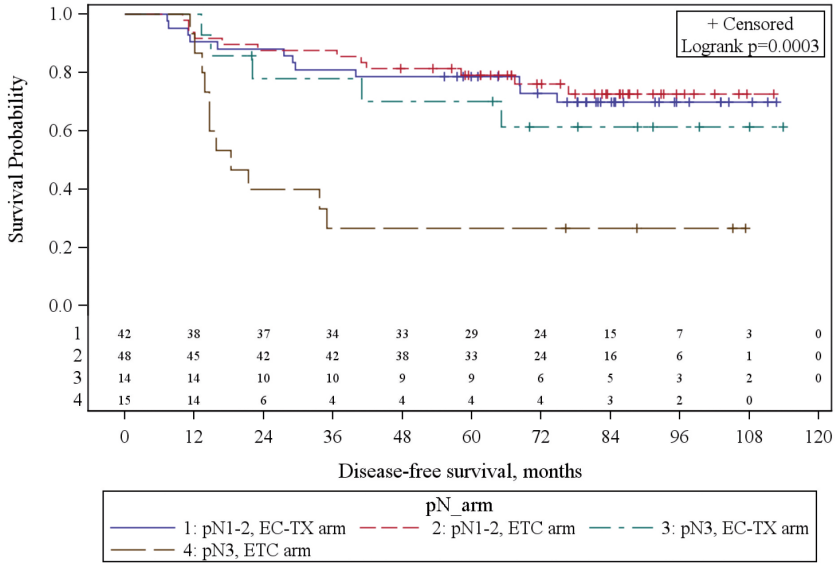


Figure S2. Survival in *BRCA1*-like patients when split into treatment and nodal status. Disease free survival (a) and overall survival (b) in *BRCA1*-like patients when split into treatment and nodal status. E=epirubicin; T=paclitaxel; C=cyclophosphamide; X=capecitabine

a



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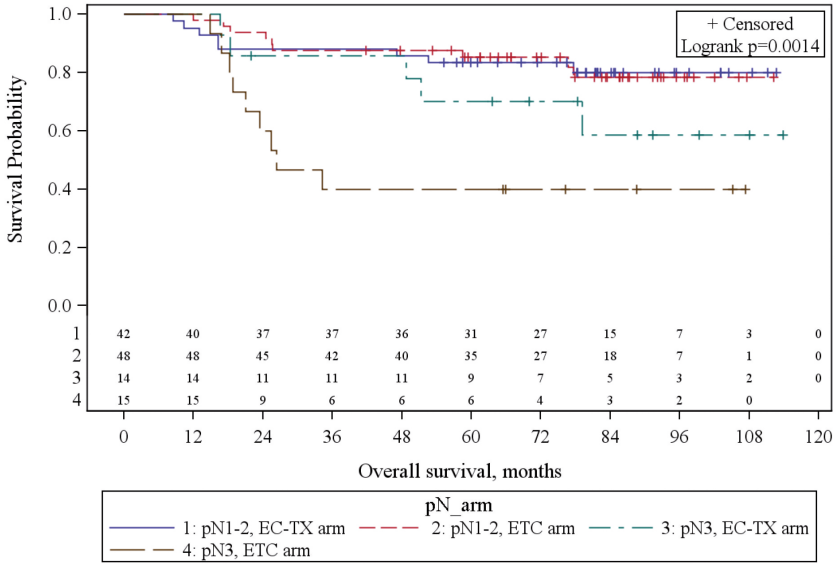
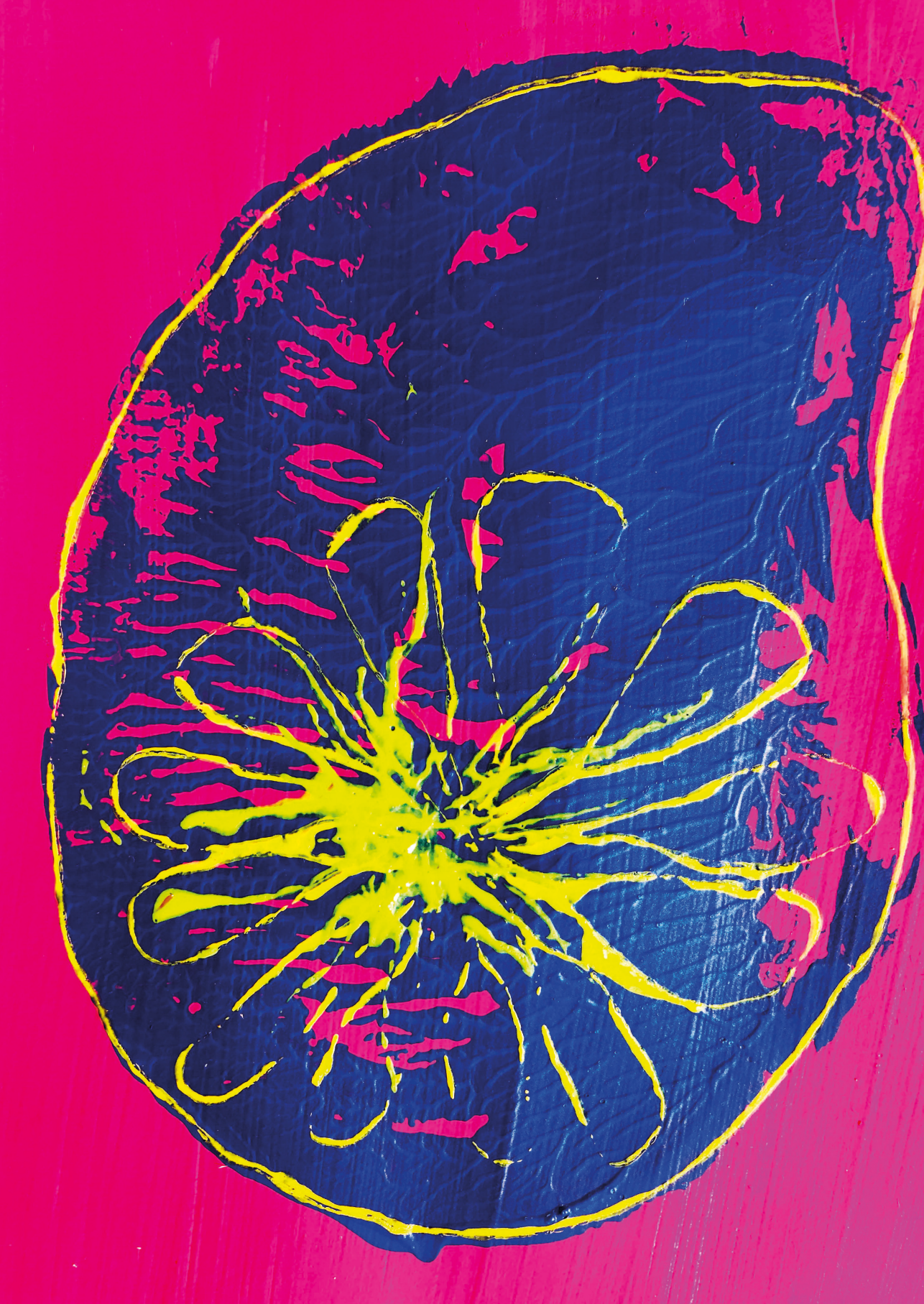




6

POSEIDON trial phase 1b results: safety, efficacy and circulating tumour DNA response of the beta isoform-sparing PI3K inhibitor taselisib (GDC-0032) combined with tamoxifen in hormone receptor positive metastatic breast cancer patients

R.D. Baird*, A.G.J. van Rossum*, M. Oliveira*, K. Beelen, M. Gao, M. Schrier, I.A.M. Mandjes, J. Garcia-Corbacho, A-L. Vallier, G. Dougall, E. van Werkhoven, C. Linossi, S. Kumar, H. van Tinteren, M. Callari, E. Beddowes, J. Perez-Garcia, H. Rosing, E. Platte, P. Nederlof, M. Schot, A. de Vries Schultink, R. Bernards, C. Saura, W. Gallagher, J. Cortes†, C. Caldas†, S.C, Linn†

*these authors contributed equally; †these authors contributed equally

Submitted.

ABSTRACT

Background: The strategy of combining endocrine therapy with PI3K-mTOR inhibition is showing promise in oestrogen-receptor (ER)-positive breast cancer, but new agents and combinations with a better therapeutic index are urgently needed. Taselisib is a potent, selective, beta-isoform sparing PI3 kinase inhibitor.

Methods: 30 patients with ER-positive, metastatic breast cancer who had failed prior endocrine therapy were treated with escalating doses of taselisib (2 or 4 mg in an intermittent or continuous schedule) combined with tamoxifen 20mg once daily in this phase 1b study using a 'rolling six' design.

Results: Taselisib combined with tamoxifen was generally well tolerated, with treatment-emergent adverse events as expected for this class of drugs, including diarrhoea (13 patients, 43%), mucositis (10 patients, 33%) and hyperglycaemia (8 patients, 27%). No dose-limiting toxicities were observed. Objective responses were seen in 6 out of 25 patients with RECIST-measurable disease (ORR 24%). Median time to disease progression was 3.7 months. 12 out of 30 patients (40%) had disease control for 6 months or more. Circulating tumour (ct)DNA studies using next-generation tagged amplicon sequencing identified early indications of treatment response and mechanistically-relevant correlates of clinical drug resistance (eg. mutations in *KRAS*, *ERBB2*) in some patients.

Conclusions: Taselisib can be safely combined with tamoxifen at the recommended phase 2 dose of 4mg given once daily on a continuous schedule. Preliminary evidence of anti-tumour activity was seen in both *PIK3CA* mutant and wild-type cancers. The randomized phase 2 part of POSEIDON (testing tamoxifen plus taselisib or placebo) is currently recruiting.

INTRODUCTION

The strategy of combining endocrine therapy with inhibitors of the phosphatidylinositol 3–kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway has shown promise in ER-positive breast cancer[1,2], where there is a high prevalence of pathway alterations. However the modest improvement in treatment efficacy when adding these agents has frequently been offset by significant increased toxicity[3].

Taselisib (GDC-0032) is an oral, potent, isoform-selective inhibitor of PI3K alpha, delta and gamma isoforms, with 30-fold less inhibition of PI3K beta relative to alpha (Ki = 0.29nM)[4]. In taselisib early clinical development, anti-tumour activity was observed in patients with ER-positive breast cancer, with proportionately more responses in *PIK3CA*-mutant compared with *PIK3CA* wild-type tumours, consistent with preclinical data[5]. This was true both for taselisib as a single agent, and also for taselisib in combination with other anti-oestrogens fulvestrant and letrozole[6,7].

Tamoxifen is well established endocrine therapy frequently used for the treatment of ER-positive breast cancer, increasingly in patients who have failed prior endocrine therapies including aromatase inhibitors and/or fulvestrant. To overcome endocrine resistance, CDK4/6 inhibitors have shown to be of added value[8], however not all patients derive benefit from a combination with CDK4/6 inhibitors. Inhibition of the PI3K pathway in combination with tamoxifen may be beneficial for a significant proportion of ER-positive patients.

We undertook a phase 1b trial to establish the safety, tolerability, and recommended phase 2 dose (RP2D) of taselisib in combination with tamoxifen, for patients with hormone receptor (HR)-positive metastatic breast cancer with progression after prior endocrine therapy. Secondary and exploratory objectives included assessment of pharmacokinetics (PK) and (preliminary) anti-tumour efficacy. Correlative translational studies were performed to identify biomarkers with potential clinical utility, including intensive plasma sampling for circulating tumour (ct)DNA analysis using next generation tagged amplicon sequencing. ctDNA monitoring in early phase clinical trials may have value in drug development[9] for the assessment of biomarkers which can: predict response to therapy[10]; provide an early indication of treatment response[11]; and shed light on potential mechanisms of acquired drug resistance[12].

PATIENTS AND METHODS

Patients

This phase 1b, multi-centre, dose-escalation study was conducted in Amsterdam, Barcelona and Cambridge, UK. The study was conducted in accordance with Good Clinical Practice and was approved by regulatory and ethics committees at each site. All patients had HR-positive breast cancer and provided written informed consent before taking part. Other key inclusion criteria: measurable or non-measurable disease according to Response Evaluation Criteria In Solid Tumours (RECIST) version 1.1; age[3] ≥ 18 years; life expectancy[3] ≥ 12 weeks; fasting glucose ≤ 120 mg/dL and HbA1c below the upper limit of normal (ULN). Key exclusion criteria: more than 5 prior chemotherapeutic regimens for metastatic breast cancer; presence of untreated, symptomatic or progressive brain metastases; diabetes mellitus requiring anti-hyperglycaemic medication; history of thrombo-embolic or inflammatory bowel disease.

Study Design and Drug Administration

The phase 1b part of the POSEIDON trial reported here used a rolling 6 design to test 3 doses/schedules of taselisib tablets in combination with 20 mg tamoxifen daily (QD). Cohort 1 tested tamoxifen plus 2mg taselisib QD in a 21 day on / 7 day off intermittent schedule; Cohort 2 tested tamoxifen plus 4mg taselisib QD in a 21 day on / 7 day off intermittent schedule; and Cohort 3 tested tamoxifen plus 4mg taselisib QD in a 28 day continuous schedule. Planned cohort expansions were undertaken in cohorts 2 and 3 to gain additional preliminary data regarding safety, tolerability and efficacy. On cycle 1 day 1, only taselisib was administered for single agent PK studies. Tamoxifen was administered in combination with taselisib from cycle 1 day 2 onwards.

Safety & Dose Intensity

Data on Adverse Events (AEs) was collected according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. All AEs were collected regardless of causality until 30-days after the last study drug administration. Dose-Limiting Toxicities (DLTs) were those treatment-emergent AEs occurring during cycle 1 (days 1-28) which warranted a dose-reduction or which were ≥ grade 3 with exceptions listed in Supplementary Methods [SM]. Relative dose intensity of both taselisib and tamoxifen was defined as the actual received dose intensity divided by the intended dose intensity.

Plasma Pharmacokinetic and Circulating Tumour (ct)DNA Studies

Details of plasma taselisib[13] and tamoxifen[14] pharmacokinetic assays, and ctDNA assays[11,12,15] are provided in [SM].

Tumour Response

Tumour response to treatment was evaluated clinically and also by CT scan assessments every 8 weeks (2 cycles of treatment), with confirmation of objective responses performed ≥ 4 weeks later. Time to progression (TTP) was calculated from start of treatment until progressive disease. All patients had progressed at the time of analysis and therefore no censoring was necessary.

RESULTS

Baseline Patient Demographics and Disease Characteristics

From November 2014 to January 2016, 30 patients were enrolled. The cut-off for data analysis was 8 February 2018. Median treatment duration was 4 months (range 1-17). Patients had a median of 2 lines of prior endocrine therapy (range 0-3) and 2 lines of prior cytotoxic chemotherapy (range 0-7) for metastatic disease. Overall 25 out of 30 patients (83%) had received a prior aromatase inhibitor for the treatment of metastatic disease, and 6/30 (20%) prior fulvestrant (Table 1).

Safety and Tolerability

No DLTs were observed. However, shortly after finishing the DLT window, one patient in cohort 1 developed diarrhoea grade 3 due to colitis, therefore the cohort was expanded. As predefined, cohorts 2 and 3 were expanded to confirm safety of these dose levels. Following independent data monitoring committee review, the RP2D of taselisib in combination with tamoxifen was set at 4 mg in a continuous schedule.

The most common treatment-emergent AEs of any grade were elevated liver enzymes (13 out of 30 patients [43%]), diarrhoea (43%), anaemia (40%) and oral mucositis (33%, Table 2). The majority of these AEs first occurred during the DLT window, persisted during study treatment, but reversed after treatment discontinuation. AEs of special interest occurred in 6 patients (20%): 3 patients had diarrhoea grade 3 due to colitis, 2 patients had rash grade 3 and 1 patient developed pneumonitis grade 4. After withholding the study drugs, and treatment with high dose corticosteroids, all recovered to ≤ grade 1.

Pharmacokinetics

The concentration-time curves for taselisib in combination with tamoxifen at cycle 1 day 15 are shown in [S1]. Samples from POSEIDON trial are displayed as individual data points against the backdrop of a population PK model from the broader taselisib clinical development programme provided by Genentech. At the taselisib 4mg daily dose level, combining patients on intermittent and continuous schedules, the cycle 1 day 15 median C_{max} for taselisib in combination with tamoxifen was 68.7 ng/mL and median AUC 1070 ng.h/mL, compared with an expected median C_{max} of 59.2 ng/mL (range 33.6-111) and

Table 1. Patient baseline characteristics.

	Cohort 1 (*N*=6) 2mg Taselisib QD 21d, 7d off + 20mg tamoxifen	Cohort 2 (*N*=13) 4mg Taselisib QD 21d, 7d off + 20mg tamoxifen	Cohort 3 (*N*=11) 4mg Taselisib continuous + 20mg tamoxifen	All patients (*N*=30)
Age in years – median (range)	51 (41–68)	54 (45–72)	54 (35–81)	53 (35–81)
ECOG Performance Status	3 (50%)	3 (23%)	5 (45%)	11 (37%)
0	3 (50%)	10 (77%)	6 (55%)	19 (63%)
1				
Histological subtype	4 (67%)	12 (92%)	8 (73%)	24 (80%)
Ductal	2 (33%)	1 (8%)	2 (18%)	5 (17%)
Lobular	0	0	1 (9%)	1 (3%)
Unknown				
***PIK3CA* mutational status**	5 (83%)	13 (100%)	8 (73%)	26 (87%)
Wild type	1 (17%)	0	2 (18%)	3 (10%)
H1047R mutation	0	0	1 (9%)	1 (3%)
E545K mutation				
Number of prior metastatic therapies	2 (1-2)	2 (1-3)	2 (0-3)	2 (0-3)
– median (range)	2 (0-7*)	1 (0-5)	2 (0-5)	2 (0-7*)
Endocrine				
Cytotoxic				
Prior endocrine therapies for	1 (17%)	4 (31%)	1 (9%)	6 (20%)
metastatic disease	0	3 (23%)	4 (36%)	7 (23%)
Tamoxifen	2 (33%)	6 (46%)	4 (36%)	12 (40%)
Aromatase inhibitor	3 (50%)	4 (31%)	2 (18%)	8 (27%)
Anastrozole	2 (33%)	2 (15%)	2 (18%)	6 (20%)
Letrozole	0	1 (8%)	0	1 (3%)
Exemestane				
Fulvestrant				
Megestrol acetate				

* after data cleaning it was found that 1 patient had received more than 5 prior lines of cytotoxic chemotherapy

Table 2. Most frequently observed treatment-emergent adverse events. Highest grade of AEs occurring at any time point, in at least 10% of patients, and thought to be at least possibly study-drug related. AE – adverse event; AST – aspartate transaminase; ALT – alanine transaminase; GGT – gamma glutamyltransaminase; QD – once daily.

	Cohort 1 (*N*=6) 2mg Taselisib QD 21d, 7d off + 20mg tamoxifen		Cohort 2 (*N*=13) 4mg Taselisib QD 21d, 7d off + 20mg tamoxifen		Cohort 3 (*N*=11) 4mg Taselisib continuous + 20mg tamoxifen		All patients (*N*=30)	
	Grade 1-2	**Grade 3-4**	**Grade 1-2**	**Grade 3-4**	**Grade 1-2**	**Grade 3-4**	**Grade 1-2**	**Grade 3-4**
AST/ALT/GGT increase	1 (17%)	0	5 (38%)	3 (23%)	4 (36%)	0	10 (33%)	3 (10%)
Diarrhoea/colitis*	2 (33%)	1 (17%)*	5 (38%)	1 (8%)*	3 (27%)	1 (9%)*	10 (33%)	3 (10%)
Anaemia	3 (50%)	0	3 (23%)	0	6 (54%)	0	12 (40%)	0
Mucositis – oral	2 (33%)	0	2 (15%)	2 (15%)	4 (36%)	0	8 (27%)	2 (7%)
Nausea	2 (33%)	0	5 (38%)	0	2 (18%)	0	9 (30%)	0
Hyperglycaemia	0	0	3 (23%)	0	4 (36%)	1 (9%)	7 (23%)	1 (3%)
Lipase/amylase increase	1 (17%)	0	2 (15%)	2 (15%)	1 (9%)	2 (18%)	4 (13%)	4 (13%)
Fatigue	0	0	3 (23%)	0	4 (36%)	0	7 (23%)	0
Headache	1 (17%)	0	5 (38%)	0	1 (9%)	0	7 (23%)	0
Thrombocytopenia	1 (17%)	0	3 (23%)	1 (8%)	1 (9%)	0	5 (17%)	1 (9%)
Alopecia	0	0	4 (31%)	0	1 (9%)	0	5 (17%)	0
Weight loss	1 (17%)	0	1 (8%)	0	3 (27%)	0	5 (17%)	0
Abdominal pain	1 (17%)	0	1 (8%)	0	2 (18%)	0	4 (13%)	0
Creatinine increase	0	0	3 (23%)	0	1 (9%)	0	4 (13%)	0
Triglyceride increase	0	0	2 (15%)	0	1 (9%)	1 (9%)	3 (10%)	1 (3%)
Pneumonitis	0	0	0	1 (8%)	0	0	0	1 (3%)
Any AE	**4 (67%)**	**1 (17%)**	**5 (38%)**	**8 (62%)**	**6 (54%)**	**5 (45%)**	**15 (50%)**	**14 (47%)**

median AUC 1190 ng.h/mL (range 630-2273) from the single agent taselisib population PK model. Endoxifen levels are shown in [S2].

Anti-tumour activity and *PIK3CA* mutational status

25 out of 30 patients had RECIST-measurable disease, and of these 6 had a confirmed RECIST partial response, yielding an objective response rate (ORR) of 24%. Best responses according are shown as a waterfall plot in Figure 1, alongside an oncoprint plot showing key gene mutations in baseline plasma or tumour tissue samples. Median TTP for the whole population was 4 months (inter-quartile range 2-8), and 8 months for patients achieving a RECIST partial response. The timecourse of responses to treatment are also visualised on a spider plot (Figure 2) and a swimmers plot [S3]. 12 out of 30 patients had disease control for 6 months or more, thus a 6-month clinical benefit rate (CBR) of 40%.

PIK3CA mutation testing was done for all patients on baseline tumour tissue and on plasma ctDNA samples. *PIK3CA* mutations were found in 8/30 (27%) of patients (see Oncoprint Figure 1 and mutation lollipop diagram [S4]). In this group of 8 patients with *PIK3CA* mutant tumours, 3 patients had a PR, and the other 5 stable disease as their best response. There was no statistically significant difference for *PIK3CA* mutant (exon 9, exon 20 or both) vs. wild-type subgroups for either ORR (38% v. 14%) or TTP (153 v. 113 days, respectively).

Circulating tumour (ct)DNA correlative studies

All patients had serial plasma sampling for ctDNA correlative studies. Here we describe four patients in whom ctDNA results illustrate molecular correlates with treatment response (Figure 3).

In the first case, the patient had previously received weekly paclitaxel and anastrozole as treatment for her *PIK3CA* mutant breast cancer metastatic to bone, lung, and subcutaneous tissues, and was treated with tamoxifen plus taselisib in the 4mg QD continuous schedule. A rapid fall in plasma ctDNA *PIK3CA*^{H1047R} fraction was observed just 1 week after starting therapy, 7 weeks before her first scheduled CT scan to assess treatment response.

Figure 1. Anti-tumour activity and pre-treatment tumour genetics (all patients, N=30).

a) Waterfall plot showing best treatment response for all 30 patients – 25 with RECIST-measurable disease and 5 with non-measurable disease (the latter marked by an asterisk*). Best RECIST response and time on treatment in months are indicated for each patient. PR - partial response, SD - stable disease, PD - progressive disease).

b) Oncoprint plot showing pre-treatment mutation status of *PIK3CA, PIK3R, PTEN, MAP3K1* and *TP53* genes. In each square, detection of a mutation in the tissue (primary or metastatic) is shown on the left side, while detection on plasma (at baseline) is shown on the right. Cases where tissue was not available are indicated in dark grey; for all the others, both tissue and plasma were tested. The black outline indicates that the mutation is present in Cosmic database. The white star indicates mutations in tissue and plasma are not in the same position. Numbers on the top indicate the exon of *PIK3CA* mutations (9 or 20); T–tumour, P–plasma.

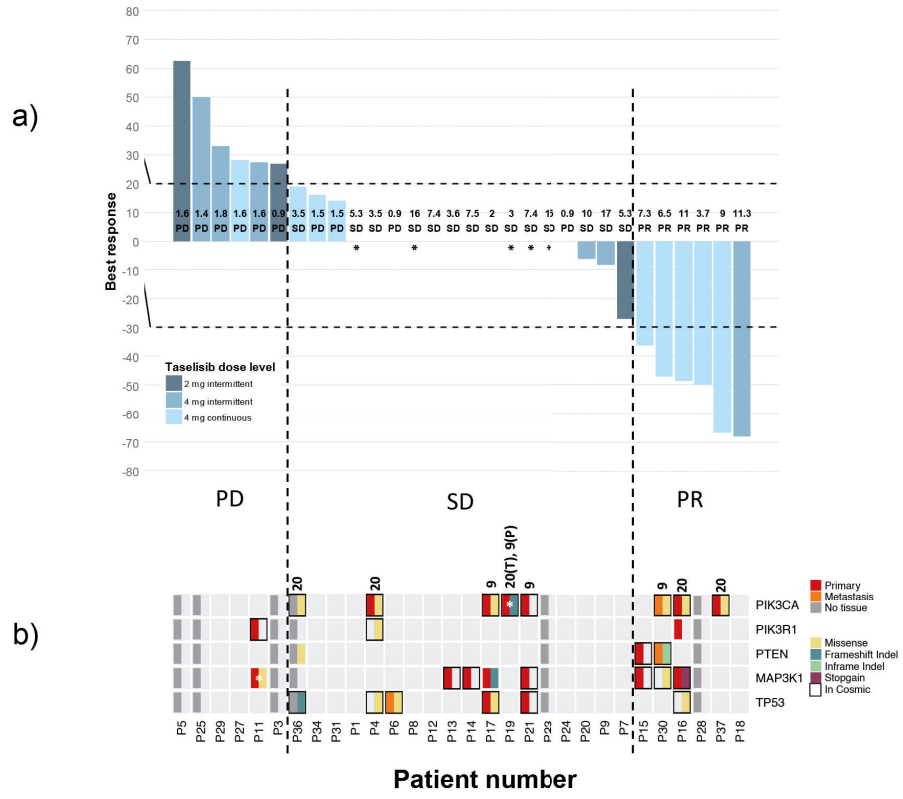
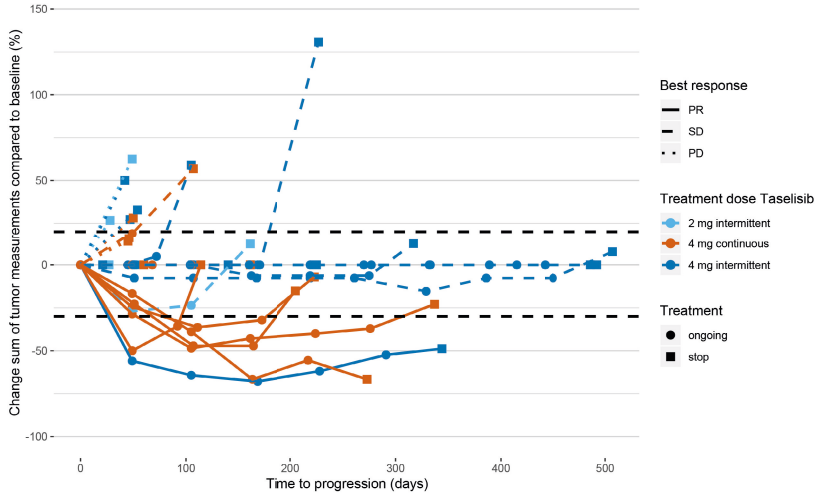


Figure 2. Spider plot showing change in tumour size over time for individual patients with RECIST-measurable disease (*N*=25). PR - partial response, SD - stable disease, PD - progressive disease, intermittent – 21 days on/7 days off

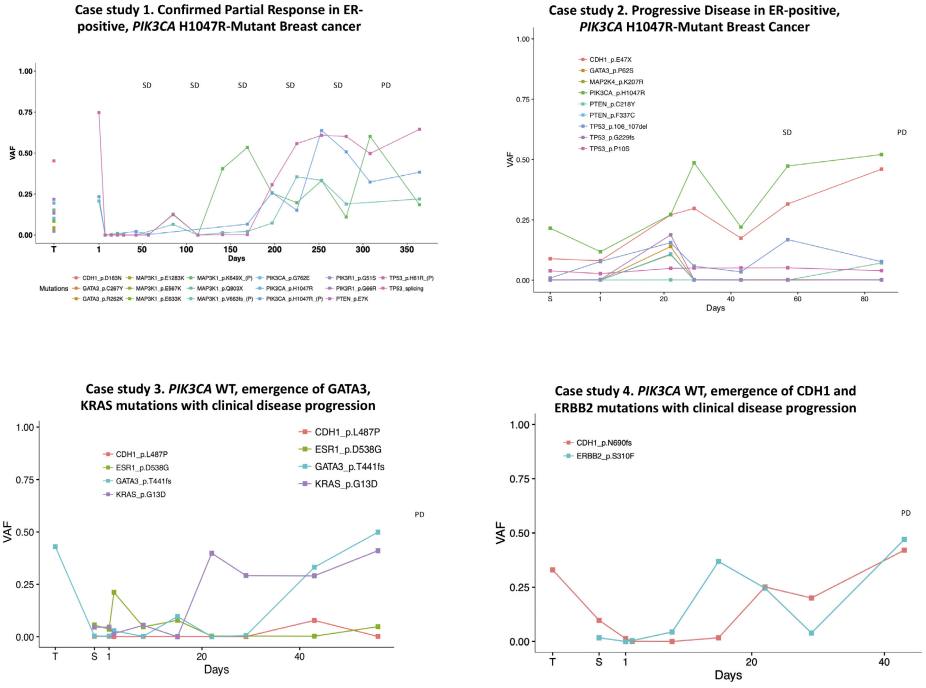


In the second case, the patient had previously received epirubicin, exemestane and capecitabine as treatment for her *PIK3CA* mutant breast cancer metastatic to liver and bone and was treated with tamoxifen plus taselisib in the 4mg QD continuous schedule. She did not respond to treatment and an increase in plasma ctDNA *PIK3CA*^{H1047R} fraction was seen on cycle 1 day 15, six weeks before her end of cycle 2 restaging CT scan.

In the third case, the patient had previously received paclitaxel, anastrozole, everolimus-exemestane, capecitabine, vinorelbine-docetaxel and letrozole to treat her *PIK3CA* wild-type breast cancer metastatic to liver and bones and was treated in the tamoxifen plus taselisib 4mg QD 21/7 intermittent cohort. She did not respond to treatment and increases in plasma ctDNA levels were found for *GATA3* and *KRAS* mutations two weeks ahead of cycle 2 CT scan.

In the fourth case, the patient had previously received paclitaxel, letrozole, docetaxel, capecitabine, exemestane and eribulin to treat her *PIK3CA* wild-type breast cancer metastatic to liver and bones and was treated in the tamoxifen plus taselisib 4mg QD continuous cohort. She did not respond to treatment and increases in plasma ctDNA levels were found for *ERBB2* and *CDH1* mutations 34 and 27 days respectively before she came off trial with disease progression.

Figure 3. Circulating tumour (ct)DNA correlative case studies. In four individual patients each having different clinical outcomes, the variant allele fraction is shown over time for gene mutations in plasma whilst on study treatment.



CDH1 – cadherin 1; CT – computed tomography; ERBB2 – Erb-B2 Receptor Tyrosine Kinase 2; GATA3 – GATA Binding Protein 3; KRAS – Kirsten ras oncogene homolog; PIK3CA – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; VAF – variant allele fraction; WT – wild type.
S = screening. T = pre-treatment tumour sample.

DISCUSSION

Taselisib in combination with tamoxifen is generally well tolerated, with a side effect profile that was manageable, and consistent with taselisib given as a single agent and in combination with other endocrine agents. In keeping with other PI3K inhibitors, the commonest side effects were diarrhoea, anaemia, nausea, mucositis and hyperglycaemia. Three out of 30 patients had grade 3 colitis, one patient was found to have grade 4 pneumonitis, all of which were reversible. The RP2D of taselisib in combination with tamoxifen was determined to be 4 mg on a daily continuous schedule.

Tamoxifen is a pro-drug that is converted to its active metabolites by cytochrome (CYP) P450 enzymes including CYP2D6, CYP3A4, CYP2B6, and CYP2C19. Taselisib is a weak inhibitor of CYP3A4 and does not inhibit any other CYPs in vitro, and did not alter the PK of midazolam, a CYP3A4 substrate, in the first-in-man study of taselisib (PMT4979g). Therefore, no change in taselisib PK was expected when given in combination with tamoxifen. Indeed, the observed taselisib concentrations at day 15 of cycle 1 were in the same range as those of a previously treated single agent taselisib cohort. Also, cycle 2 day 1 Z-endoxifen levels were on average above the laboratory threshold of 5.9 ng/mL[16] in all dose levels.

Preliminary evidence of anti-tumour activity was observed, with confirmed partial responses seen in 6/25 patients with RECIST-measurable disease (ORR 24%). Responses were seen in patients with *PIK3CA*^{H1047R} mutant, *PIK3CA*^{E545K} mutant and *PIK3CA*^{WT} tumours.

A strong rationale exists to explore the combination of PI3K inhibitors with endocrine therapy for the treatment of ER+ breast cancer. In addition to the POSEIDON trial combination with tamoxifen, taselisib is given in clinical trials together with fulvestrant (NCT02340221)[17] and letrozole (NCT02273973)[7]. Although *PIK3CA* mutations have been implicated in primary endocrine resistance and their prevalence is relatively high (20-25% in ductal breast cancer and 40% in lobular breast cancer), results are conflicting[18,19] and the outcome might depend on the specific mutation that is studied[20].

In the SANDPIPER randomised phase 3 trial (NCT02340221)[17], patients with or without a *PIK3CA* mutation were randomised between taselisib plus fulvestrant and placebo plus fulvestrant. Taselisib dose and schedule were the same as recommended for phase 2 of the POSEIDON study (ie. taselisib 4mg daily continuous). Median PFS with taselisib plus fulvestrant in patients with a *PIK3CA* mutation was significantly longer (7.4 months) than

with placebo plus fulvestrant (5.4 months; HR 0.70). No significant PFS difference was observed in patients who had a *PIK3CA* wildtype tumour (median PFS 5.6 months vs 4.0 months). However, information about a test for interaction is lacking. Adverse events grade 3 or higher were observed in almost half of the patients. The toxicity profile seen in POSEIDON is consistent to that reported in previous trials testing taselisib plus endocrine therapy in the metastatic setting.

Despite these encouraging results, *PIK3CA* mutational status may not on its own be sufficient to identify which ER-positive breast cancer patients will benefit most from the addition of a PI3K inhibitor to endocrine therapy. Individual patients with *PIK3CA* wild-type tumours can respond, and some patients with *PIK3CA* mutant tumours do not. Further studies are required to identify the optimal biomarker profile for PI3K combination therapy, and how best to use the results of real-time plasma ctDNA monitoring for the management of individual patients. These questions are being addressed in the randomised phase 2 part of POSEIDON which is ongoing.

To conclude, the RP2D of taselisib in combination with tamoxifen 20 mg daily is 4 mg QD in a continuous schedule. Phase 2 of POSEIDON (NCT02301988) is currently recruiting and randomises patients (*N*=280 in total) to receive tamoxifen 20 mg daily with either taselisib 4 mg or placebo once daily; including a specific focus on patients with lobular breast cancer (*N*=110); and a major translational effort to identify predictive biomarkers to help select which patients are most likely to benefit from the addition of a PI3K inhibitor to their endocrine therapy.

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SUPPLEMENTARY MATERIAL

METHODS

Patients

Patients had histologically or cytologically confirmed oestrogen receptor (ER) and / or progesterone receptor (PR)- positive carcinoma of the breast, based on the most recent assessment of ER and PR status from primary breast cancer or from recurrent metastatic disease with 10% or more stained cells considered positive.

Dose-Limiting Toxicity (DLT) Definitions

Dose-Limiting Toxicities (DLTs) were those treatment-emergent AEs occurring during cycle 1 (days 1-28) which warranted a dose-reduction or which were ≥grade 3 with the following exceptions:

- grade ≥ 3 non-haematologic AE, excluding grade 3 nausea, vomiting, or diarrhoea that resolved to grade ≤ 1 within 7 days
- grade 3 rash that resolved to grade ≤ 2 within 7 days
- grade ≥ 3 febrile neutropenia; grade ≥ 4 neutropenia lasting > 7 days
- grade ≥ 4 thrombocytopenia lasting > 48 hours
- grade ≥ 4 anaemia
- grade ≥ 3 total bilirubin, hepatic transaminase (alanine transaminase [ALT], aspartate transaminase ([AST]), amylase, or lipase lasting > 72 hours except for patients with grade 1 hepatic transaminase levels at baseline as a result of metastases
- hepatic transaminase ≥ 7.5× ULN
- any fasting grade 4 hyperglycaemia or fasting grade 3 hyperglycaemia lasting more than 7 days despite appropriate treatment with an oral hypoglycaemic agent.

Per-protocol defined adverse events of special interest (AESI) were:

- DLTs occurring during the DLT assessment window
- Grade 4 hyperglycaemia
- Grade ≥ 3 symptomatic hyperglycaemia
- Grade ≥ 3 diarrhoea
- Grade ≥2 colitis or enterocolitis
- Grade ≥ 3 rash; Grade ≥ 2 pneumonitis
- Cases of potential drug-induced liver injury that include an elevated ALT or AST in combination with either an elevated bilirubin or clinical jaundice, defined by Hy's law
- Suspected transmission of an infectious agent by the study drug

Pharmacokinetic methods

To determine the plasma taselisib pharmacokinetics, plasma PK samples were taken after a single dose (cycle 1 day 1), then subsequently in combination with tamoxifen (cycle 1 days 2, 3, 15 and 16; then on day 1 of cycles 2, 3 and 5 and at disease progression). Taselisib concentrations was were determined at Covance laboratories (Geneva, Switserland) using a validated LC/-MS-/MS assay with a lower limit of quantitation of 0.87 nmol/L[1]. Tamoxifen PK was evaluated from serum samples taken at cycle 1 days 2, 3 and 15, day 1 of cycle 2, 3 and 5 and at disease progression, and analysed at the Department of Pharmacy & Pharmacology of the Slotervaart HospitalNetherlands Cancer Institute, Amsterdam, the Netherlands, also using a validated HPLC/-MS-/MS assay with a lower limit of quantitation of 5 ng/mL for tamoxifen and 1 ng/mL for Z-endoxifen[2]. The PK analyses were performed using standard non-compartmental methods. Endoxifen Z-endoxifen concentrations were compared between taselisib dose levels using a Kruskal-Wallis test.

Circulating tumour (ct)DNA methods

Serial blood samples were collected in EDTA tubes and centrifuged within 1 hour at 820g to separate the plasma from the peripheral blood cells. The plasma was then centrifuged at 1420 g for 10 minutes to pellet any remaining cellular debris. Plasma aliquots were stored at -80°C. DNA was extracted from aliquots of plasma using the QIAsymphony (Qiagen). Tumour DNA was isolated from FFPE and frozen samples using DNeasy Blood and Tissue kits from Qiagen. At the Cancer Research UK Cambridge Institute, *PIK3CA* mutation hotspot (H1047R, E545K) were analysed by digital PCR using the BiomarkTM microfluidic system (Fluidigm), and Next-Generation Tagged-Amplicon Sequencing (NG-TAS) was performed as previously described. For the sequencing lane, quality control of raw data was done using Fast QC. Picard Tool (v 1.140) was used for the alignment and bam metrics computation. The Genome Analysis Toolkit (GATK, v3.5) was used for local realignment of the bam files. For mutation calling, it was run separately for each amplicon in the panel, and the core mutation calling was performed using Mutect 2. The same filtering and criteria for somatic mutation calling was used as previously described[3–5].

References for Methods

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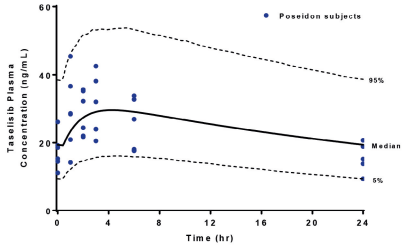
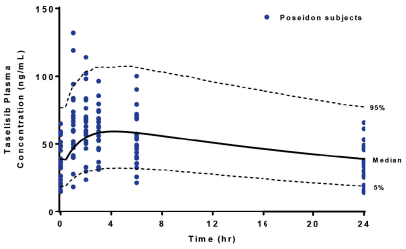
S1. Supplementary Figure 1. Taselisib pharmacokinetics in combination with tamoxifen.



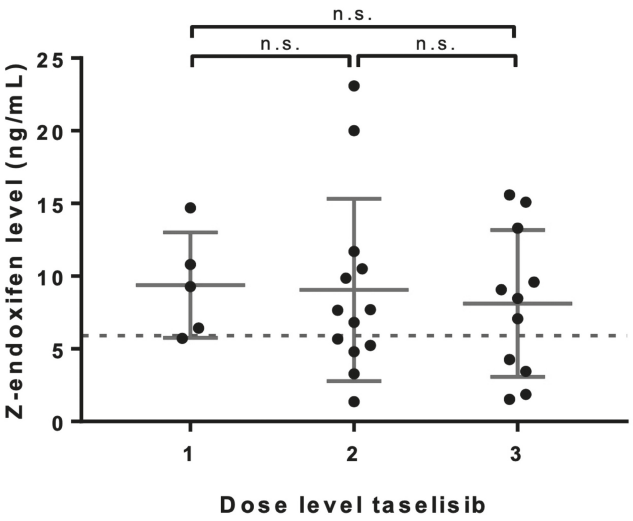
	POSEIDON study	Single agent study
C_{max} median	32.5	29.6
AUC median	418	595



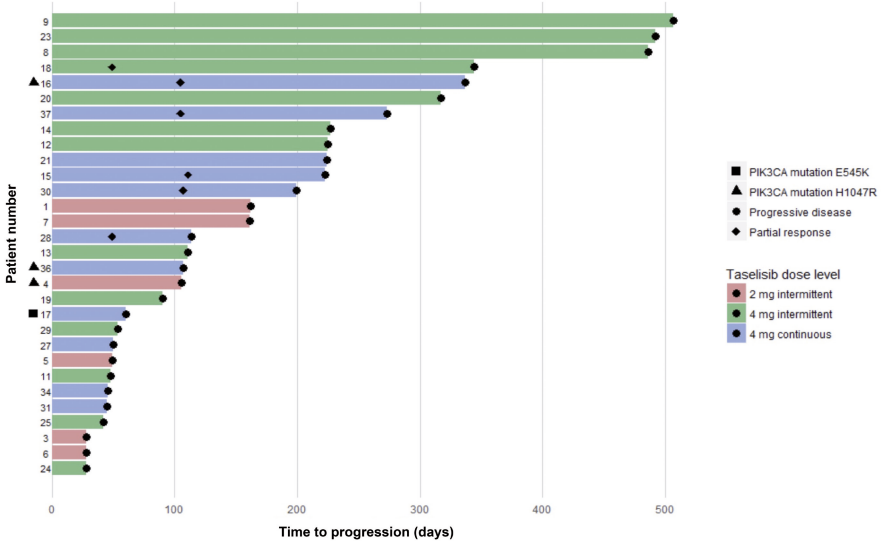
	POSEIDON study	Single agent study
C_{max} median	68.7	59.2
AUC median	1070	1190

Plasma taselisib concentrations for POSEIDON trial patients (in combination with tamoxifen) are displayed as individual data points against the backdrop of a broader single agent taselisib population PK model derived from the broader Genentech phase I programme at two different dose levels (left – 2 mg; right 4 mg). This population PK model was kindly provided by Genentech.

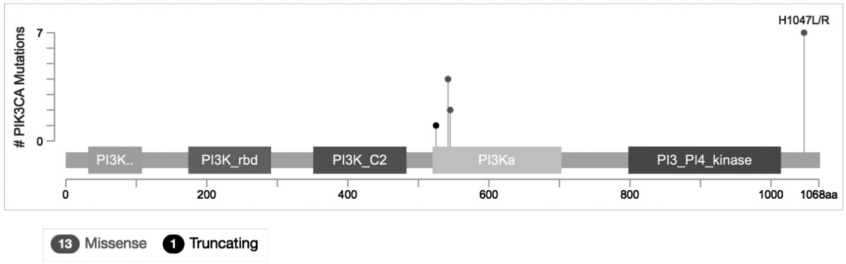
S2. Supplementary Figure 2. Pharmacokinetics: Z-endoxifen levels per taselisib dose level.



S3. Supplementary Figure 3. Time to progression per patient in a swimmers plot (all patients, N=30).



S4. Supplementary Figure 4. *PIK3CA* mutations detected at baseline (in samples from 8 out of 30 patients).



Patient	Tissue	Disease	AA change	Type	Chrmosome	Start Position	End Position	Ref	Var
P4	plasma	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P4	PT	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P16	plasma	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P16	PT	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P17	plasma	SD	E545K3D	Missense	3	178936091	178936091	G	A
P17	PT	SD	E545K3D	Missense	3	178936091	178936091	G	A
P19	plasma	SD	E525fs3D	FS del	3	178936031	178936032	GA	-
P19	PT	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P21	PT	SD	E542K3D	Missense	3	178936082	178936082	G	A
P30	plasma	PR	E542K3D	Missense	3	178936082	178936082	G	A
P30	MT	PR	E542K3D	Missense	3	178936082	178936082	G	A
P30	PT	PR	E542K3D	Missense	3	178936082	178936082	G	A
P36	plasma	SD	H1047R3D	Missense	3	178952085	178952085	A	G
P37	PT	PR	H1047L3D	Missense	3	178952085	178952085	A	T

PT = Primary tumour, MT = Metastatic tumour, SD = Stable disease, PR = Partial response disease

7

Carboplatin-cyclophosphamide or paclitaxel without or with bevacizumab as first line treatment for metastatic triple negative breast cancer (BOOG 2013-01)

A.G.J. van Rossum, I.A.M. Mandjes, E. van Werkhoven, H. van Tinteren, J.M. Rademaker - Lakhai, P. Nederlof, J.E.A. Portielje, R.J. van Alphen, E. Platte, D. van den Broek, M. Kok, S.C. Linn and H.M. Oosterkamp, on behalf of the Triple-B trialists' group

Submitted.

ABSTRACT

Background: While the addition of bevacizumab to chemotherapy conferred a modest progression-free survival (PFS) benefit in metastatic triple negative breast cancer (mTNBC), no overall survival (OS) benefit has been reported. However, a combination with carboplatin-cyclophosphamide has never been investigated.

Methods: The Triple-B study is a multicentre, randomised phase IIb trial which aims to prospectively validate predictive biomarkers. Here we report on a pre-planned safety and preliminary efficacy analysis after the first 12 patients had been treated with carboplatin-cyclophosphamide (CC) and bevacizumab (B). mTNBC patients (n=58) were randomised in first line between CC and paclitaxel (P) without or with bevacizumab (CC ± B or P ± B). In addition, results of baseline plasma vascular endothelial growth factor receptor-2 (pVEGFR-2) level as predictive biomarker for bevacizumab benefit are reported.

Results: Median follow up was 22.1 months. Toxicity was manageable and consistent with what is known for each agent separately. There was a trend for prolonged PFS with bevacizumab compared with chemotherapy only (7.0 vs 5.2 months; adjusted hazard ratio [HR] 0.60, 95% confidence interval [CI] 0.33-1.08; p=0.09), but no effect on OS. In this small study, pVEGFR-2 levels did not predict for bevacizumab PFS benefit. Both the intention-to-treat analysis as well as the per-protocol analysis did not yield a significant treatment-by-biomarker test for interaction ($p_{interaction}$=0.69).

Conclusions: CC without or with bevacizumab is safe as first-line treatment for mTNBC and side effects are consistent with those known for each individual agent.

INTRODUCTION

Triple negative breast cancer (TNBC) accounts for 10-15% of all breast cancers and has a particularly poor prognosis.[1] The time from diagnosis to distant recurrences is shorter than for other breast cancer subtypes and median survival of patients with metastatic TNBC (mTNBC) is on average only one year.[2] Although current treatment strategies are limited to conventional cytotoxic chemotherapy, several TNBC subtypes with distinct biological features and putative novel targets for therapy have been described[1,3,4]. There are indications that TNBCs that are homologous recombination deficient (HRD)[5] are more sensitive to bifunctional alkylating and platinum agents than non-HRD TNBCs[6,7], and relatively resistant to taxanes[8].

Angiogenesis is important for tumour growth and development, particularly in TNBC. Levels of angiogenesis mediator vascular endothelial growth factor A (VEGF-A) were found to be higher in TNBC than in non-TNBC.[9] Therefore, inhibiting angiogenesis might be a potentially effective therapeutic target in this particular subtype.[3,10] The first results of bevacizumab, a monoclonal antibody against VEGF-A, in combination with chemotherapy were promising[11]. However, others found less pronounced treatment effects[12-14] and no overall survival (OS) benefit was seen in either of these trials. These modest results led to a search for biomarkers of bevacizumab benefit. Significant associations between plasma VEGF-A (pVEGF-A) levels and survival benefit of bevacizumab were observed in retrospective analyses of breast cancer trials[14,15]. These findings led to the development of the MERiDiAN trial in which the predictive value of pVEGF-A was prospectively evaluated[16].

Baseline plasma VEGFR-2 (pVEGFR-2) level was identified as another potential predictive biomarker for bevacizumab benefit in retrospective analyses[15]. The Triple-B trial aimed to prospectively analyse baseline pVEGFR-2 level as a predictive biomarker for bevacizumab efficacy. A co-primary objective was to validate the *BRCA1*-like classifier as biomarker for efficacy of alkylating chemotherapy and platinum compounds. mTNBC patients were treated in first-line with either carboplatin-cyclophosphamide (CC) or paclitaxel (P) with or without bevacizumab (B). Since carboplatin-cyclophosphamide had never been combined with bevacizumab before, a safety interim analysis had been planned after 12 patients had been randomised in the CC + B arm.

With the emerging evidence of only modest bevacizumab efficacy and the results of the MERiDiAN trial demonstrating the limited utility of baseline pVEGFR-2 level as biomarker for bevacizumab efficacy, we deemed it necessary to adapt the Triple-B design and

replace add-on bevacizumab with a different add-on. Therefore, we also report on the preliminary efficacy of bevacizumab addition.

METHODS

Patients

The Triple-B (Biomarker discovery randomised phase IIb trial with carboplatin-cyclophosphamide versus paclitaxel with or without Bevacizumab as first-line treatment in advanced triple negative Breast cancer; NCT01898117) study is a randomised, multicentre, open label, phase 2b trial. Patients with histologically confirmed locally advanced or metastatic TNBC were eligible. ER was considered negative when <10% of the tumour cells showed nuclear staining. The tumour was negative for HER2 when immunohistochemical staining was of 0 or 1+ intensity. In equivocal cases (2+), an *in-situ* hybridization assay was performed to determine *HER2* amplification status. Further eligibility criteria are listed in Supplementary appendix.

All patients gave written informed consent. The study was performed in accordance with the Declaration of Helsinki. The study protocol and its amendments were reviewed and approved by the ethical committee of the Netherlands Cancer Institute and the institutional boards of the participating centres. The REMARK (Reporting Recommendations for Tumour Marker Prognostic Studies) criteria were used to report this study.[17]

Treatment

Patients were randomised between four treatment arms: 1. carboplatin area under curve (AUC) 5 and cyclophosphamide 600 mg/m^2 on day 1 every 4 weeks (CC); 2. carboplatin AUC 5 and cyclophosphamide 600 mg/m^2 on day 1 and bevacizumab 10 mg/kg on day 1 and 15 every 4 weeks (CC + B); 3. paclitaxel 90 mg/m^2 on day 1, 8 and 15 every 4 weeks (P); and 4. paclitaxel 90 mg/m^2 on day 1, 8 and 15 and bevacizumab 10 mg/kg on day 1 and 15 every 4 weeks (P + B). Treatment continued until progressive disease, unacceptable toxicity or upon patient's request. In case of an ongoing response and good tolerance after 6 cycles, it was allowed to either continue or stop treatment with chemotherapy and/or bevacizumab. Stratification factors were (neo)adjuvant systemic treatment (yes vs no), (neo)adjuvant taxane treatment (yes vs no) and treating centre.

Design and objectives

The Triple-B study was designed as a marker-by-treatment interaction trial with two primary objectives. The primary objective that we report here was to test whether the baseline pVEGFR-2 level could indicate which patients have longer progression free survival (PFS) with the addition of bevacizumab to first-line chemotherapy for TNBC. PFS was defined as the time from randomisation until progressive disease or death due to any cause, whichever occurred first. The other primary objective of the trial was to validate the *BRCA1*-like profile as a predictive marker for PFS benefit of carboplatin-cyclophosphamide compared with paclitaxel. Because validation of the *BRCA1*-like profile as predictive marker is still a primary objective in the ongoing trial, it will be discussed in later reports.

Secondary endpoint OS was defined as time from randomisation until death by any cause. Toxicity was scored using the Common Toxicity Criteria for Adverse Events (CTCAE) version 4.03.

Plasma VEGFR-2 concentration

pVEGFR-2 level was measured using the Quantikine Human VEGFR-2/KDR enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems, Minneapolis, USA). The cohort was split, based on a prespecified cut-off derived from the AVADO trial[15], into a pVEGFR-2 low subgroup (≤ 7.15 ng/ml) and a pVEGFR-2 high subgroup (> 7.15 ng/ml). Details on measurement of pVEGFR-2 are provided in the Supplementary appendix.

Statistics

Details on the sample size calculation are provided in the Supplementary appendix.

Clinicopathological characteristics of the four treatment arms were compared using a Kruskal-Wallis test, Chi-square test or Fisher's exact test. All patients who received at least one cycle of the allocated treatment were evaluated for toxicity. Adverse events (AEs) and serious adverse events (SAEs) were described per treatment arm.

Efficacy was assessed in the intention-to-treat (ITT) population. The ITT population consisted of all patients who were allocated to one of the treatments. PFS and OS were estimated using the Kaplan-Meier method and compared using the log-rank test. The association between clinicopathologic variables and survival endpoints was tested in

univariable Cox regression models. If the Wald p-value was smaller than 0.2, the variable was included in a multivariable Cox regression model. Multivariable Cox regression models were constructed to derive adjusted hazard ratios.

The association between baseline pVEGFR-2 level and PFS was assessed using the Kaplan-Meier method and compared with a log-rank test in the ITT population and the per-protocol treatment (PPT) population. The PPT population included all eligible patients who received at least one cycle of the allocated treatment. Cox regression models were made to assess the hazard and to test for the interaction.

Two-sided p-values of less than 0.05 were considered statistically significant. Analyses were performed using R software (version 3.3.1).

RESULTS

From October 2013 until January 2018, 58 patients were enrolled in the bevacizumab-part of the trial in 22 centres in the Netherlands (Table 1). Baseline characteristics were balanced between the treatment groups. The majority of patients (46 out of 58 patients [79.3%] received (neo)adjuvant taxane-containing chemotherapy. Only two patients were treated with carboplatin, one in the CC-arm and one in the P + B-arm. Also, 36 patients [62%] had a distant recurrence free interval (DRFI) of more than 24 months. Figure 1 shows the number of patients included in the analyses for toxicity, ITT and PPT.

Adverse events

The most common grade 2 or higher AEs that were at least possibly related to the study treatment are listed in Table 2. As expected, we observed more AEs in the bevacizumab-containing treatment arms than in the chemotherapy-only arms (Supplemental table 1). Hypertension (11 out of 28 patients [39.3%] vs 2 out of 29 patients [6.9%], p<0.01) and fatigue (11 patients [39.3%] vs 4 patients [13.8%], p=0.04) were observed more frequently in the bevacizumab-containing treatment arms compared with the chemotherapy-only treatment arms.

Anaemia (11 out of 27 patients [40.7%] vs 2 out of 30 patients [6.7%], p<0.01), nausea (7 patients [25.9%] vs 0 patients, p<0.01) and vomiting (7 patients [25.9%] vs 0 patients, p<0.01) were more frequent in the CC ± B arms compared with the P ± B arms (Supplemental table 2). In contrast, alopecia (0 patients vs 6 patients [20.0%], p=0.03)

and peripheral neuropathy (2 patients [7.4%] vs 10 patients [33.3%], p=0.02) were more common adverse events in the P ± B arms.

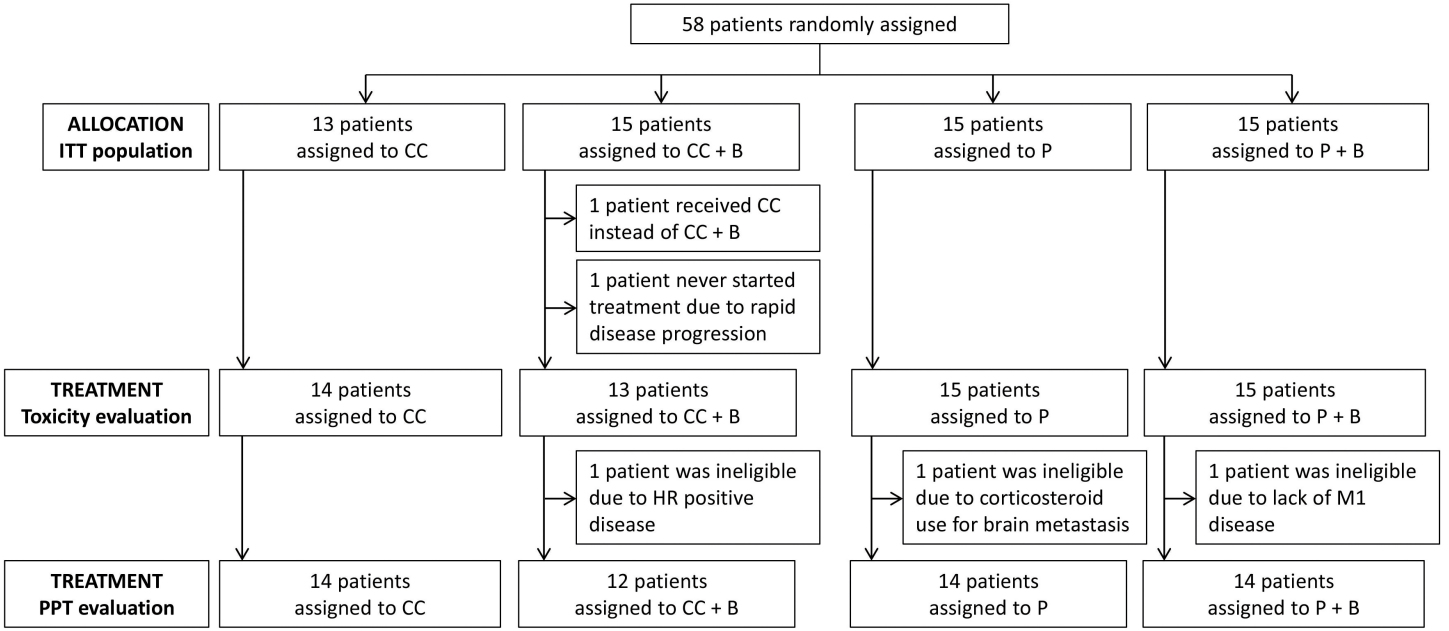
Table 1. Patient characteristics. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel; IQR = interquartile range, BCS = breast conserving surgery; * split by prespecified cut-off into a low VEGFR-2 level (≤ 7.15 ng/ml) subgroup and a high VEGFR-2 level (> 7.15 ng/ml) subgroup

	CC	CC+B	P	P+B
	n=13	n=15	n=15	n=15
Age – median (IQR range)	59 (51-65)	55 (52-66)	51 (46-60)	50 (46-58.5)
Surgery – n (%)				
none	2 (15.3)	2 (13.3)	0	1 (6.7)
BCS	5 (38.5)	7 (46.7)	5 (33.3)	1 (6.7)
mastectomy	6 (46.2)	6 (40.0)	10 (66.7)	13 (86.7)
Previous (neo)adjuvant chemotherapy – n (%)				
no	2 (15.4)	4 (26.7)	2 (13.3)	4 (26.7)
yes	11 (84.6)	11 (73.3)	13 (86.7)	11 (73.3)
Previous (neo)adjuvant taxanes – n (%)				
no	4 (30.8)	7 (46.7)	5 (33.3)	6 (40.0)
yes	9 (69.2)	8 (53.3)	10 (66.7)	9 (60.0)
Disease free interval – n (%)				
≤ 24 months	4 (30.8)	4 (26.7)	4 (26.7)	10 (66.7)
> 24 months	9 (69.2)	11 (73.3)	11 (73.3)	5 (33.3)
Number of metastatic sites – n (%)				
≥ 3 sites	9 (69.2)	9 (60.0)	7 (46.7)	5 (33.3)
< 3 sites	4 (30.8)	6 (40.0)	8 (53.3)	10 (66.7)
Localisation of disease – n (%)				
locoregional	1 (7.7)	1 (6.7)	1 (6.7)	3 (20.0)
bone only	2 (15.4)	3 (20.0)	2 (13.3)	4 (26.7)
visceral	3 (23.1)	3 (20.0)	4 (26.7)	3 (20.0)
mixed	7 (53.8)	8 (53.3)	8 (53.3)	5 (33.3)
Disease evaluation – n (%)				
measurable	11 (84.6)	14 (93.3)	11 (73.3)	9 (60.0)
non-measurable	2 (15.4)	1 (6.7)	4 (26.7)	6 (40.0)
Plasma VEGFR-2 level – n (%)*				
low	2 (15.4)	3 (20.0)	4 (26.7)	5 (33.3)
high	10 (76.9)	9 (60.0)	10 (66.7)	6 (40.0)
missing	1 (7.7)	3 (20.0)	1 (6.7)	4 (26.7)

Table 2. Adverse events per treatment arm, at least possibly related to the study treatment and observed in at least 10% of all patients. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel

	CC (n=14)		**CC + B (n=13)**		**P (n=15)**		**P + B (n=15)**	
	grade 2	grade 3-4	grade 2	grade 3-4	grade 2	grade 3-4	grade 2	grade 3-4
Alopecia	0	0	0	0	3 (20.0%)	0	3 (20.0%)	0
Anemia	6 (42.9%)	0	3 (21.4%)	2 (14.3%)	0	0	2 (13.3%)	0
Fatigue	3 (21.4%)	0	3 (21.4%)	3 (21.4%)	1 (6.7%)	0	5 (33.3%)	0
Hypertension	1 (7.1%)	0	1 (7.1%)	5 (35.7%)	0	1 (6.7%)	1 (6.7%)	4 (26.7%)
Nausea	2 (14.3%)	1 (7.1%)	4 (28.6%)	0	0	0	0	0
Neutrophil count decreased	0	6 (42.9%)	3 (21.4%)	8 (61.5%)	5 (33.3%)	3 (20.0%)	5 (33.3%)	2 (13.3%)
Peripheral sensory neuropathy	0	0	2 (14.3%)	0	5 (33.3%)	0	3 (20.0%)	1 (6.7%)
Vomiting	2 (14.3%)	0	5 (35.7%)	0	0	0	0	0
White blood cell decreased	1 (7.1%)	6 (42.9%)	4 (28.6%)	5 (38.5%)	5 (33.3%)	1 (6.7%)	3 (20.0%)	1 (6.7%)
Total number of adverse events	24	19	42	29	24	8	35	15

Figure 1. CONSORT diagram. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel, ITT = intention-to-treat, PPT = per protocol treated



Serious adverse events

All SAEs that were at least possibly related to the study treatment are listed in Table 3. The most common SAEs were fever (3 patients), anaemia (2 patients) and diarrhoea (2 patients). Four CC + B treated patients encountered at least one SAE compared with 2 patients in the CC and P + B arm, respectively and none in the subgroup of patients treated with P. No major bevacizumab-related SAEs were observed.

Table 3. Serious adverse events at least possibly related to study treatment. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel

	CC n=14	CC + B n=13	P n=15	P + B n=15
Anaemia		2 (14.3%)		
Diarrhoea		2 (14.3%)		
Fever	1 (7.1%)			2 (13.3%)
Neutrophil count decreased		1 (7.1%)		
Platelet count decreased		1 (7.1%)		
Thromboembolic event	1 (7.1%)			
White blood cell decreased		1 (7.1%)		
Total number of patients with ≥ 1 SAE	2 (14.3%)	4 (28.6%)	0 (0.0%)	2 (13.3%)

Treatment exposure

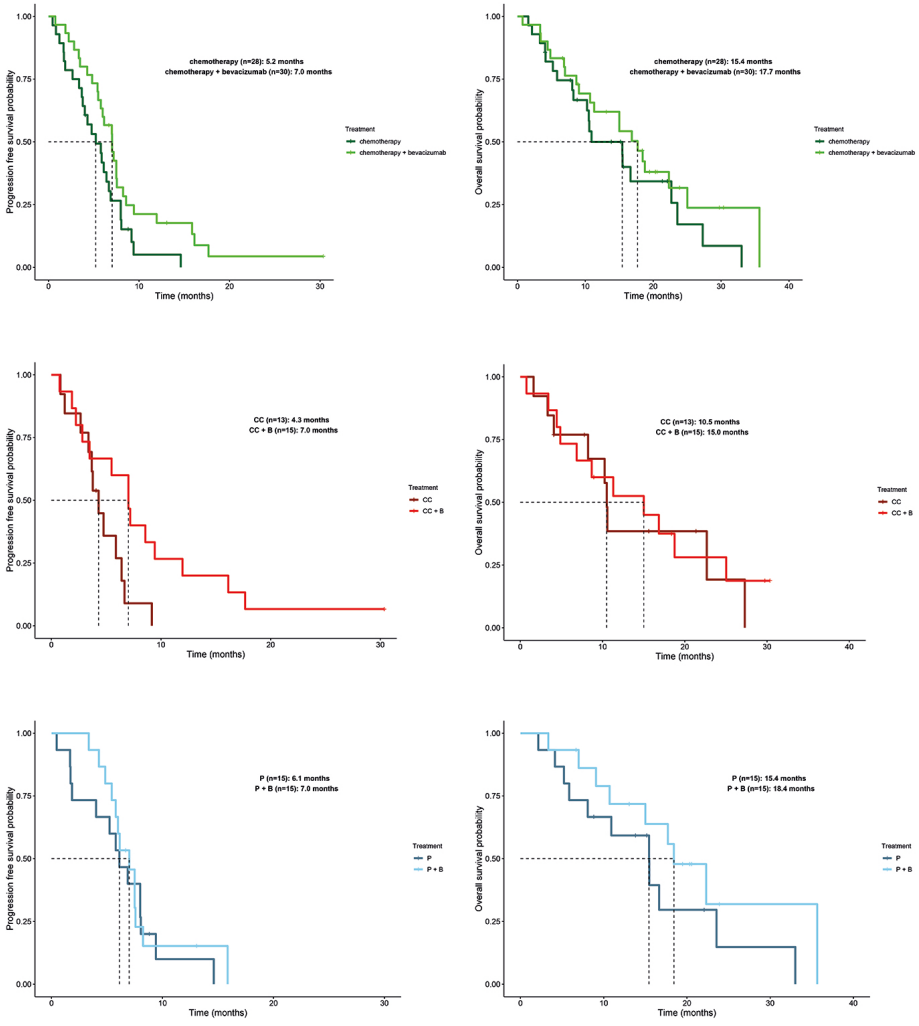
Patients received on average 4 to 6 cycles of treatment and 2 additional cycles of bevacizumab single agent in the CC + B treated arm (Supplemental table 3). Dose reductions and delays occurred more often in the treatment arms with bevacizumab than in the chemotherapy only arms. The relative total dose intensity (RTDI) of patients who were treated with CC + B was lower than the RTDI of the CC treated subgroup (89.6% vs 96.9%; student's T-test p=0.04). Four patients out of 57 patients (7%) discontinued treatment due to toxicity: one due to bone marrow toxicity, one due to peripheral sensory neuropathy, one due to ongoing pruritus and one due to multiple side effects.

Efficacy

Median follow up was 22.1 months (interquartile range [IQR] 18.4-30.4). The patients treated with bevacizumab had a significantly longer PFS than the chemotherapy-only subgroup (median PFS: 7.0 months vs 5.2 months; unadjusted hazard ratio [HR] 0.56, 95% confidence interval [CI] 0.32-0.98, p=0.04; Figure 2a). The only clinicopathologic

variables that were significant at the 0.2 level in univariable analysis were age and use of neoadjuvant or adjuvant chemotherapy. When corrected for these factors, PFS was not significantly different anymore (adjusted HR 0.60, 95% CI 0.33-1.08; p=0.09).

Figure 2. Association between treatment and progression free survival (PFS; a, b, c) and overall survival (OS; d, e, f) of the intention to treat population. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel



When split by chemotherapy backbone, PFS was significantly longer for CC + B-treated patients than for CC-treated patients (median PFS 7.0 months vs 4.3 months; unadjusted HR 0.39, 95% CI 0.16-0.95, p=0.04; Figure 2b). However, the difference was no longer significant when corrected for prognostic factors (adjusted HR 0.45, 95% CI 0.18-1.10, p=0.08). We did not observe a significant difference in PFS between P-treated patients and the P + B-treated subgroup (6.1 months vs 7.0 months; adjusted HR 0.74, 95% CI 0.32-1.70, p=0.48; Figure 2c). An overview of time to progression of all patients is given in Supplemental figure 1.

OS was not significantly different for patients who received chemotherapy with bevacizumab compared with the chemotherapy-only subgroup (15.4 months vs 17.7 months; Figure 2d), when corrected for age, use of neoadjuvant or adjuvant chemotherapy, use of neoadjuvant or adjuvant taxanes, and the number of metastatic sites (adjusted HR 0.88, 95% CI 0.46-1.69, p=0.70). Similarly, no significant difference in OS was observed when treatments were split by chemotherapy backbone (Figure 2e and 2f).

pVEGFR-2

For 49 out of 58 PPT patients (84.5%; Table 1), baseline pVEGFR-2 levels were measured. pVEGFR-2 level was not significantly associated with PFS (adjusted for age and use of neoadjuvant or adjuvant chemotherapy HR 1.16, 95% CI 0.59-2.29, p=0.67) or correlated with other clinicopathologic factors. Patients with a low pVEGFR-2 level had a significantly longer PFS with bevacizumab-treatment compared with chemotherapy only (median PFS: 7.0 months vs 3.5 months; adjusted HR 0.23, 95% CI 0.06-0.91, p=0.04; Supplemental figure 2a). However, PFS was not significantly different in patients with a high pVEGFR-2 level when treated with chemotherapy with bevacizumab compared with chemotherapy only (7.0 months vs 5.8 months; adjusted HR 0.61, 95% CI 0.27-1.36, p=0.23; Supplemental figure 2b). Also, the interaction between treatment (chemotherapy without or with bevacizumab) and pVEGFR-2 level was not significant ($p_{interaction}$=0.69). pVEGFR-2 analysis in the PPT population is displayed in Supplemental figure 2c and 2d (low pVEGFR-2 subgroup: adjusted HR 0.38, 95% CI 0.15-0.94, p=0.04; high pVEGFR-2 subgroup: adjusted HR 0.26, 95% CI 0.06-1.09, p=0.07). Also here, the test for interaction was not significant ($p_{interaction}$=0.75).

DISCUSSION

Here we report on the toxicity and efficacy of two chemotherapy backbones (carboplatin-cyclophosphamide and paclitaxel) without or with the addition of bevacizumab as first-line treatment of mTNBC patients in the randomised Triple-B study. We showed that it is safe to add bevacizumab to CC and that toxicity is slightly different from P + B. The addition of bevacizumab to these chemotherapy regimens resulted in a trend towards longer PFS. OS was not significantly prolonged after bevacizumab-containing treatment.

The efficacy results of the bevacizumab addition are in line with the hormone receptor-negative or TNBC subgroup analyses[18] of the E2100 trial[11], the AVADO trial[13] and the RIBBON-1 trial[12]. The AVADO trial and the RIBBON-1 trial showed that adding bevacizumab to chemotherapy prolonged PFS, but not OS. Although an explanation remains elusive, it might be caused by the selective inhibition of VEGF-A by bevacizumab. Since other isoforms of VEGF are still able to bind to their receptor on endothelial cells[19], blocking VEGF-A might result in a temporary effect on tumour progression. In time other isoforms might take over to stimulate angiogenesis and tumour growth, thereby impairing the efficacy of later lines of chemotherapy.

To our knowledge, this is the first trial that randomises breast cancer patients to the combination of carboplatin and cyclophosphamide. Furthermore, this is the first time that bevacizumab is added to this combination. CC has previously been applied as a safe and effective treatment for ovarian cancer.[20-22] In agreement with these reports, anaemia, nausea and vomiting occurred more often in the CC-arms than in the P-arms. Although we did observe more toxicity when bevacizumab was added to CC, this combination was considered safe by the independent data safety monitoring board.

The CC-arms resulted in similar PFS and OS as the P-arms in our cohort, with PFS of 5.5 months for CC ± B and 6.5 months for P ± B. Interestingly, three patients treated with CC + B had a PFS of more than 16 months. Considering that the average overall survival of mTNBC patients is one year[2], these patients responded remarkably well to the treatment.

We prospectively tested baseline pVEGFR-2 level as predictive biomarker for PFS and OS benefit of the addition of bevacizumab to first-line chemotherapy for TNBC. Previously, two groups showed in a retrospective analysis that baseline pVEGFR-2 level was a promising biomarker for PFS benefit of bevacizumab in HER2-negative breast cancer

patients.[14,15] In our relatively small study, we could not confirm these earlier findings, suggesting limited predictive value for baseline pVEGFR-2 levels. Our findings are in line with the results from the MERiDiAN study[23] in which the interaction between pVEGFR-2 level and bevacizumab treatment was also not significant.

A major limitation to the analyses of this report is that the number of patients is lower than planned in advance. Designed as a marker-by-treatment interaction trial, sample size calculations were based on the anticipated treatment effect and the size of the biomarker subgroups. Due to low rate of accrual because of emerging evidence that bevacizumab was less promising than expected, and after the MERiDiAN trial had demonstrated that baseline pVEGFR-2 level was not a suitable biomarker for bevacizumab benefit, we were forced to amend the protocol and to bring the bevacizumab part of the trial to a close. Therefore, the number of patients in these analyses is limited.

The second primary objective of this trial was to validate the *BRCA1*-like profile as predictive biomarker for survival benefit of alkylating agents and platinum compounds compared with taxanes and will be addressed later when the main study has been accomplished

The bevacizumab-part of the Triple-B study presented here demonstrates that carboplatin-cyclophosphamide without or with bevacizumab is a safe first-line treatment for mTNBC. The difference in toxicity profile between CC and P can be useful to guide treatment choices in the management of mTNBC. Although this result was obtained from a cohort that was stopped at interim and should therefore be interpreted with caution, bevacizumab addition to paclitaxel or carboplatin-cyclophosphamide prolonged PFS. For OS, no benefit was observed. In this small cohort, we were not able to validate baseline pVEGFR-2 level as a predictive biomarker for bevacizumab benefit. Given the biological heterogeneity and variation in responses, predictive biomarkers for treatment efficacy in TNBC are needed. Marker-by-treatment interaction trials, such as the ongoing Triple-B study, are required to validate these biomarkers and consequently optimize treatment decisions.

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SUPPLEMENTARY MATERIAL

METHODS

Eligibility criteria

Patients had to have a WHO performance status of 0 or 1, adequate bone marrow, liver and kidney function and APTT and PT/INR within normal range. Main exclusion criteria were prior cytotoxic treatment for metastatic disease, metastatic recurrence within 12 months after the last (neo)adjuvant paclitaxel administration, or within 6 months after the last (neo)adjuvant docetaxel administration, history of uncontrolled hypertension and clinically relevant cardiovascular (aneurysm, thrombosis) or gastrointestinal (perforation, fistula) disorders.

Plasma vascular endothelial growth factor receptor 2 (pVEGFR-2)

Blood (1 x 10 mL) was collected from all participating patients at baseline and at progression to measure the pVEGFR-2 concentration. The plasma was isolated and the VEGFR-2 level measured using the Quantikine Human VEGFR-2/KDR enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems, Minneapolis, USA) following the manufacturer's instructions.

Baseline pVEGFR-2 level was dichotomized based on the results of a pilot study of 30 patients. We compared pVEGFR-2 level using two different assays: the immunological multiparametric chip technique (IMPACT) from Roche Diagnostics (Risch-Rotkreuz, Switserland) and the Quantikine Human VEGFR-2/KDR ELISA kit (R&D systems, Minneapolis, USA). Spearman's correlation was 0.93. Applying the cut-off used in the AVADO trial[15], the cohort was split into a pVEGFR-2 low subgroup (≤ 7.15 ng/ml) and a pVEGFR-2 high subgroup (> 7.15 ng/ml).

Sample size calculation

The sample size of the study was calculated based on the interaction between *BRCA1*-like status and the type of chemotherapy (CC vs. P) treatment according to the method of Peterson and George[20]. The following assumptions were made: 1. the prevalence of *BRCA1*-like status in triple negative disease is 50%, 2. the PFS hazard ratio in the CC arm is 0.7 for *BRCA1*-like compared with non-*BRCA1*-like, and 3. the estimated median PFS is 5.4 months for standard-of-care chemotherapy in TNBC. For the interaction between

pVEGFR-2 and bevacizumab, the assumptions were the same except that the hazard ratio of high versus low pVEGFR-2 would be 0.5 in the bevacizumab-containing arms versus 1.00 in the chemotherapy only arms. A total of 304 patients would be sufficient to observe the 269 required events to demonstrate the interaction with *BRCA1*-like status and 286 events for the pVEGFR-2 interaction with 90% power and two-sided α of 0.10. Two interim analyses in addition to the final analysis were planned for comparison of efficacy of the treatments. The analyses were planned at equally spaced information fractions. After bevacizumab was replaced by atezolizumab, the boundaries were adjusted for a single interim analysis. This analysis was planned to be performed when all patients who received bevacizumab had finished their treatment. The Hwangh-Shih-DeCani spending function was used with a parameter yielding an O'Brien-Fleming-like boundary. The associated one-sided nominal p-values (for treatment comparison) correspond to respectively 0.0011 and 0.0489 (i.e., final analysis two-sided $p<2*0.0489$).

RESULTS

Supplemental table 1. Adverse events, split by addition of bevacizumab, that were at least possibly related to the study treatment and observed in at least 10% of all patients. * Two-sided chi-square test or Fisher's exact test

	No bevacizumab (n=29)				With bevacizumab (n=28)				
	grade 2	grade 3	grade 4	all grades	grade 2	grade 3	grade 4	all grades	p-value*
Alopecia	3 (10.3%)	0	0	3 (10.3%)	3 (10.7%)	0	0	3 (10.7%)	1.00
Anemia	6 (20.7%)	0	0	6 (20.7%)	5 (17.9%)	2 (7.1%)	0	7 (25.0%)	0.70
Fatigue	4 (13.8%)	0	0	4 (13.8%)	8 (28.6%)	3 (10.7%)	0	11 (39.3%)	**0.04**
Hypertension	1 (3.4%)	1 (3.4%)	0	2 (6.9%)	2 (7.1%)	9 (32.1%)	0	11 (39.3%)	**<0.01**
Nausea	2 (6.9%)	1 (3.4%)	0	3 (10.3%)	4 (14.3%)	0	0	4 (14.3%)	0.71
Neutrophil count decreased	5 (17.2%)	5 (17.2%)	4 (13.8%)	14 (48.3%)	8 (28.6%)	4 (14.3%)	6 (20.7%)	18 (64.3%)	0.22
Peripheral sensory neuropathy	5 (17.2%)	0	0	5 (17.2%)	6 (21.4%)	1 (3.4%)	0	7 (25.0%)	0.47
Proteinuria	0	0	0	0	1 (7.1%)	0	0	1 (3.6%)	0.49
Thrombo-embolic event	0	1 (3.4%)	0	1	1 (3.6%)	0	0	1 (3.6%)	1.00
Vomiting	2 (6.9%)	0	0	2 (6.9%)	5 (17.9%)	0	0	5 (17.9%)	0.25
White blood cell decreased	7 (20.7%)	7 (24.1%)	0	14 (48.3%)	7 (24.1%)	4 (13.8%)	2 (6.9%)	13 (44.8%)	0.89
Total number of adverse events	49	21	6	76	78	35	9	122	

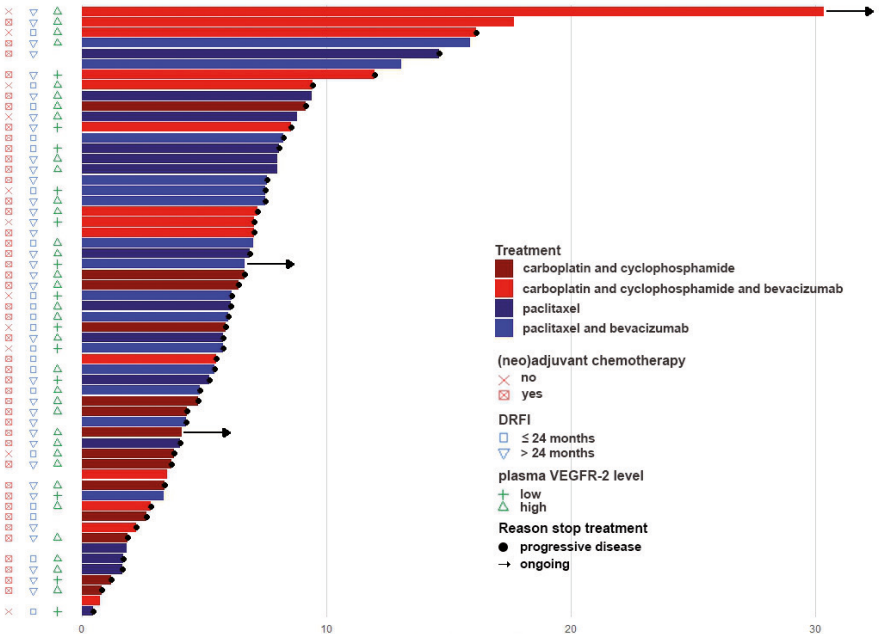
Supplemental table 2. Adverse events, split by chemotherapy backbone, that were at least possibly related to the study treatment and observed in at least 10% of all patients. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel; * Two-sided chi-square test or Fisher's exact test

	CC ± B (n=27)				**P ± B (n=30)**				
	grade 2	grade 3	grade 4	all grades	grade 2	grade 3	grade 4	all grades	p-value
Alopecia	0	0	0	0	6 (20.0%)	0	0	6 (20.0%)	**0.03**
Anemia	9 (33.3%)	2 (7.4%)	0	11 (40.7%)	2 (6.7%)	0	0	2 (6.7%)	**<0.01**
Fatigue	6 (22.2%)	3 (11.1%)	0	9 (33.3%)	6 (20.0%)	0	0	6 (20.0%)	0.25
Hypertension	2 (7.4%)	5 (18.5%)	0	7 (25.9%)	1 (3.3%)	5 (16.7%)	0	6 (20.0%)	0.59
Nausea	6 (22.2%)	1 (3.7%)	0	7 (25.9%)	0	0	0	0	**<0.01**
Neutrophil count decreased	3 (11.1%)	4 (14.8%)	10 (37.0%)	17 (63.0%)	10 (33.3%)	5 (16.7%)	0	15 (50.0%)	0.33
Peripheral sensory neuropathy	2 (7.4%)	0	0	2 (7.4%)	9 (30.0%)	1 (3.3%)	0	10 (33.3%)	**0.02**
Vomiting	7 (25.9%)	0	0	7 (25.9%)	0	0	0	0	**<0.01**
White blood cell decreased	6 (22.2%)	9 (33.3%)	2 (7.4%)	17 (63.0%)	8 (26.7%)	2 (6.7%)	0	10 (33.3%)	0.03
Total number of adverse events	67	34	14	115	60	22	1	83	

Supplemental table 3. Treatment exposure. CC = carboplatin and cyclophosphamide, B = bevacizumab, P = paclitaxel

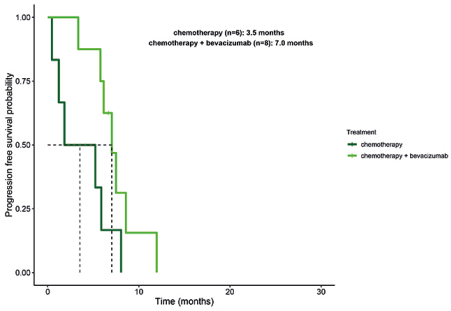
	CC (n=14)	CC + B (n=13)	P (n=15)	P + B (n=15)
Median number of cycles (IQR)				
carboplatin	4 (2.8-6)	6 (5-7)		
cyclophosphamide	4 (2.8-6)	6 (5-7)		
paclitaxel			5 (4-7)	6 (4-7)
bevacizumab		8 (5-11.5)		6 (5-8)
Mean relative total dose intensity, % (SD)	96.9 (7.6)	89.6 (14.9)	94.6 (8.5)	88.3 (14.1)
Total no. of cycles				
carboplatin	61	82		
cyclophosphamide	61	82		
paclitaxel			84	84
bevacizumab		123		88
No. of cycles reduced in dose due to toxicity	8	20	10	39
No. of cycles delayed due to toxicity	4	9	1	4
No. of cycles omitted due to toxicity	0	7	3	10
No. of patients continuing B after stop chemotherapy		6		3
Reasons of treatment discontinuation				
progressive disease	13	11	11	10
adverse event(s)		1	2	1
patient's request			2	2
physician's choice				1
ongoing	1	1		1

Supplemental figure 1. Time to progression of the per-protocol-treated population. DRFI = distant recurrence free interval

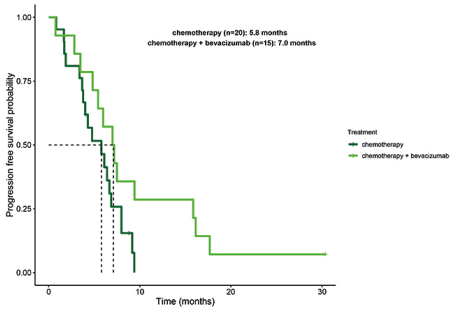


Supplemental figure 2. Association between treatment and progression free survival (PFS) of the intention-to-treat population (**a, b**) and the per-protocol treated population (**c, d**), split by prespecified cut-off into a low VEGFR-2 level (≤ 7.15 ng/ml) subgroup and a high VEGFR-2 level (> 7.15 ng/ml) subgroup.

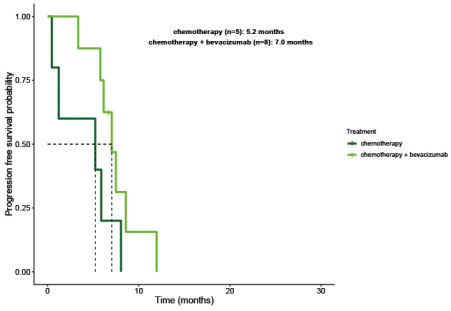
a. low VEGFR-2 level



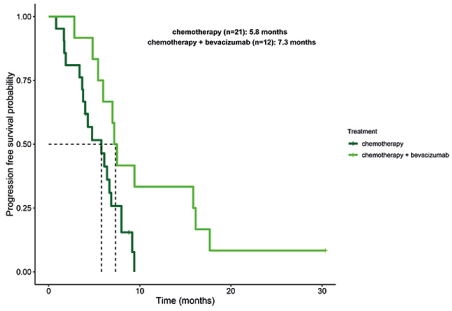
b. high VEGFR-2 level



c. low VEGFR-2 level



d. high VEGFR-2 level



8

General discussion

The aim of this thesis was to identify biomarkers that predict efficacy or toxicity of systemic treatment for early or metastatic breast cancer. We analyzed markers in four clinical trials. In this general discussion, we will highlight our main findings and put them in the light of current practice. We will discuss the assets, shortcomings and possible solutions of our research and those of other investigations.

Efficacy of adjuvant chemotherapy

Efficacy of adjuvant chemotherapy has improved substantially over the past decades. The first-generation schedules reduced breast cancer mortality by 35% compared with no chemotherapy. Second and third generation regimens each diminished mortality by another 20% compared with the previous chemotherapy generation.[1] Currently, four third-generation chemotherapeutic regimens are used in the clinic in the Netherlands. All of these regimens were associated with improved survival when directly compared with a second generation schedule. Direct comparison of two treatment regimens in a randomized, clinical trial setting provides the highest single-study level of evidence for superiority of one treatment over another, or the lack thereof.

The BCIRG 001 trial showed that 6 cycles of docetaxel, doxorubicin and cyclophosphamide (TAC) resulted in longer 5-year disease free survival (DFS) and overall survival (OS) compared with a second-generation regimen of 6 cycles of fluorouracil, doxorubicin and cyclophosphamide (FAC) in breast cancer patients with lymph node positive disease.[2,3]

To find equally effective or superior regimens, third-generation TAC was used in several other studies, including the BCIRG 005 trial. The BCIRG 005 trial compared 6 cycles of TAC with 4 cycles of AC followed by 4 cycles docetaxel (AC-T), both conventionally scheduled.[4] Due to the additional 2 cycles, the cumulative dose of the chemotherapeutic agents in the TAC arm was higher. Yet, the efficacy of the combination regimens was similar. With emerging evidence that sequential administration of anthracycline and taxane-based chemotherapy might be superior than concurrent, the AC-T regimen of the BCIRG 005 trial was used by Sparano et al to investigate other promising treatment regimens. Patients were randomized between 4 cycles 3-weekly and 12 weekly cycles of docetaxel or paclitaxel after AC. Weekly paclitaxel improved DFS and OS compared with 3-weekly paclitaxel. Also 3-weekly docetaxel was superior regarding DFS than 3-weekly paclitaxel, but not regarding OS. Although weekly paclitaxel caused more neuropathy of any grade compared with the other treatment arms, it was associated with less grade 3 and 4 adverse events in general compared with 3-weekly docetaxel. These results were confirmed after a median follow up of 12.1 years.[5] Both 4 cycles

AC followed by 12 weekly cycles of paclitaxel and 4 cycles AC followed by 4 cycles of 3-weekly docetaxel are currently-used third-generation chemotherapeutic regimens.

Finally, three cycles of fluorouracil-epirubicin-cyclosphosphamide followed by 3 cycles of docetaxel ($FE_{100}C$-D) is a currently-used, third-generation treatment that showed improved outcome compared with a second generation regimen of 6 cycles $FE_{100}C$ in breast cancer patients with lymph node positive disease.[6]

In **chapter 1**, we reported on the direct comparison of the efficacy of a third-generation regimen and a more experimental, dose dense treatment. Patients were randomized between 6 cycles of dose dense scheduled doxorubicin and cyclophosphamide (ddAC) and 6 cycles of docetaxel, doxorubicin and cyclophosphamide (TAC). With 7 years of median follow up, the MATADOR trial showed that recurrence free survival (RFS) and OS were not significantly different between 6 cycles ddAC and 6 cycles TAC. This study was the first to describe a head-to-head comparison of these regimens.

Although comparing the efficacy of a particular treatment in one study with the results of that particular treatment in another may be useful, it is crucial to take the case mix of patients into consideration. Even if the treatment and the endpoint are similar, comparisons of efficacy may be hampered by the characteristics of the included patients. For TAC, we compared our findings with the results of the BCIRG001 trial.[2,3] Although the 5-year OS rate in the TAC-treated group of our study was substantially higher than in the TAC-treated BCIRG001 trial, 19% of the patients in the MATADOR cohort had lymph node negative disease. To our knowledge, the CALGB 40101 trial[7] is the only study that assessed the toxicity and efficacy of 6 cycles ddAC. Patients with operable breast cancer were initially randomized between 4 or 6 cycles of either AC or paclitaxel (T) every 3 weeks. Based on convincing evidence from the CALGB 9741 trial that dose dense chemotherapy (either sequential or concurrent 4 cycles A and C followed by paclitaxel, every 2 weeks) leads to improved outcome, AC and T were administered every 2 weeks.[8] The trial did not show non-inferiority of T over AC. Although the survival rates of the CALGB 40101 trial and our study were similar, all patients included in the CALGB 40101 trial had 0-3 positive lymph nodes compared with only 18% of the patients in the MATADOR trial. Also, a separate analysis on the efficacy of 6 cycles of ddAC was lacking, hampering the comparison. Even though subgroup analyses on the comparisons of 3 weekly versus 2 weekly chemotherapy and of 4 cycles versus 6 cycles did not change the conclusions, the comparison should be interpreted with caution.

Many other adjuvant chemotherapy regimens exist. However, direct comparisons with clinically-used treatments are sometimes lacking. For these regimens, it may be challenging to determine its value in current practice. A recent meta-analysis confirmed the increased survival rates after sequential anthracycline and taxane-based chemotherapy.[9] Also, dose dense administration of chemotherapy appeared to result in a better outcome than conventionally scheduled chemotherapy.[9] Sequentially given, dose dense chemotherapy was investigated in **chapter 4** of this thesis. In the German Adjuvant Intergroup Node-positive Study 2 (GAIN-2), the investigators intended to improve on the dose dense principle by increasing the dose of the sequentially given drugs (dose intensified epirubicin, paclitaxel and cyclophosphamide, iddETC) or by adding a fourth compound to the treatment (epirubicin-cyclophosphamide followed by paclitaxel-capecitabine, EC-TX). Although the addition of capecitabine led to more toxicity, iddETC and EC-TX were not significantly different regarding disease free survival.[10] The position of these treatments in the current chemotherapeutic landscape is difficult to assess, since iddETC has not been compared with clinically-used treatments. A previous head-to-head comparison of iddETC and 4 cycles of epirubicin and cyclophosphamide followed by 4 cycles of paclitaxel every 3 weeks (EC-P) showed that iddETC improved overall survival.[11,12] Assuming that EC-P is not substantially different from AC-T as used by Sparano et al[13], these results may indicate that iddETC has at least similar efficacy as the currently used regimens.

Adjuvant versus neoadjuvant chemotherapy

The chemotherapy treatments mentioned above were administered in the adjuvant setting, e.g. after locoregional treatment consisting of surgery and radiotherapy. However, neoadjuvant chemotherapy given before surgery has gained interest in the Netherlands.[14] Neoadjuvant chemotherapy has two major advantages: 1. it can downsize the tumor, providing a higher chance of breast conserving surgery or enabling mastectomy to an initially irresectable tumor; 2. it may provide insight in the sensitivity or resistance pattern of the tumor to chemotherapy.[15] A large meta-analysis of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) showed that indeed patients who were treated with neoadjuvant chemotherapy had a higher frequency of breast conserving surgery compared with patients who received adjuvant chemotherapy.[16] However, use of neoadjuvant chemotherapy was associated with higher rates of locoregional recurrences. Besides, distant recurrences and breast cancer mortality were not significantly different between neoadjuvant and adjuvant chemotherapy. Why would we want to use neoadjuvant chemotherapy? During neoadjuvant treatment, the response of a tumor may be assessed by imaging. If a tumor does not diminish

in size, the chemotherapy regimen may be switched to increase the response rate.[17] Also, neoadjuvant chemotherapy may result in a pathologic complete response, e.g. no detectable tumor tissue at surgery, which is associated with favorable outcome.[18] In contrast, tumors without a pathologic complete response may benefit from additional adjuvant chemotherapy. The CREATE-X study showed that adjuvant capecitabine in patients with residual disease after neoadjuvant treatment with anthracyclines, taxanes or both resulted in longer survival, particularly for the subgroup with triple negative disease.[19] In addition, changes in expression of hormone receptors and the human epidermal growth factor receptor 2 (HER2) after neoadjuvant chemotherapy may have implications for adjuvant endocrine or anti-HER2 treatment.[20]

Predictive biomarkers

Most currently used treatments have proven their efficacy in a general breast cancer population. The clinical trials primarily aimed to directly compare two treatments to show superiority of one treatment over another and to determine the position of these treatments in guidelines. However, patients respond differently to the various treatments. Due to inconclusive subgroup analyses based on clinicopathologic characteristics or blood or tissue-derived biomarkers, guidelines still lack a tailored treatment advice. However, growing knowledge on the heterogeneity of tumors forces us to invest in acquiring additional information on treatment sensitivity or resistance. Every newly developed trial should contain at least one predefined objective, either primary or secondary, to find predictive biomarkers for the investigated treatments. Even if the subgroups that arise from the biomarkers in these trials may be too small to draw conclusions, meta-analyses that use all available information may provide information on the true value of a predictive biomarker.

The level of evidence of biomarker investigations is determined by several aspects. Whereas the highest evidential value is obtained when a biomarker is validated as primary objective of a prospective trial, secondary analyses of a retrospective cohort could also identify a potential biomarker.[21] The use of prospectively collected and processed samples are preferred over archived samples and the need to validate findings depends on the level of evidence. However, an important and often neglected aspect of biomarker evaluation is prior knowledge on the tumor biology and the mechanism of action of a drug.

In this thesis, we investigated the predictive value of various biomarkers in different settings, without or with a biological rationale. In **chapter 2 and 3**, we described the

search for predictive biomarkers for efficacy and toxicity of adjuvant chemotherapy in a prospective trial without a clear hypothesis on suitable candidates. **Chapter 4, 5 and 6** contain hypothesis-driven biomarkers investigations, either as retrospective analysis of a clinical trial, as secondary objective of a phase 1b study or as primary objective of a prospective biomarker trial.

Predicting survival benefit and toxicity of chemotherapy schedules in a prospective biomarker trial

Survival benefit

Although taxane-containing regimens are currently used as standard of care for high-risk, lymph node negative or lymph node positive breast cancer patients, some patients derive no benefit from the addition of a taxane. In some cases, it might even be harmful. Predicting which patients derive benefit and which patients do not, would help to tailor treatment. Although several biomarkers for taxane benefit have been described, most investigations were performed in a single arm study.[22-24] The associations found between the biomarkers and outcome in these trials are in fact reflecting the natural course of the disease, e.g. the prognosis of these patients after receiving the investigated treatment. In other studies patients were treated with two or more different chemotherapy regimens, but the investigators did not evaluate the biomarker for these treatments separately.[25,26] Therefore, these explorations are in fact also single arm studies describing prognostic profiles instead of predictive signatures. Despite the shortcoming of a single arm study to provide indisputable evidence for the predictive value of biomarkers, these studies might help to identify predictive signatures. If a biomarker is identified in a single arm study, the predictive capacity of these signatures should be assessed in a prospective biomarker study randomizing between the treatment of interest and at least one other treatment. To our knowledge, only one group of investigators used this approach. In a single arm study, 133 stage I-II breast cancer patients were treated with neoadjuvant treatment of weekly paclitaxel followed by fluorouracil, doxorubicin and cyclophosphamide (T/FAC).[27] A training set of patients was used to build a Diagonal Linear Discriminant Analysis (DLDA) multifactor predictor of pathologic complete response (pCR) to T/FAC. The multifactor predictor consisted of 3 clinical variables (age, estrogen receptor [ER] status and histologic grade) and 30 genes (DLDA-30). In the validation set, the predictor appeared promising with a sensitivity of 92% and a negative predictive value (NPV) of 96%. As a next step, the investigators designed a prospective, randomized trial with the primary objective to validate the DLDA-30 for the prediction of pCR after T/FAC chemotherapy.[28] While

validating the treatment specific predictive value of the profile was the primary aim of the study, the profile-by-treatment interaction was only a secondary objective. The sensitivity of the predictor for pCR was 63% and the NPV 88% in the T/FAC treated patients and only 29% and 92% in the FAC treated subgroup. However, the limited overall sample size and the low number of pCR events (n=7) in the FAC treated subgroup reduced the post hoc determined power to detect a significant interaction to only 14%-50%. Despite the two-step design of the studies to obtain a predictive classifier, it remains elusive whether the DLDA-30 predictor is specific for pCR after T/FAC or predicts pCR in general.

The MATADOR trial was designed as a prospective biomarker study and aimed to find a gene expression profile that could predict survival benefit of either of two treatments. Power calculations were based on the anticipated treatment effects. Also, patients were randomized between two equally effective third-generation adjuvant chemotherapy regimens and prognostic characteristics were balanced between the treatment groups. We used a supervised approach with a specific aim to identify significant associations between gene expression-treatment interactions and outcome. Although we identified a gene expression profile, it appeared to have more prognostic than predictive value.

An important explanation for these findings is related to the type of data. Next generation sequencing of RNA provides information on thousands of genes. To assess the predictive value of the genes, we used a Cox regression-based analysis with gene expression-treatment interaction variables and outcome as main input. Inevitably, the model also requires the expression of the individual genes and treatment as input variables. Whereas significant associations between interactions and survival provide predictive information, significant associations between individual genes and outcome indicate their prognostic value. The abundance of data requires penalization, selecting only the strongest associations to build the model. Since our model contained more individual gene-survival associations than interaction-survival associations, the prognostic signal in our data might have been more pronounced than the predictive signal. Even after correction for known prognostic clinicopathologic factors the profile appeared to have independent prognostic value.

Other explanations are tumor related. In our cohort, a multiplicity of resistance mechanisms against the combination regimens may exist. Identifying one profile that comprises all of these mechanisms is complicated, particularly if resistance mechanisms are not shared by a substantial part of the tumors.[29] Moreover, heterogeneity within tumors might cause varying degrees of sensitivity or resistance to a combination

treatment. Bulk analysis of gene expression data takes the most prevalent types of tumor cells into account, but does not reflect the smaller subclones. If these smaller subclones are indeed the drivers of resistance, the identified profile will not accurately predict survival benefit of a specific treatment. Altogether, a supervised, data-driven approach using gene expression data to find a predictive biomarker is challenging. Next generation sequencing provides an extensive amount of information. Analyzing and interpreting this information requires a multidisciplinary team of translational researchers, including bioinformaticians, statisticians and clinicians. Moreover, without prior knowledge on potentially involved mechanisms, a search for predictive biomarkers in genome-wide data could be considered "a shot in the dark" with limited chance of success. Instead, a more biology-driven way of testing associations between gene expression and survival may provide directions. There are several methods to analyze gene expression data in a biology-driven way using gene set testing methods[30], including the globaltest[31] with the hallmark gene sets as input.

Hallmark gene sets derived from the Molecular Signatures Database (MSigDB) describe 50 biological processes and pathways.[32] These gene sets can be used to explore which processes are associated with a state of disease or with outcome, as has been shown in breast cancer for specific phenotypes[33,34], for fibroblasts[35], and for survival[36]. In our analyses we used the hallmark gene sets to assess the association between expression and survival separately for the ddAC-treated patients and the TAC-treated subgroup. Since the addition of docetaxel was the most evident distinction between the treatments, we hypothesized that the mitotic spindle gene set and possibly the apoptosis gene set would predict which patients would derive a better outcome after TAC, but would not predict survival benefit from ddAC. Instead, we observed that enriched gene expression of immune-related pathways (interferon gamma, allograft rejection, interferon alpha, IL6-JAK-STAT3 signaling, inflammatory response and complement) was associated with longer RFS in patients treated with TAC, but not in the ddAC-treated subgroup of patients with a basal tumor according to the PAM50 subclassification[37,38]. These results raised the question whether the endogenous anti-cancer immune response could predict benefit of docetaxel.

In the early 2000's Chan and Yang argued that docetaxel indeed has an effect on the immune system by changing the levels of cytokines and lymphocytes to stimulate an antitumor response.[39] Preclinical investigations showed that docetaxel enhanced T cell-mediated tumor cell kill[40] and that docetaxel was able to promote differentiation of cultured monocytes of metastatic breast cancer patients into antitumor M1 macrophages[41]. In addition, docetaxel increased the ratio between effector T cells and

regulatory T cells (Treg) in metastatic breast cancer patients[42] and it increased the serum levels of interferon-γ (IFN-γ), interleukin-2 (IL-2) and IL-6 and enhanced the activity of natural killer cells in patients with advanced breast cancer[43]. Although assessed in breast cancer patients treated with combination regimens, gene expression signatures of immune activation have also been linked to improved outcome after docetaxel-based chemotherapy. High expression of an immune signature at baseline was associated with pCR after docetaxel-containing neoadjuvant chemotherapy in hormone receptor positive, HER2 negative breast cancer.[44,45] Two other studies showed that particularly basal tumors or immunohistochemistry-based triple negative breast cancer tumors were associated with high expression of immune signatures.[46,47] TNBC patients treated with docetaxel-containing neoadjuvant treatment had a better outcome if their tumor showed high expression of cytotoxic molecules, T cell receptor signaling pathway components, Th1-related cytokines and B cell markers.[48] Others showed that expression of PD-L1 and CD80 were associated with improved survival after docetaxel-containing chemotherapy in TNBC.[49]

A predictive biomarker should accurately and reliably identify patients with improved survival after a particular treatment. Also, it should be easy and cheap to be clinically applicable. Over the last few years, next generation sequencing has become easier, faster and cheaper. However, the turn-around time and interpretation of genome-wide data limit its use in the clinic. A clinically applicable and easy way of assessing the endogenous anti-cancer immune response is the scoring of tumor-infiltrating lymphocytes (TILs) on a hematoxylin and eosin (H&E) staining. Due to the association between enrichment in immune-related gene expression and RFS in the basal subgroup we evaluated the predictive value of TILs in the TNBC subgroup of our cohort. Patients with a high level of TILs (≥ 20%) in their tumor had a numerically longer RFS after TAC than after ddAC. The opposite was observed for patients with low levels of TILs who derived more benefit from ddAC. In a cohort of patients from two neoadjuvant studies TILs were an independent predictor of response to anthracycline-and-docetaxel-based chemotherapy.[50] However, as many others aiming to assess the predictive value of TILs, this investigation lacked a biomarker-by-treatment interaction calculation. Formally testing such interaction is required to distinguish a potential prognostic marker from a predictive marker.[51] The interaction test between TILs and treatment in the MATADOR study was significant when corrected for known prognostic factors. Validation of our results in an independent cohort is needed to assess whether pretreatment level of TILs might be used to select patients for docetaxel-based or dose dense chemotherapy. In order to validate, the same methods should be applied. The discrepancies between our results and a previous trial that did not find a significant interaction between TILs and

docetaxel-containing chemotherapy[52] might be explained by differences in treatment schedules (A or AC followed by CMF [BIG 02-98 trial: cyclophosphamide – methotrexate – 5-fluorouracil] and A-docetaxel[T] or AT followed by CMF, MATADOR trial: TAC and ddAC), differences in the cut-off for ER positivity (BIG 02-98 trial: > 1%, MATADOR trial: ≥ 10%), or by the differences in cut-off of TILs. Although several studies used the lymphocyte-predominant breast cancer (LPBC) phenotype with ≥ 50% or ≥ 60% TILs[49,50,53], leading to small subgroups, an established threshold to distinguish low and high levels of TILs is lacking. Due to the variation in abundance of TILs in different breast cancer subtypes[54], it seems sensible to define subtype specific cut-offs. The optimal cut-off should have the highest predictive value and, at the same time, should comprise a reasonable proportion of patients. In the MATADOR study, we used a cut-off based on the median, which is similar to the median found in other TNBC cohorts.[52,55] Validating our results in an independent cohort using the same cut-off, and an internationally recognized, reproducible method of TIL scoring[56], would bring us one step closer to using TILs to tailor treatment.

Toxicity

Many associations between toxicity of anthracycline and/or taxane-containing chemotherapy and single nucleotide polymorphisms (SNPs) have been described.[57-64] Although most SNPs were selected based on their role in drug metabolism, none have made it to the clinic yet. In **chapter 3**, we described exploratory analyses of associations between clinicopathologic factors or SNPs and toxicity of the adjuvant chemotherapy regimens used in the MATADOR study. By selecting three frequently occurring, clinically meaningful side effects and replicating previously described associations instead of finding new links, we aimed to contribute to the existing evidence on the associations. The toxicities that we focused on were anemia, febrile neutropenia and peripheral neuropathy. Compared with 4 cycles of ddAC[65] or 6 cycles of conventionally scheduled or dose dense AC[7], anemia was observed more often in our cohort, while the rates of febrile neutropenia were similar[65] or somewhat lower[7]. Peripheral neuropathy was observed in the TAC treated patients as well as in the ddAC treated subgroup at similar rates as in other trials.[66,67] We assessed the association between toxicity and 4 clinical factors as well as 13 SNPs. Higher age and lower baseline platelet count were associated with anemia in the total cohort. *FGFR4* was not significantly associated with febrile neutropenia in all patients, but it had a significant interaction with treatment with a higher risk of febrile neutropenia in wildtype or heterozygous variant carriers treated with TAC and in homozygous variant carriers treated with ddAC. Variants in *TECTA* and to a lesser extend *GSTP1* were associated with peripheral neuropathy. Despite

the confirmation of the associations[60,63,68,69] in our dataset, the underlying biological mechanisms remain largely elusive. Further research is needed to understand the role of these proteins in the occurrence of adverse events.

However, most of the investigated associations were not significant in the MATADOR study, indicating the difficulty of finding clinically meaningful associations. They may partly be explained by limitations in our study. Most associations studies used germline DNA of patients to assess the genetic variants, while we used tumor tissue. Despite the high concordance between genetic variants in 15 pairs of tumor tissue and normal tissue within our cohort, we cannot exclude the possibility that tumor tissue might deviate genetically from the normal tissue in which side effects arise. Also, ethnic origin is linked to the frequencies of genetic variants. Although the majority of our Dutch cohort is likely from Caucasian decent, some of the original studies identified the predictive SNP in a cohort of patients with a different ethnic background. Finally, some genetic variants were infrequent. Even if an association between these variants and toxicity would exist, it would be hard to find the link due to the low numbers of patients with a variant genotype who also encountered the adverse event of interest. Consequently, the power to find significant associations between the toxicity and the SNPs was limited.

Some limitations are more general and shared by most biomarker investigations that aim to predict toxicity. First, many of the biomarker studies had a primary objective other than predicting toxicity. Also, the MATADOR study was not primarily designed to find predictive markers for adverse events. These studies lack power to find associations between toxicity and SNPs. Validation of observed associations in independent datasets is essential to test the clinical value of the findings. Second, some studies assessed a large number of genetic variants and thereby increased the risk of type 1 error. Genome wide association studies (GWAS) are designed to find associations between toxicity and SNPs. However, these studies aim to test thousands of associations, leading to a higher rate of false positive findings. New analytical methods to control for the type 1 error have been proposed.[70,71] Finally, a biological rationale for the association between toxicity and a genetic variant was often lacking or too weak. To our knowledge, the only genetic variant that is currently used in the clinic has a strong biological rationale based on the mechanism of action of the drug. Dihydropyrimidine dehydrogenase (DPD) plays an important role in the metabolism of capecitabine. DPD activity is used to assess the risk of toxicity[72] and employed to adjust treatment strategy. Therefore, DPD is an example of a clinically valuable biomarker. It accurately predicts potentially life-threating or severe long-term complications and changes treatment decisions.

Summarizing, there is still a lot to gain in the field of predicting toxicity. Investigations should start off with a hypothesis based on a biological rationale, the sample size should be sufficient, efforts should be made to control for type 1 error, and independent validation is essential to find predictive markers of toxicity.

Predicting survival benefit of adjuvant chemotherapy schedules in a post-hoc analysis

In **chapter 4**, we tested a hypothesis-based biomarker, the *BRCA1*-like profile, for the efficacy of adjuvant chemotherapy in a post-hoc manner. The *BRCA1*-like profile is a DNA copy number-based classifier reflecting the genomic instability that arises when the BRCA1 protein is inactive. An inactive BRCA1 protein can be the result of a mutation in the *BRCA1* gene, hypermethylation of the *BRCA1* promotor or a yet unknown cause. Previous reports showed that tumors that displayed a *BRCA1*-like profile were more sensitive to high dose alkylating agents and platinum compounds than to conventionally dosed chemotherapy.[73-75]

The GAIN-2 study randomized lymph node positive breast cancer patients between 3 x 3 cycles of non-myeloablative intensified, dose dense, sequential epirubicin, paclitaxel and cyclophosphamide (ETC) and 4 cycles of dose dense epirubicin combined with cyclophosphamide followed by 10 cycles of weekly paclitaxel and 4 cycles of capecitabine (EC-TX). Due to the intensified nature of the ETC arm, the cumulative dose of alkylating agent cyclophosphamide was higher than in the EC-TX arm. In our retrospective study we aimed to validate the *BRCA1*-like profile as predictive marker for survival benefit of intensified alkylating agent cyclophosphamide. However, DFS was not significantly different between ETC or EC-TX in patients with a *BRCA1*-like tumor, nor in their non-*BRCA1*-like counterparts. On the contrary, EC-TX resulted in a numerically longer DFS in patients with *BRCA1*-like tumor and ETC in patients with a non-*BRCA1*-like tumor with a borderline non-significant interaction between *BRCA1*-like status and treatment. When the *BRCA1*-like subgroup was split by lymph node status, DFS was even significantly longer after EC-TX than after ETC in patients with 10 or more positive lymph nodes. These findings suggest that not ETC, but EC-TX is more effective in patients with a *BRCA1*-like tumor, particularly in the event of extensive lymph node involvement. These findings may be explained by three treatment-related factors. First, increasing the dose and intensity of cyclophosphamide to higher than standard treatment may not improve outcome, as was indicated by other groups[76,77]. Even if a sensitive subgroup exists, the *BRCA1*-like profile might not be able to indicate which patients would benefit from a higher than standard dose of cyclophosphamide. Secondly, *BRCA1*-like tumors

have been associated with relative resistance to taxanes.[78] With sequential ETC, single agent paclitaxel during 6 weeks might have provided *BRCA1*-like tumors time to regrow. Because in the EC-TX arm paclitaxel was combined with capecitabine, the latter might have eradicated taxane-resistant cells. Thirdly, capecitabine may be of added value to a combination treatment with epirubicin, cyclophosphamide and paclitaxel in patients with a *BRCA1*-like tumor. Metabolites of capecitabine have been associated with disrupted RNA synthesis and imbalances in the deoxynucleotide pool that is needed to repair DNA damage.[79] In this way, capecitabine could increase the amount of DNA damage, which will affect the cell viability. Because repair of DNA damage is impaired in *BRCA1*-like tumors, capecitabine may be very effective in this subgroup of tumors.

Evaluating the predictive value of the *BRCA1*-like profile in the GAIN-2 trial cohort was a retrospective, post-hoc analysis. With a sample size based on the primary endpoint, retrospective biomarker analyses are hardly ever sufficiently powered. However, expanding the proportion of biomarker positive patients could be used to improve the efficiency of the analyses.[80] By selecting only patients with TNBC, we tried to enrich for patients with a *BRCA1*-like tumor. However, the size of the analyzed subgroups was limited by the number of enrolled TNBC patients and by the availability of tumor tissue to determine the *BRCA1*-like status. Therefore, our findings should be interpreted with caution.

Although we could not confirm our initial theory, these analyses may have led to another hypothesis. Given its role in repair of DNA damage, it seems credible that capecitabine could be of added value in the treatment of *BRCA1*-like disease. Further research may elucidate the exact mechanism of action of capecitabine in *BRCA1*-like tumors. Validation in an independent cohort is needed to confirm that capecitabine is an effective drug in patients with a *BRCA1*-like tumor. Although using different chemotherapy backbones, the CREATE-X trial[19] and the FinXX trial[81] both investigated the addition of capecitabine and would therefore be suitable to test the predictive capacity of the *BRCA1*-like profile. If the retrospective analyses of the *BRCA1*-like profile in these trials are inconclusive, a final step could be to evaluate the *BRCA1*-like profile as primary objective of a prospective biomarker trial in which TNBC patients are randomized between a capecitabine-containing treatment and a non-capecitabine-containing therapy.

Predicting survival benefit of targeted treatment in a phase 1b study

Phase 1 studies generally aim to find the recommended dose and schedule of a drug or combination of drugs for phase II investigations.[82] Multiple stepwise approaches are used to escalate the dose and test different schedules. The most frequently used method is the traditional 3+3 design. In **chapter 5**, we used an algorithm-based extension of this design, the rolling 6 design, to determine the recommended phase 2 dose and schedule of phosphatidylinositol 3–kinase (PI3K) inhibitor taselisib when combined with standard dose of tamoxifen. In addition, we tried to identify biomarkers that might predict survival benefit of taselisib, including a biomarker with a strong biological rationale.

In ER positive breast cancer, the PI3K pathway has been described as an important escape route for tumor cells to grow and proliferate.[83-85] Simultaneously blocking both the ER pathway and the PI3K pathway has shown to improve survival at the expense of toxicity.[86,87] In order to reduce toxicity, taselisib was designed to specifically target the α isoform of the p110 catalytic subunit of PI3K. In line with other phase 1b trials that investigated taselisib combined with endocrine treatment, we found that taselisib and tamoxifen is a safe combination for the treatment of ER-positive breast cancer and should be dosed at 4 mg QD in tablet formulation.

Taselisib binds to PI3K at the ATP-binding pocket to block its interaction with other PI3K pathway components. In vitro studies have shown that taselisib has a greater potency against cell lines with a mutation in the gene that encodes for the p110α subunit of PI3K, the *PIK3CA* gene.[88,89] Also, a preclinical study indicated that taselisib selectively reduced protein levels of mutant p110α.[90] Therefore, we and others hypothesized that patients with *PIK3CA* mutant breast cancer would derive more benefit from taselisib compared with patients with wildtype disease.

After dose finding, the combination of fulvestrant and taselisib was further tested in a single arm phase II study.[91] Twenty out of 47 evaluated patients (42.5%) had a *PIK3CA* mutation. In patients with measurable disease, the clinical benefit rate (CBR; complete response, partial response or stable disease as best response) was higher for patients whose tumor harbored a *PIK3CA* mutation than for patients with *PIK3CA* wildtype disease or patients with unknown tumor *PIK3CA* mutation status (38.5% vs 23.8% vs 20.0%, respectively). The subsequent phase 3 SANDPIPER trial[92] was designed as a subgroup-specific study[93] with the focus on patients with *PIK3CA* mutant disease. ER positive, HER2-negative advanced or metastatic breast cancer patients were randomized

(2:1) between fulvestrant and taselisib and fulvestrant and placebo. Out of 631 patients, 516 patients (81.7%) had *PIK3CA* mutant disease. In this subgroup, the objective response rate (ORR; complete response or partial response as best response) was significantly higher in the fulvestrant and taselisib arm compared with the fulvestrant and placebo arm (28% vs 11.9%). Also, progression free survival was significantly longer in the fulvestrant and taselisib arm. In an exploratory analysis of the patients with *PIK3CA* wildtype disease, responses followed the same trend, but were not significant. In the double-blind, phase 2 LORELEI trial[94], patients were randomized between letrozole and taselisib or letrozole and placebo as neoadjuvant treatment. Using a randomized phase II screening design[93], the sample size calculation was based on the anticipated ORR and the pCR rate in the patients with a *PIK3CA* mutation. Most grade 3 or higher adverse events were observed in the letrozole and taselisib arm. *PIK3CA* mutations were observed in 152 patients (45.5%). The ORR was higher for letrozole and taselisib (56.2%) compared with letrozole and placebo (38%) in the patients with a *PIK3CA* mutation, and in all patients (50% versus 39.3%). There were no significant differences in pCR rates.

In the small group of patients treated in the phase 1b part of the POSEIDON study, we identified a *PIK3CA* mutation in 8 (27%) out of 30 metastatic breast cancer patients. An objective response was observed in 6 (24%) out of 25 patients with measurable disease. In the subgroup with a *PIK3CA* mutation, 3 patients (37.5%) had a confirmed partial response.

Although these studies showed that responses were more evident and more abundant in the patients with *PIK3CA* mutant disease, responses were also observed in patients with *PIK3CA* wildtype disease. Moreover, the subgroup-specific design of the SANDPIPER trial hampers the interpretation of the analyses in the *PIK3CA* wildtype subgroup. The LORELEI trial based the overall sample size on the treatment effect in the patients with *PIK3CA* mutant disease. A smaller but significant treatment effect in the patients with *PIK3CA* wildtype disease may therefore be missed. Recently, the SOLAR-1 study showed that a different α isoform-specific inhibitor of PI3K alpelisib combined with fulvestrant substantially prolonged progression free survival (PFS) of patients with *PIK3CA* mutated disease compared with placebo and fulvestrant.[95] Also in this study the focus was on patients with *PIK3CA* mutated disease. Although the difference in progression free survival was not significant in the non-mutated subgroup, we cannot rule out that this subgroup will not benefit. The phase 1b part of our study was not designed to detect a different treatment effect based on *PIK3CA* mutation status. Therefore, discarding taselisib for patients with *PIK3CA* wildtype disease may be too early. Phase 2 of the POSEIDON study will include both patients with *PIK3CA* mutant

disease and patients with *PIK3CA* wildtype disease and will be able to assess whether the association between *PIK3CA* mutation status and taselisib treatment is significant for PFS. Because activating *PIK3CA* mutations are more common in lobular breast cancer (40%) than in ductal breast cancer (25%), the second part of phase 2 will focus on lobular breast cancers.

To gain insight into genomic alterations, including mutations, tumor tissue is required. Apart from resected material during surgery, tumor tissue can be obtained in the form of a biopsy. However, biopsies comprise only a part of one lesion and might therefore not reflect the heterogeneity of the disease. Furthermore, taking a biopsy is an invasive procedure. Circulating tumor DNA (ctDNA) is present in the plasma or serum of patients and can be used as a liquid biopsy.[96,97] ctDNA might be of value at different stages of disease: in screening to detect early signs of cancer, in molecular profiling to determine prognosis, in detecting residual disease, in monitoring response to treatment, and in mapping clonal evolution.[98] In metastatic breast cancer, serial measurements in 30 patients showed that ctDNA had a wider dynamic range than tumor marker CA15.3 and circulating tumor cells to estimate the burden of disease.[99] Also, changes in ctDNA levels were the first sign of treatment response in more than half of the patients. Others showed that quantification of mutant allele fractions in ctDNA provide information on possible resistance mechanisms that may arise after exposure to a certain drug.[97] When selecting the right application, ctDNA might even be used to predict treatment benefit.

In phase 1b of the POSEIDON study, we performed serial plasma sampling for ctDNA analyses of all patients. In two patients, change in the allele fraction of mutant *PIK3CA* was indicative of treatment response. In two other patients, an increase in mutant allele fraction of several genes preceded evaluation scans that showed progressive disease. Although the sample size is limited, our analyses underline the evidence that ctDNA can provide valuable information on treatment response, progression, and potentially targetable mutations. In phase 2 of the POSEIDON study, serial ctDNA samples will be obtained in 180 patients. In addition to evaluating baseline *PIK3CA* mutation status as predictive biomarker for taselisib, these samples may provide more insight in early signs of progression and resistance mechanisms.

Besides mutation analyses, ctDNA can be used to detect loss of heterozygosity (LOH)[100], to assess the integrity of the tumor DNA[101], and to determine the methylation status of specific genomic regions[102]. Also, the number of circulating nucleosomes in ctDNA samples might provide information on the extend of tumor cell death.[103,104] ctDNA analysis a highly interesting field of research due to the minimally invasive method of

sampling and the abundance of information that can be obtained from it. However, sample handling and processing need to be optimized. Furthermore, a hypothesis with a clear biological rationale is needed to use ctDNA for biomarker development. Finally, a team of experts is required to interpret and assess the clinical utility of ctDNA-derived biomarkers.

Predicting survival benefit of targeted treatment in a prospective biomarker trial

Although validation of a previously described biomarker instead of identifying a new biomarker increases the chance of success, prior knowledge on tumor biology and the mechanism of action of a drug are pivotal for biomarker identification. However, even if a clear biological rationale exists, it may be challenging to validate potential biomarkers. In **chapter 6** we described a prospective trial that aimed to validate a biology-based biomarker for bevacizumab addition.

Monoclonal antibody bevacizumab inhibits angiogenesis by blocking vascular endothelial growth factor A (VEGF-A).[105] Although bevacizumab appeared to have only a modest effect on survival in patients with various molecular breast cancer subtypes[106-108], it was shown that angiogenesis may be particularly important in TNBC.[109-111] Therefore, we evaluated bevacizumab added to first-line chemotherapy for metastatic TNBC. We described the results of the interim analyses of the phase 2b Triple-B study in which patients were randomized between four treatment regimens: carboplatin and cyclophosphamide without (CC) or with bevacizumab (CC + B) and paclitaxel without (P) or with bevacizumab (P + B). CC and CC + B appeared to be safe as first-line treatment for metastatic TNBC. In line with previous reports[107,108], we found that bevacizumab addition resulted in a significantly longer PFS in univariable analyses. However, corrected for known prognostic factors PFS was no longer significantly different. Also, we found no significant OS benefit of bevacizumab. Designed as a biomarker-by-treatment interaction trial, the primary objective was to assess the predictive value of two biomarkers: 1. validate the *BRCA1*-like profile as predictor of improved outcome after alkylating agents and platinum compounds compared with taxane treatment, and 2. assess the predictive capacity of baseline plasma VEGF receptor 2 (pVEGFR-2) level for survival benefit of the addition of bevacizumab to first-line chemotherapy for metastatic TNBC. Whereas retrospective studies showed that pVEGF-A and pVEGFR-2 level could predict which patients would derive benefit from bevacizumab[112,113], the prospective MERiDiAN trial could not confirm the predictive capacity of pVEGF-A for improved outcome after bevacizumab.[114] Due to slow accrual and emerging evidence from the

MERiDiAN trial that also pVEGFR-2 level had limited predictive value[115], we deemed it necessary to discard the primary objective on pVEGFR-2 and close off the bevacizumab part of the trial. Therefore, our analyses of pVEGFR-2 were performed in only 58 of the anticipated 304 patients. We could not validate pVEGFR-2 as predictive biomarker for survival benefit of bevacizumab addition. However, the limited size of our cohort urges us to carefully draw conclusions.

Together with the previous investigations on the value of bevacizumab for breast cancer, our study raises two important questions: 1. why does bevacizumab improve only PFS, but not OS; and 2. if both VEGF-A and VEGFR-2 are not the imperative predictive markers for bevacizumab, which markers do predict a survival advantage. Bevacizumab seems to have a temporary effect on disease progression without prolonging overall survival. This may be explained by the use of other angiogenic pathways than the VEGF pathway to form new blood vessels.[116] Targeting only the VEGF pathway might work initially, but will be insufficient to impair all angiogenic signals. A tumor could even employ an alternative route within the VEGF pathway to promote angiogenesis. With bevacizumab blocking only VEGF-A, other VEGF isoforms might be able to take over. Also, VEGF levels have been associated with impaired maturation of dendritic cells via VEGFR-1.[117,118] Blocking VEGF-A with bevacizumab stimulated the differentiation of dendritic cells and increased T cell proliferation rates.[117,119] Because other isoforms of VEGF, such as VEGF-B[120], also binds to VEGFR-1, the initial antitumor response starting with maturation of dendritic cells may be canceled out when these isoforms attach to the receptor.

Despite the possibly temporal effect of bevacizumab, it clearly modulates the immune microenvironment. This alternative effect of antiangiogenic treatment might shed light on a different way to employ this class of drugs. In a breast cancer xenograft model, anti-VEGF treatment inhibited the infiltration of suppressive immune cells while it increased the mature dendritic cell fraction.[121] Others showed that low dose anti-VEGFR-2 treatment polarized tumor-associated macrophages from protumor M2-like to antitumor M1-like and that the combination of low dose anti-VEGFR-2 treatment and cancer vaccine therapy elicited a CD8 T cell dependent anticancer effect.[122,123] Also, it has been reported that antiangiogenic therapy upregulated adaptive immunosuppressive pathways (programmed death 1 [PD-1]/ programmed death ligand 1 [PD-L1]) that may be targeted by immune checkpoint inhibitors.[124] Considering these findings, antiangiogenic treatment could be used as induction therapy to improve the efficacy of immunotherapy. The combination of bevacizumab and atezolizumab without or with chemotherapy has shown to improve survival in other tumor types.[125,126] Recently, the combination was approved as first-line treatment for metastatic non-squamous non-

small cell lung cancer by the Food and Drug Administration (FDA). Currently, several trials are ongoing investigating the combination of antiangiogenic treatment with immune checkpoint inhibition (NCT03395899, NCT03424005) for treatment of breast cancer.

When the primary objective on pVEGFR-2 was discarded, the Triple-B study was amended to replace add-on bevacizumab with programmed death-ligand 1 (PD-L1) inhibitor atezolizumab. TNBC expresses PD-L1 or PD-1 to evade the immune system.[127,128] Binding of PD-L1 or PD-1 to its counterpart on T cells inhibits T cell proliferation and cytokine production.[129,130] Blocking this interaction through administration of a PD-1 or PD-L1 antibody elicits an antitumor response.[131-133] Several phase 1 and 2 studies have shown that PD-L1 or PD-1 blockade without or with chemotherapy has promising antitumor activity.[131-134] Recently, the phase 3 IMpassion130 trial confirmed the additive value of atezolizumab to chemotherapy.[135] Metastatic TNBC patients who were randomized to receive atezolizumab and nab-paclitaxel had a significantly longer PFS than patients who were treated with placebo and nab-paclitaxel. Subgroup analyses showed that the differential treatment effect seemed more pronounced in the PD-L1 positive tumors. Also, an interim analysis of OS showed a substantial benefit of atezolizumab in the PD-L1 positive subgroup. The Triple-B study aims to confirm these findings and is the first study to assess the added value of atezolizumab to carboplatin-cyclophosphamide.

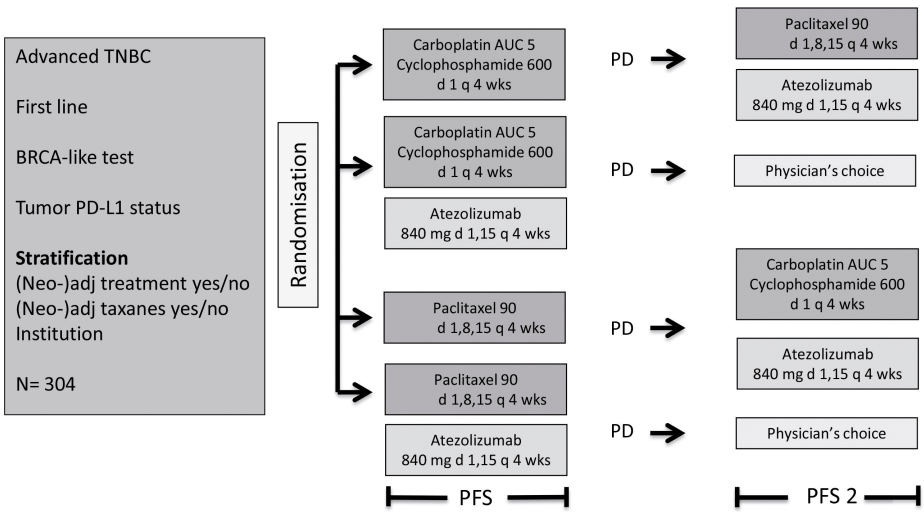


Figure 1. Design of the Triple-B study after the amendment.

CONCLUSION

In conclusion, finding biomarkers that predict efficacy or toxicity of systemic treatments for early or metastatic breast cancer is challenging. Although the amount of studies is overwhelming, the level of evidence of the investigations varies widely. Retrospective analyses of a breast cancer cohort may serve to identify a potential biomarker. However, an adequately powered prospective trial that primarily aims to validate a biomarker is often missing. To have the potential to make it to the clinic, a biomarker should meet three criteria[136]: 1. it should be able to accurately identify patients with the same genotype or phenotype, called analytic validity, 2. it should be able to detect or predict a disorder, named clinical validity, and 3. its assessed risk should warrant a change in disease management, known as clinical utility. On top of that, each biomarker evaluation should start with a biological rationale. This requires prior knowledge on the biology of a tumor and on the mechanism of action of a particular drug. The only currently used predictive biomarkers for survival benefit were derived from an evident link between the biomarker and the mechanism of action of the drug, e.g. ER for endocrine therapy and HER2 for anti-HER2 treatment. Also, DPD had a strong rationale to predict toxicity of capecitabine. However, as indicated in this thesis, even if a biology-based hypothesis exists, validation of a biomarker may not always be successful. Identifying clinically useful predictive biomarkers is challenging. However, joined efforts of prospective trials to validate promising hypothesis-based predictive biomarkers will bring personalized treatment an important step closer.

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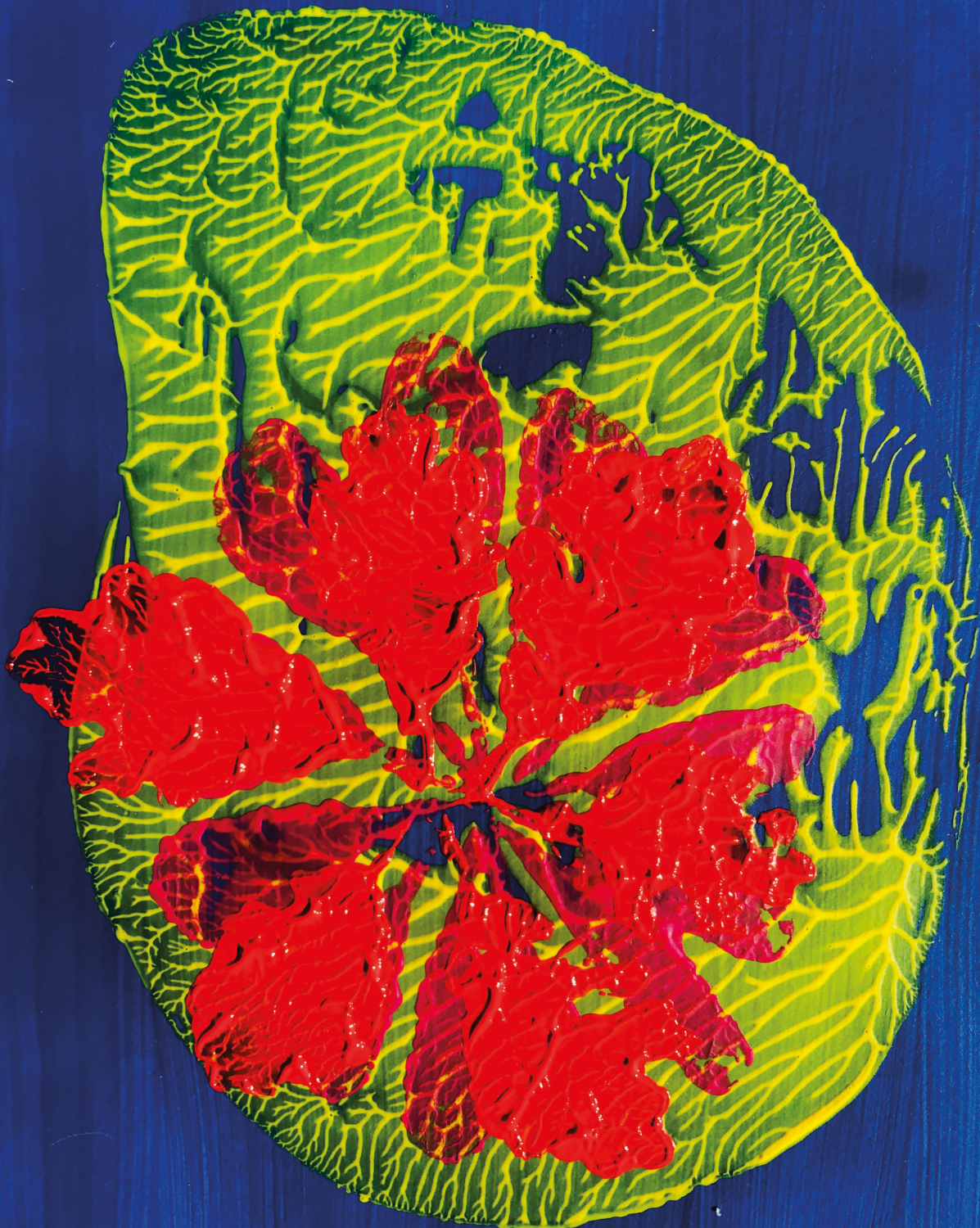

A

Appendix

SUMMARY

Breast cancer is one of the most common types of cancer worldwide.[1] Despite advances in systemic treatment leading to increased survival rates[2], a substantial number of patients still dies of the disease. A personalized treatment strategy is needed to further improve breast cancer survival. Prognostic biomarkers can be used to assess the risk of developing a disease recurrence and thereby the need for systemic treatment.[3] Predictive biomarkers indicate whether a patient derives survival benefit of a specific treatment and are used to tailor therapy.[4] Both types of biomarkers are indispensable for an individualized treatment plan. Although several prognostic markers are currently used in the clinic[5,6], there are only two predictive markers: the estrogen receptor (ER) for endocrine therapy and the human epidermal growth factor receptor 2 (HER2) for anti-HER2 treatment.

In this thesis the identification of putative predictive biomarkers for survival benefit of systemic treatment for early or metastatic breast cancer patients are described. In **chapter 1, 2 and 3** we report on predictive biomarkers for survival benefit and toxicity of adjuvant chemotherapy in the MATADOR trial. **Chapter 4** comprises the assessment of the *BRCA1*-like profile as predictive biomarker for intensified chemotherapy in the GAIN-2 trial. Finally, the first analyses on biomarkers for efficacy of targeted therapy for metastatic breast cancer are presented in **chapters 5 and 6**.

Biomarkers for adjuvant chemotherapy

The addition of taxanes and dose dense scheduling of adjuvant chemotherapy improved breast cancer specific survival substantially.[2,7] However, it is not known whether an individual patient will benefit from taxane-containing chemotherapy, from a dose dense treatment, or both. Therefore, biomarkers are needed to predict treatment benefit. The multicenter, phase 3 MATADOR trial was designed to find predictive biomarkers for taxane benefit. Between 2004 and 2012, 664 patients with pT1-3, pN0-3 breast cancer were randomized between 6 cycles of dose dense scheduled doxorubicin and cyclophosphamide (ddAC) and 6 cycles of conventionally scheduled docetaxel, doxorubicin and cyclophosphamide (TAC). The primary aim of the trial was to identify a gene expression profile that predicts survival benefit of either ddAC or TAC. In **chapter 1** we compared the survival of the two treatments. With a median follow up of 7 years, recurrence free survival (RFS) was not significantly different between ddAC and TAC. Also, overall survival (OS) was not significantly different. To our knowledge, the MATADOR trial is the first trial to directly compare 6 cycles ddAC and 6 cycles TAC. Our

findings indicate that 6 cycles of ddAC would be a valuable alternative for patients who have a contraindication for taxanes.

In **chapter 2** we described the results on the primary objective of the MATADOR trial. Using RNA-sequencing data of 528 patients, we identified a gene expression profile with prognostic value, but limited predictive capacity. This might be explained by the efficacy of other adjuvant treatments (radiotherapy, endocrine therapy) and by the variety in resistance mechanisms. If a resistance mechanism is not shared by a large proportion of the tumors, it is highly unlikely that a predictive gene expression profile will be found.[8] In addition, bulk RNA-sequencing data will only reflect the most abundant tumor cell. If smaller subclones are drivers of resistance, the identified profile will not accurately predict survival benefit.[8] In contrast to this data-driven approach, we employed a biology-driven analysis using the hallmark gene sets.[9] Enrichment in immune-related gene expression appeared to be associated with favorable outcome after TAC, but not after ddAC in patients with a basal tumor. We assessed the clinical applicability of this association by analyzing the abundance of tumor infiltrating lymphocytes (TILs assessed on H&E) in the triple negative breast cancer (TNBC) patients. Patients with high TILs (≥ 20%) had a numerically longer RFS when treated with TAC than treated with ddAC, while patients with low TILs (< 20%) derived more benefit from ddAC. The interaction between TILs and treatment was significant, indicating that TILs might predict RFS benefit from docetaxel-containing or dose dense adjuvant chemotherapy. Validation of the interaction between TILs and docetaxel-containing or dose dense treatment in an independent cohort is required.

Potential biomarkers for toxicity of 6 cycles ddAC and 6 cycles TAC were described in **chapter 3**. In the MATADOR study, anemia, hand-foot syndrome, cough and phlebitis were observed more frequently in ddAC treated patients. Diarrhea, edema of the limb and peripheral neuropathy were more common in the TAC treated subgroup. For biomarker analyses, we selected three frequently occurring and clinically relevant toxicities: anemia, febrile neutropenia and peripheral neuropathy. We aimed to validate previously described associations between toxicity and clinicopathologic factors or single nucleotide polymorphisms (SNPs). We were able to replicate five associations[10-13]. Anemia was associated with age at diagnosis and baseline platelet count. Although genetic variants in *FGFR4* were not associated with febrile neutropenia in all patients, a significant interaction was found between *FGFR4* and treatment with homozygous variant carriers having a higher risk of febrile neutropenia after ddAC and wildtype or heterozygous variant carriers after TAC. Also, genetic variants in *TECTA* and *GSTP1* were associated with peripheral neuropathy in all patients. However, most associations

could not be validated in our cohort. This might be explained by differences in methods, e.g. the type of patient material on which the genetic variants were determined or the background of the investigated population. In addition, the majority of these associations lack a biological rationale, which may increase the chance of an incidental finding. Also, most genomic studies are not designed to find significant associations and therefore lack enough patients and statistical power. Future studies with an adequate design and a sufficient number of patients are needed to validate our results.

Sequential administration of chemotherapeutics allows for increasing drug dose without inducing additional toxicity. In **chapter 4**, a dose dense scheduled, sequentially given chemotherapeutic regimen with increased drug dose (intensified chemotherapy) was discussed. Previous analyses of the German Adjuvant Intergroup Node-positive Study 2 (GAIN-2) showed that 3 x 3 cycles of dose intensified, sequential epirubicin, paclitaxel and cyclophosphamide (ETC) resulted in similar disease free survival (DFS) and OS as four cycles concurrently given epirubicin and cyclophosphamide followed by 10 cycles weekly paclitaxel and 4 cycles capecitabine (EC-TX).[14] A predictive biomarker could indicate which patients would benefit from dose intensified ETC. In view of the predictive value for survival benefit of high dose alkylating and platinum agents[15,16], we assessed the *BRCA1*-like profile as predictor of superior survival of intensified ETC in 163 TNBC patients of the GAIN-2 study. With a median follow up of 83 months, DFS and OS were not significantly different between the *BRCA1*-like subgroups. Also, there was no survival difference between the treatments when split by *BRCA1*-like subgroup. One explanation for our results could be that capecitabine is a highly effective drug in *BRCA1*-like tumors due to its role in DNA repair. To test this hypothesis, the *BRCA1*-like profile will be assessed as predictive biomarker in an independent cohort, e.g. CREATE-X study[17] or FinXX study[18] in which capecitabine improved outcome of the triple negative breast cancer subgroup.

Biomarkers for targeted treatment

The phosphatidylinositol 3–kinase (PI3K) pathway is an important escape route of endocrine resistant tumor cells to grow and proliferate. Simultaneously blocking the estrogen receptor (ER) and the PI3K pathway could overcome endocrine resistance. Two clinical trials showed that PI3K pathway inhibition combined with endocrine therapy prolonged progression free survival (PFS) and OS compared with endocrine treatment only in metastatic ER positive breast cancer patients.[19,20] However, it also caused substantial toxicity. To reduce toxicity and maintain efficacy, isoform selective PI3K inhibitor taselisib was developed. As single agent[21] and combined with endocrine

treatment[22,23], it had promising antitumor activity, particularly in *PIK3CA* mutant breast cancer. In phase 1b of the POSEIDON study as described in **chapter 5**, we aimed to find the recommended phase 2 dose of taselisib combined with standard dose tamoxifen using a rolling 6 design. Thirty patients with ER positive breast cancer were treated at 3 different dose levels. The recommended phase 2 dose of taselisib was 4mg QD. An objective response was observed in 6 out of 25 patients (24%) with measurable disease. A *PIK3CA* mutation was found in 8 patients (27%) of whom three had a confirmed partial response. In line with other reports[24-26], responses were more abundant in patients with *PIK3CA* mutant disease. However, antitumor activity of taselisib was also observed in patients with *PIK3CA* wildtype tumors. Phase II of the POSEIDON study will answer the questions whether taselisib and tamoxifen prolong PFS compared with placebo and tamoxifen and whether *PIK3CA* mutation status can be used as predictive biomarker for survival benefit of taselisib.

Angiogenesis plays a pivotal role in tumor growth. A key mediator in this process is vascular endothelial growth factor A (VEGF-A). Previous studies showed that monoclonal antibody against VEGF-A bevacizumab prolonged PFS when added to chemotherapy as treatment for metastatic breast cancer.[27-29] However, it remained elusive which patients would benefit most from bevacizumab addition. Retrospective analyses suggested VEGF-A and VEGF receptor 2 (VEGFR-2) as potential predictive biomarkers.[30] In the Triple-B study, we aimed to evaluate two biomarkers: baseline plasma VEGF receptor 2 (pVEGFR-2) levels as predictive biomarker for survival benefit of bevacizumab and the *BRCA1*-like profile as predictor of survival benefit of alkylating chemotherapy combined with a platinum compound. In **chapter 6**, we report on the results of an interim analysis of 58 metastatic TNBC patients who were randomized to carboplatin and cyclophosphamide with (CC + B) or without bevacizumab (CC) and paclitaxel with (P + B) or without bevacizumab (P). Hypertension and fatigue were more frequent in the bevacizumab-arms. CC caused more anemia, nausea and vomiting, while P was associated with more alopecia and neuropathy. With a median follow up of 22 months, PFS was significantly longer in patients who were treated with bevacizumab compared with patients who were treated with chemotherapy only. However, the difference was no longer significant when corrected for prognostic variables. OS was not significantly different between the treatment subgroups, which is in line with previous reports.[28,29] In this small cohort, we were not able to validate baseline pVEGFR-2 level as predictive biomarker for survival benefit of bevacizumab. However, CC with or without bevacizumab is a safe combination as first-line treatment for metastatic TNBC. Due to the modest additive value of bevacizumab and with emerging evidence that VEGFR-2 level had limited predictive value[31], the study protocol was amended to replace add-on

bevacizumab with programmed-death ligand 1 (PD-L1) antibody atezolizumab. In addition to the *BRCA1*-like profile, the ongoing part of the Triple-B study will focus on identification of a predictive biomarker for survival benefit of atezolizumab.

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SAMENVATTING (DUTCH SUMMARY)

Wereldwijd is borstkanker een van de meest voorkomende vormen van kanker.[1] Ondanks de geboekte vooruitgangen in systemische therapie die geleid hebben tot verbeterde overleving[2], overlijdt jaarlijks nog steeds een aanzienlijk aantal patiënten aan de gevolgen van de ziekte. Een gepersonaliseerde behandelstrategie is nodig om stappen te kunnen maken in het verder verbeteren van de borstkanker-specifieke overleving. Prognostische markers kunnen helpen bij het inschatten van het risico op terugkeer van de ziekte en kunnen gebruikt worden om de indicatie voor systemische therapie te bepalen.[3] Predictieve markers geven informatie over de overlevingswinst die een patiënt door een specifieke therapie kan behalen en kunnen ingezet worden om een individueel behandelplan samen te stellen.[4] Hoewel er verschillende prognostische markers gebruikt worden in de kliniek[5,6], zijn er slechts twee predictieve markers: de oestrogeen receptor (ER) voor endocriene therapie en human epidermal growth factor receptor 2 (HER2) voor anti-HER2-behandeling.

In dit proefschrift streven wij ernaar om predictieve biomarkers te identificeren die een overlevingsvoordeel van systemische therapie bij primaire of uitgezaaide borstkanker kunnen voorspellen. In **hoofdstuk 1, 2 en 3** beschrijven wij de zoektocht naar predictieve biomakers voor overlevingswinst en bijwerkingen van adjuvante chemotherapie in de MATADOR studie. **Hoofdstuk 4** bevat de analyse van het *BRCA1*-like profiel als predictieve marker voor geïntensiveerde chemotherapie in de GAIN-2 studie. Tot slot worden de eerste analyses omtrent biomarkers voor effectiviteit van doelgerichte therapie voor uitgezaaide borstkanker bediscussieerd in **hoofdstuk 5 en 6**.

Biomarkers voor adjuvante chemotherapie

De toevoeging van taxanen aan en de dose dense toediening van adjuvante chemotherapie heeft de borstkanker-specifieke overleving aanzienlijk verbeterd.[2,7] Het is echter nog onbekend welke individuele patiënten baat hebben van taxaan-bevattende chemotherapie, van dose dense toegediende behandeling, of van beide. Derhalve zijn er biomarkers nodig die een overlevingsvoordeel van een specifieke behandeling kunnen voorspellen. De multicenter, fase 3 MATADOR studie was ontworpen om predictieve biomarkers voor een overlevingsvoordeel van taxanen te vinden. Tussen 2004 en 2012 zijn 664 patiënten met pT1-3, pN0-3 borstkanker gerandomiseerd tussen 6 cycli dose dense doxorubicine en cyclofosfamide (ddAC) en docetaxel, doxorubicine en cyclofosfamide (TAC). Het primaire doel van de studie was om een genexpressieprofiel te ontwikkelen dat een overlevingsvoordeel van ddAC of TAC kan

voorspellen. In **hoofdstuk 1** vergeleken wij de overleving van patiënten die met een van deze twee therapieën behandeld waren. Bij een mediane follow up van 7 jaar was er geen verschil in ziektevrije overleving tussen ddAC en TAC. Ook de totale overleving was niet verschillend tussen de twee behandelarmen. Voor zover nu bij ons bekend is de MATADOR studie de eerste studie waarin een directe vergelijking tussen 6 cycli ddAC en 6 cycli TAC wordt gemaakt. Onze bevindingen impliceren dat 6 cycli ddAC een waardvol alternatief is voor patiënten met een contra-indicatie voor taxanen.

In **hoofdstuk 2** hebben wij de resultaten omtrent het primaire doel van de MATADOR studie beschreven. Gebruikmakend van RNA sequencing data van 528 patiënten hebben wij een genexpressieprofiel ontwikkeld met prognostische betekenis, maar slechts beperkte predictieve waarde. Een verklaring hiervoor zou kunnen liggen in de variatie aan resistentiemechanismen. Als een resistentiemechanisme niet gedeeld wordt door de meerderheid van de tumoren, is het onwaarschijnlijk dat er een predictief genexpressieprofiel kan worden gevonden.[8] Daarnaast geven bulk RNA sequencing data voornamelijk een weerspiegeling van de meest voorkomende tumorcellen. In het geval dat kleinere subklonen de belangrijkste veroorzaker van resistentie zijn, zal het ontwikkelde profiel geen accurate predictie kunnen maken van het therapie-effect.[8] In tegenstelling tot deze op data gebaseerde aanpak, hebben wij een biologie-gedreven analyse gedaan met de hallmark genensets.[9] Verrijking in immuun-gerelateerde genexpressie bleek geassocieerd te zijn met een overlevingsvoordeel na TAC, maar niet na ddAC, in patiënten met een basale tumor. Vervolgens hebben wij de praktische klinische toepasbaarheid van deze associatie getest door de aanwezigheid van tumor infiltrerende lymfocyten (TILs) te meten in patiënten met triple negatieve borstkanker (TNBC). Patiënten met veel TILs (≥ 20%) in hun tumor hadden een numeriek langere ziektevrije overleving wanneer zij behandeld waren met TAC, terwijl patiënten met weinig TILs (< 20%) in de tumor meer baat hadden van ddAC. De interactie tussen TILs en behandeling was significant, wat erop zou kunnen wijzen dat veel TILs een voordeel in ziektevrije overleving van docetaxel-bevattende chemotherapie voorspelt, terwijl bij weinig TILs mogelijk ddAC beter is. Validatie van de interactie tussen TILs en docetaxel-bevattende or dose dense chemotherapie in een onafhankelijk cohort is noodzakelijk.

Potentiele biomarkers voor toxiciteit van 6 cycli ddAC en 6 cycli TAC zijn beschreven in **hoofdstuk 3**. In de MATADOR studie werden anemie, hand-voet syndroom, hoesten en flebitis vaker beschreven in de ddAC-behandelde patiënten. Diarree, oedeem aan een van de ledematen en perifere neuropathie werden frequenter gezien in de TAC-behandelde groep. Voor de biomarker analyses hebben wij 3 frequent optredende en klinisch relevante bijwerkingen geselecteerd: anemie, febriele neutropenie en perifere

neuropathie. Het doel was om eerder beschreven associaties tussen toxiciteit en klinisch-pathologische factoren of enkel-nucleotide polymorfismen (SNPs) te valideren. Wij hebben 5 associaties kunnen repliceren.[10-13] Anemie was geassocieerd met leeftijd bij diagnose en de uitgangswaarde van de bloedplaatjesconcentratie. Hoewel de genetische varianten in *FGFR4* niet geassocieerd waren met febriele neutropenie in alle patiënten, was de interactie tussen *FGFR4* en behandeling wel significant, waarbij dragers van een homozygoot variant allel een hoger risico op febriele neutropenie hadden na ddAC en dragers van een wildtype of heterozygoot variant allel na TAC. Daarnaast waren genetische varianten in *TECTA* en *GSTP1* gerelateerd aan het optreden van perifere neuropathie in alle patiënten. Echter, de meeste associaties konden niet worden gevalideerd. Dit zou verklaard kunnen worden door verschillen in methodes, zoals het type patiëntmateriaal waarop de genetische varianten waren bepaald of de genetische achtergrond van de onderzochte populatie. Daarnaast ontbreekt voor de meerderheid van deze associaties een biologische rationale, waardoor de kans op incidentele bevindingen verhoogd kan zijn. Ook zijn veel genetische studies niet opgezet om significante associaties te vinden met als gevolg dat de subgroepen met patiënten te klein zijn en de statistische power tekortschiet. Toekomstige onderzoeken met een adequaat ontwerp en voldoende patiënten zijn nodig om onze resultaten te kunnen valideren.

Sequentiële toediening van chemotherapeutica maakt het mogelijk om de dosis van middelen te verhogen zonder additionele toxiciteit te veroorzaken. In **hoofdstuk 4** wordt een dose dense, sequentieel toegediende chemotherapeutische behandeling met verhoogde dosis (geïntensiveerde chemotherapie) bediscussieerd. Eerder analyses van de German Adjuvant Intergroup Node-positive Study 2 (GAIN-2) toonden aan dat 3 x 3 cycli van geïntensiveerde, sequentieel toegediende epirubicine, paclitaxel en cyclofosfamide (ETC) resulteerden in gelijke ziektevrije overleving en totale overleving als 4 cycli gelijktijdig gegeven epirubicine en cyclofosfamide gevolgd door 10 cycli wekelijks paclitaxel en 4 cycli capecitabine (EC-TX).[14] Een predictieve biomarker zou kunnen helpen om patiënten te selecteren die een overlevingsvoordeel van geïntensiveerde ETC hebben. Gezien de aangetoonde waarde om overlevingsvoordeel te voorspellen van hoge dosis alkylerende en platinum-bevattende chemotherapie[15,16], hebben wij het *BRCA1*-like profiel onderzocht als voorspeller van verbeterde overleving na geïntensiveerde ETC in 163 triple negatieve borstkanker patiënten van de GAIN-2 studie. Bij een mediane follow up van 83 maanden waren ziektevrije overleving en totale overleving niet significant verschillend tussen de *BRCA1*-like subgroepen ongeacht behandeling. Ook werd er geen verschil in overleving waargenomen tussen de behandelgroepen wanneer deze gesplitst waren op *BRCA1*-like status. Een verklaring

hiervoor zou kunnen zijn dat capecitabine een erg effectieve behandeling voor *BRCA1*-like tumoren zou kunnen zijn door haar rol in het herstellen van DNA schade. Om deze hypothese te testen zal de predictieve waarde van het *BRCA1*-like profiel onderzocht worden in een onafhankelijke cohort, zoals de CREATE-X studie[17] of de FinXX studie[18] waarin capecitabine verbeterde overleving in de subgroep met triple negatieve borstkanker liet zien.

Biomarkers voor doelgerichte therapie

De fosfatidylinositol 3–kinase (PI3K) signaleringsroute is een belangrijke ontsnappingsroute voor tumorcellen die resistent zijn tegen endocriene therapie om te groeien en prolifereren. Gelijktijdige blokkering van de oestrogeen receptor (ER) en de PI3K signaleringsroute zou endocriene therapieresistentie kunnen opheffen. Twee klinische trials hebben aangetoond dat het remmen van de PI3K signaleringsroute in combinatie met endocriene therapie een langere progressievrije overleving oplevert dan endocriene therapie alleen in gemetastaseerde ER positieve borstkankerpatiënten.[19,20] Echter, dit ging gepaard met aanzienlijke toxiciteit. Om toxiciteit te reduceren en de effectiviteit te behouden, is een *isoform*-specifieke remmer van PI3K, taselisib, ontwikkeld. Zowel als monotherapie[21] als in combinatie met endocriene therapie[22,23] toonde taselisib veelbelovende antitumor activiteit, met name in *PIK3CA* gemuteerde borstkanker. In fase 1b van de POSEIDON studie zoals beschreven in **hoofdstuk 5** was het doel om de beoogde dosering van taselisib voor fase 2 in combinatie met standaard gedoseerde tamoxifen te vinden in een rolling 6 studieopzet. Dertig patiënten met ER-positieve borstkanker werden behandeld op 3 verschillende doseringsniveaus. De beoogde fase 2 dosering van taselisib was 4mg QD. Een objectieve respons werd waargenomen in 6 van de 25 patiënten (24%) met meetbare ziekte. Een *PIK3CA* mutatie werd gevonden in 8 patiënten, van wie 3 patiënten een bevestigde partiële respons hadden. Overeenkomstig met andere publicaties[24-26] werden er vaker responsen gezien in patiënten met *PIK3CA* gemuteerde ziekte. Echter, antitumor activiteit werd ook waargenomen in patiënten met *PIK3CA* wildtype borstkanker. Fase 2 van de POSEIDON studie zal de vragen beantwoorden of taselisib gecombineerd met tamoxifen een langere progressievrije overleving geeft dan placebo en tamoxifen en of *PIK3CA* mutatie status gebruikt kan worden als predictieve biomarker voor overlevingsvoordeel van taselisib.

Angiogenese speelt een cruciale rol in tumorgroei. Een belangrijke factor in dit proces is vasculaire endotheliale groeifactor A (VEGF-A). Eerder studies toonden aan dat monoclonaal antilichaam tegen VEGF-A bevacizumab de progressievrije overleving

verlengde wanneer deze gecombineerd werd met chemotherapie voor gemetastaseerde borstkanker.[27-29] Echter, het bleef onduidelijk welke patiënten het meeste baat zouden hebben bij de toevoeging van bevacizumab. Retrospectieve analyses suggereerden dat VEGF-A en VEGF receptor 2 (VEGFR-2) potentiele predictieve biomarkers zouden kunnen zijn.[30] In de Triple-B studie was het doel om twee biomarkers te evalueren: de plasma uitgangswaarde van VEGFR-2 (pVEGFR-2) als predictieve biomarker voor overlevingsvoordeel van bevacizumab en het *BRCA1*-like profiel als voorspeller van overlevingsvoordeel van een alkylerend en een platinummiddel. In **hoofdstuk 6** beschrijven wij de resultaten van een interimanalyse van 58 gemetastaseerde TNBC patiënten die gerandomiseerd werden tussen carboplatin en cyclofosfamide zonder (CC) of met bevacizumab (CC + B) en paclitaxel zonder (P) of met bevacizumab (P + B). Hypertensie en vermoeidheid werden vaker gerapporteerd in de bevacizumab-behandelarmen. CC veroorzaakte vaker anemie, misselijkheid en braken, terwijl P vaker geassocieerd was met alopecia en neuropathie. Bij een mediane follow up van 22 maanden was de progressievrije overleving significant langer in patiënten die met bevacizumab behandeld waren in vergelijking met patiënten die alleen chemotherapie toegediend hadden gekregen. Echter, het verschil in progressievrije overleving was niet meer significant na correctie voor prognostische variabelen. De totale overleving was niet significant verschillend tussen de behandelgroepen, wat in overeenstemming is met andere publicaties[28,29]. In dit kleine cohort kon pVEGFR-2 concentratie niet gevalideerd worden als predictieve biomarker voor overlevingsvoordeel van bevacizumab. Desalniettemin bleek CC met of zonder bevacizumab een veilige eerstelijnsbehandeling voor gemetastaseerde TNBC. Door de beperkte effectiviteit van bevacizumab en nieuw bewijs dat VEGFR-2 beperkte predictieve waarde heeft[31], is het studieprotocol inmiddels geamendeerd om bevacizumab te vervangen door het programmed-death ligand 1 (PD-L1) antilichaam atezolizumab. Naast het *BRCA1*-like profiel zal de focus in het huidige deel van de Triple-B studie liggen op het identificeren van predictieve biomarkers voor overlevingsvoordeel van atezolizumab.

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CURRICULUM VITAE

Annelot Geerke Jantine van Rossum was born on 9 April 1987 in Geldermalsen, the Netherlands. She grew up in a loving family with two sisters and a brother. After secondary school at the Gymnasium Camphusianum in Gorinchem, she started medical training at the University of Amsterdam. From the second until the fourth year she did a clinical research project on the use of the electronic nose (eNose) in patients with chronic obstructive pulmonary disease (COPD). After a senior internship at the Department of Medical Oncology of the Netherlands Cancer Institute – Antoni van Leeuwenhoek, she obtained her medical degree in March 2012. Subsequently, she worked as resident-not-in-training at the Department of Medical Oncology at the Netherlands Cancer Institute – Antoni van Leeuwenhoek. In April 2014 she started her PhD at the Division of Molecular Pathology at the Netherlands Cancer Institute. She was award with a Susan G. Koomen Scholar-In-Training Award at the San Antonio Breast Cancer Symposium in 2015 for the work presented in chapter 4 of this thesis and a Merit Award at the European Society for Medical Oncology Congress in 2018 for the work described in chapter 3 of this thesis. In January 2019 she will start her training to specialize in Internal Medicine at the Tergooi Ziekenhuis in Hilversum under supervision of M.E.M. Rentinck, MD, and continue at the University Medical Centers Amsterdam, location Academic Medical Center under supervision of Prof. S.E. Geerlings, MD, PhD.