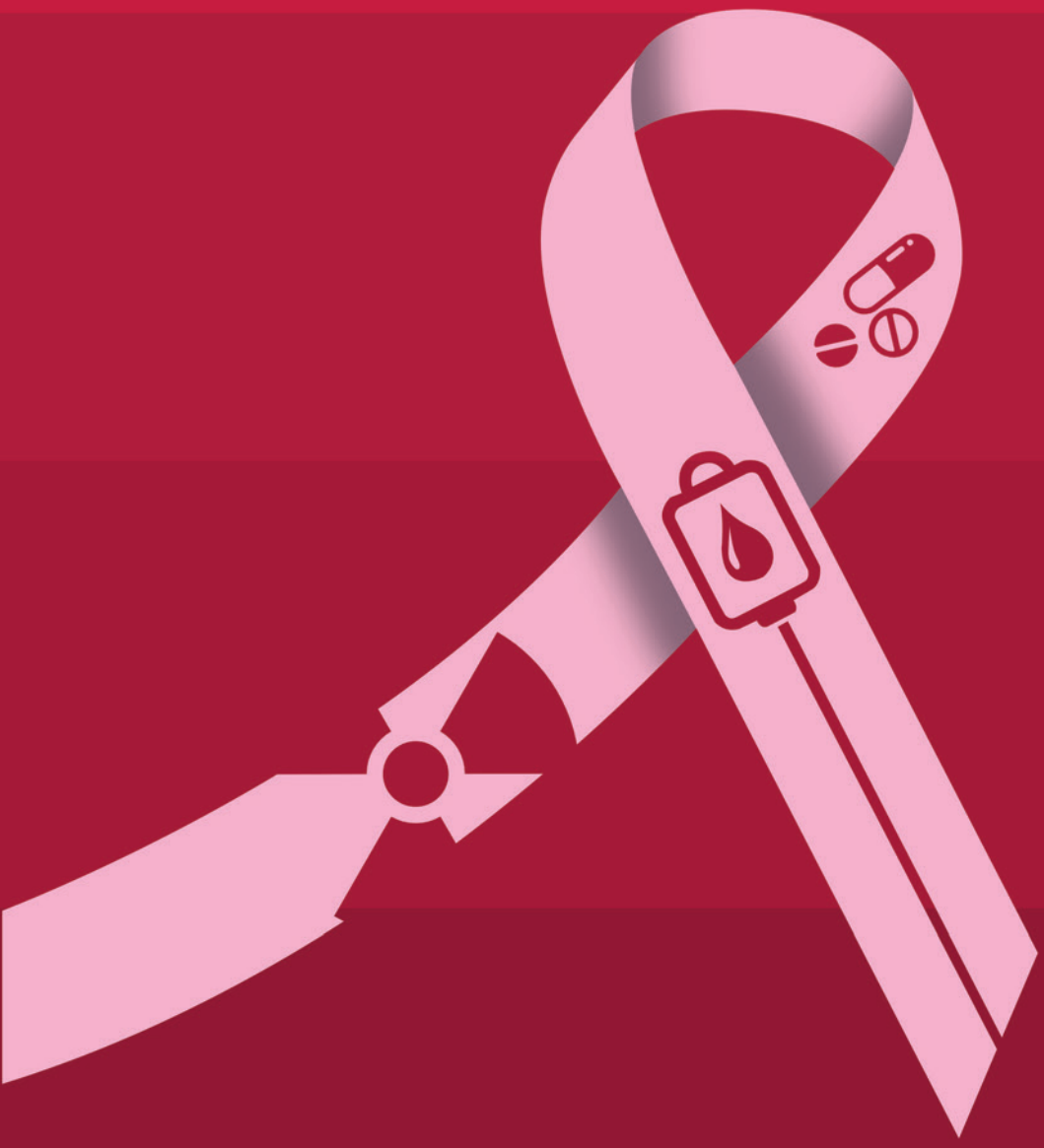
TREATMENT OF BREAST CANCER

DEALING WITH TOXICITY



JAN DROOGER

Treatment of breast cancer: dealing with toxicity

Behandeling van borstkanker:
omgaan met toxiciteit

Jan Cornelis Drooger

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Behandeling van borstkanker: omgaan met toxiciteit

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1

Introduction

GENERAL INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and more than 500,000 deaths in 2012 [1]. Moreover, the incidence of breast cancer rapidly increases, which can partly be explained by the increased life expectancy in the past decades and the ageing of the population. Breast cancer alone already accounts for 25% of all cancer cases and 15% of all cancer deaths among females worldwide. Europe, Northern America, Australia, New Zealand and Japan account for about one half of all breast cancer cases and 38% of deaths worldwide and currently the life time breast cancer risk in these countries is increasing to 12% [1].

Early breast cancer

Most breast cancer patients are diagnosed with early stage breast cancer and treated with a curative intent including local therapy (surgery and in many cases adjuvant radiotherapy) and often perioperative systemic treatment (chemotherapy, endocrine therapy and/or targeted therapy). These systemic treatments can be given before (neoadjuvant) or after (adjuvant) resection of the tumor. Neoadjuvant is the treatment of choice especially in case of locally advanced breast cancer and in case of need for downstaging in order to perform breast conserving surgery instead of mastectomy.

Systemic therapies are effective both in reducing the risk of distant and local recurrence. The decision whether or not and if so, which (neo)adjuvant systemic therapy should be used, takes many factors into account such as age, life expectancy (defined based on age, comorbidities and WHO performance status), expression of estrogen and/or progesterone receptors, overexpression of human epidermal growth factor receptor 2 (HER2), histomorphological grade, tumor size and nodal status. Genomic analysis of primary tumor tissue is increasingly used, mainly in hormone receptor positive, HER2-negative breast cancer to better assess the risk of relapse and thereby to determine whether or not chemotherapy should be added to endocrine therapy in these patients [2,3].

Postoperative radiotherapy to the breast/chest wall and/or locoregional lymph nodes reduces the risk of local recurrence and improves survival in specific patients as well [4,5]. A large proportion of patients treated for early stage breast cancer therefore receives postoperative radiotherapy.

Besides anticancer treatment aiming to exert direct antitumor effects, also supportive measures are important in the treatment of early breast cancer. Especially in patients with cancer treatment induced bone loss, osteoclast inhibitors (bisphosphonates or denosumab) decrease the risk of skeletal related complications [6].

Despite taking all above mentioned factors into account when deciding whether or not to apply adjuvant systemic therapy or radiotherapy, still a large (undefined) proportion of patients are overtreated and exposed to therapy-associated toxicity while they have no survival advantage of the therapy as they are cured by surgery alone.

Metastatic breast cancer

Unfortunately, a number of the patients who are treated with curative intent for early breast cancer develop local recurrence, a second primary breast cancer or metastatic disease. In addition to patients who experience metastatic disease after being treated for a primary tumor, approximately five percent of the patients with breast cancer present with metastatic disease at the initial diagnosis. Once patients have developed metastatic disease, cure is in general not possible and treatment is aimed at prolongation of survival, and preservation or improving quality of life. Overall survival widely varies between patients based on subtype of tumor and on sites and burden of metastases. Also in metastatic breast cancer, treatment highly depends on tumor and patient characteristics.

Besides anticancer treatment aiming to exert direct antitumor effects, also supportive measures are important in the treatment of metastatic breast cancer. Especially in patients with bone metastases osteoclast inhibitors (bisphosphonates or monoclonal antibodies against RANKL) decrease the risk of skeletal related complications [7].

Especially in the treatment of metastatic breast cancer, where cure is no longer possible, it is of utmost importance to take quality of life into account. Unfortunately, most anticancer treatments are associated with toxicity, which might impair quality of life.

AIMS AND OUTLINE OF THE THESIS

Two of the major challenges in breast cancer research are to increase efficacy of anticancer treatments without increasing clinical relevant toxicity and/or to decrease toxicity of anticancer treatment without decreasing its efficacy. To achieve this, improved insight into the mechanisms underlying treatment-induced toxicity is crucial. This thesis describes studies in which it is aimed to optimize therapy for patients with breast cancer in various stages of the disease. This might be reached by dealing with toxicity in several ways: (1) decreasing toxicity, while maintaining efficacy, (2) increasing efficacy, without increasing clinical relevant toxicity, (3) increasing efficacy by toxicity-based dose escalation and (4) prevention of (complications of) toxicity of anticancer treatments.

Impaired DNA repair mechanism related to increased treatment-associated toxicity

Carriers of a germline *BRCA1* or *BRCA2* (*BRCA1/2*) mutation face an increased lifetime risk of developing breast cancer, estimated to range from 47 to 66% for *BRCA1* mutation carriers and from 40 to 57% for *BRCA2* mutation carriers [8,9]. Mean age at breast cancer diagnosis in this subgroup of patients is 45 years, which is substantially lower than in the general population [9].

BRCA1/2-associated breast cancer is characterized by homologous recombination deficiency, leading to inadequate repair of double strand DNA breaks [10-12]. Both ionizing radiation (diagnostic radiation and radiotherapy) and chemotherapy induce DNA damage by several mechanisms including induction of double strand DNA breaks. Especially platinum derivates are strong inducers of double strand DNA breaks. The vulnerability of cells for ionizing radiation and chemotherapy largely depends

on the capability of the cells to repair DNA damage. Because BRCA1 and BRCA2 proteins play an important role in the repair of DNA damage, the acute and late toxicity of ionizing radiation and chemotherapy might be increased in *BRCA1/2* mutation carriers compared to sporadic controls.

Ionizing radiation

Occurrence of breast cancer is a well-known long term side effect of ionizing radiation [13]. As a consequence, also adjuvant radiotherapy for primary breast cancer might increase the risk of a second (ipsilateral or contralateral) breast cancer. In **Chapter 2** we describe and discuss the current literature on the association between ionizing diagnostic and/or therapeutic radiation and the risk of developing a first or second primary breast cancer, with particular attention for patients with a *BRCA1/2* mutation. Relevant data regarding the association between adjuvant radiotherapy and contralateral breast cancer risk in younger patients with a *BRCA1/2* mutation are sparse. Knowledge about the possible increased risk of contralateral breast cancer by radiotherapy might be of great importance for optimal shared decision making regarding mastectomy without radiotherapy versus breast conserving surgery including radiotherapy at primary breast cancer diagnosis. Therefore, we studied the impact of radiotherapy on the risk of contralateral breast cancer among *BRCA1/2*-associated breast cancer patients in a retrospective cohort study with special attention for patients younger than 40 years at breast cancer onset (**Chapter 3**). Over the years, an increasing proportion of *BRCA1/2* mutation carriers after developing breast cancer seems to opt for bilateral mastectomy instead of unilateral mastectomy or breast conserving surgery without radiotherapy. These trends might influence the proportion of patients at risk for radiation-induced breast cancer. We therefore also explored potential tendencies in locoregional treatments and the rates of contralateral risk-reducing mastectomies over the past decades.

Chemotherapy

Platinum derivates, which are strong inducers of double strand DNA breaks, showed higher efficacy in breast cancer patients with a *BRCA1/2* mutation compared to sporadic breast cancer patients [14-18]. Increased sensitivity for chemotherapeutic regimens other than platinum-based has also been suggested, which might be explained by the induction of double strand DNA breaks through these agents but also by the functions of BRCA1 and BRCA2 proteins in the cell cycle [19-22].

Thus far, two small studies investigated the acute toxicity of mainly older (neo)adjuvant chemotherapy regimens in *BRCA1/2* mutation carriers, compared to sporadic breast cancer patients, with inconsistent results [23,24]. We therefore performed a larger single center retrospective cohort study to examine potential differences in (neo)adjuvant chemotherapy-associated toxicity between *BRCA1/2*-associated and sporadic breast cancer patients (**Chapter 4**).

Could toxicity be used to optimize treatment?

Both anthracyclines and cyclophosphamide are highly effective drugs in the treatment of breast cancer, although not all patients benefit from anthracycline- and cyclophosphamide-containing

chemotherapy, both in (neo)adjuvant and metastatic setting [25,26]. The cumulative dose of anthracyclines administered is important. From randomized controlled trials, it is clear that higher 'standard dose' of anthracyclines for early breast cancer improves patient survival compared to lower 'standard dose' [27]. On the other hand, a reason for differences in efficacy among patients who have had a similar dose of anthracyclines administered could be the large inter-individual (between patients) as well as the intra-individual (within patients) variability in pharmacokinetic parameters [28]. Interestingly, some retrospective studies showed that breast cancer patients given adjuvant chemotherapy but not attaining at least moderate hematological toxicity have a worse prognosis compared to those with more toxicity [29-32]. The current standard of dosing of chemotherapy is guided by body surface area with an a posteriori dose reduction in case of excessive toxicity. Dose escalation among patients without toxicity is, however, not standard of care. The administration of an inappropriately low dose of chemotherapy is therefore not recognized, leaving patients that might benefit from an increased dose unidentified. The percentage of breast cancer patients receiving a suboptimal dose is unknown, as well as the amount of underdosing in these individuals. We, therefore, addressed the feasibility of a simple tool for neutrophil-guided dose adaptation of anthracycline-cyclophosphamide-containing chemotherapy in female breast cancer patients (**Chapter 5**). In this study we aimed to reach nadir absolute neutrophil count of ≤1.0 x 10e9/L with recovery to ≥1.5 x 10e9/L at the time of the planned next treatment cycle, without excessive hematological or non-hematological toxicity.

Treatment related toxicity, compliance and therapeutic drug monitoring

In **Chapter 6**, we focused on endocrine therapy. Tamoxifen, a selective estrogen receptor modulator, is an important drug in the treatment of hormone receptor positive breast cancer. Since tamoxifen often has to be used for a longer period of time, therapy adherence, change in environmental factors and possibly resistance mechanisms might influence the systemic exposure over time [33]. Prevalence of full adherence ranged from 41 to 72% determined at the end of five years of treatment in a recent systematic review [34]. In particular treatment side effects were negatively associated with adherence. Inter-individual variation in metabolism of tamoxifen, which is influenced by both genetic and environmental factors, contributes to the differences in efficacy and toxicity of tamoxifen. Endoxifen is believed to be the principal active metabolite of tamoxifen. The best way forward for individualization of tamoxifen therapy seems to be therapeutic drug monitoring (TDM). When a 'therapeutic window' has been established, TDM can not only be used to increase efficacy (by increasing the dose in patients below threshold) but also to decrease toxicity without decreasing efficacy adherence by decreasing the dose in patients with side effect who have high endoxifen concentrations. The often used method of TDM by serial measurements of plasma concentrations of drugs and/or metabolites over time might be difficult to incorporate in clinical practice. A potential method, which gives in retrospect information about the course of an anticancer drug and/or its metabolites could be to measure these concentrations in scalp hair. Scalp hair grows with an average rate of one centimeter

per month and so, segmental analysis of hair allows the determination of the historic pattern of drug concentration [35]. **Chapter 6** describes the validation of an earlier developed high-performance highly sensitive ultra performance liquid chromatography/tandem mass spectrometry method for quantification of tamoxifen and its three main metabolites (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen) in scalp hair.

Prevention and treatment of toxicity

Cancer treatment induced bone loss is an important toxicity of breast cancer treatment. Several causes of bone loss due to cancer treatment have been identified, among which estrogen deprivation (by chemotherapy induced amenorrhea and/or treatment with endocrine therapy) and effects on the bone of chemotherapy and supportive drugs, such as steroids. One of the newer supportive care drugs to prevent bone loss is denosumab, a fully humanized monoclonal antibody against the receptor activator of nuclear factor kappa-B ligand (RANKL) [36]. This drug has proven efficacy in the treatment of cancer treatment induced bone loss in patients with early breast cancer [37]. Furthermore, denosumab was found to be superior to zoledronic acid in delaying or preventing of skeletal related complications in patients with bone metastases [38]. In **Chapter 7** we describe the current indications for denosumab in both early and metastatic breast cancer treatment, with special attention for efficacy and short and long term toxicity.

Postponing (cyto)toxic therapy an option in metastatic HER2-positive breast cancer?

In patients with metastatic breast cancer it is not only important to find new treatment options which increase efficacy but also to find new treatment options which decrease treatment-associated toxicity in order to maintain or improve quality of life. An important way to improve quality of life might be by reducing treatment-induced toxicity, for example by delaying the start of chemotherapy. Likewise, in patients with hormone receptor positive metastatic breast cancer it is common practice to start with endocrine therapy only, whenever possible. For patients with metastatic breast cancer overexpressing HER2, standard first line treatment consisted until recently of the combination of trastuzumab with a taxane [39,40]. Bevacizumab is a fully humanized monoclonal antibody against vascular endothelial growth factor (VEGF) and seems to increase the efficacy of trastuzumab in preclinical research [41,42]. In **Chapter 8** we describe an open label randomized, non-comparative, phase 2 study of concomitant trastuzumab, bevacizumab and paclitaxel versus trastuzumab and bevacizumab, followed by trastuzumab, bevacizumab and paclitaxel at progression as first line treatment for patients with HER2-positive metastatic breast cancer. Primary endpoint was progression free rate at one year. Secondary endpoints included progression free survival, overall survival, response, safety and toxicity.

Finally, the results of this thesis are discussed and suggestions for further research are mentioned in **Chapter 9**.

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2

Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for *BRCA1* and *BRCA2* mutation carriers: A critical review of the literature

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ABSTRACT

Occurrence of breast cancer is a well-known long term side effect of ionizing radiation (both diagnostic and therapeutic). The radiation-induced breast cancer risk increases with longer follow-up, higher radiation dose and younger age of exposure. The risk for breast cancer following irradiation for lymphomas is well known. Although data regarding the carcinogenic risk of adjuvant radiotherapy for a primary breast cancer are sparse, an increased risk is suggested with longer follow-up mainly when exposed at younger age. Particularly, patients with a *BRCA1/2* mutation might be more sensitive for the deleterious effects of ionizing radiation due to an impaired capacity of repairing double strand DNA breaks. This might have consequences for the use of mammography in breast cancer screening, as well as the choice between breast conserving therapy including radiotherapy and mastectomy at primary breast cancer diagnosis in young *BRCA1/2* mutation carriers. Good data regarding this topic, however, are scarce, mainly due to constraints in the design of performed studies. In this review, we will discuss the current literature on the association between ionizing radiation and developing breast cancer, with particular attention to patients with a *BRCA1/2* mutation.

INTRODUCTION

Both normal breast tissue and breast cancer tissue are sensitive to ionizing radiation. Although adjuvant radiotherapy for early breast cancer reduces the risk of local recurrence and improves breast cancer specific survival [1,2], the commonly used dose also entails potentially carcinogenic scattered radiation to the surrounding healthy tissue possibly leading to an increased risk of a second malignancy.

The earliest evidence that ionizing radiation is associated with an increased risk of breast cancer has been obtained from studies among atomic bombing survivors of Hiroshima and Nagasaki [3-5]. The relative risk (RR) of developing any form of cancer depends on the dose received, age at exposure and gender and is as high as 4.5 for women exposed to a high dose of radiation at young age [6]. Further evidence for the association of ionizing radiation and breast cancer risk was shown in epidemiological studies conducted among patients with scoliosis or tuberculosis intensively monitored by X-rays [7-10], and in studies performed in patients treated with radiotherapy for benign breast disease, postpartum mastitis and skin hemangioma, reviewed by Preston et al. [10]. The strongest and most reliable evidence, however was generated from studies conducted in patients treated with radiotherapy for hematological malignancies, mainly for Hodgkin's lymphoma [11-15]. For example survivors of Hodgkin's lymphoma treated with mantle field irradiation had a 2.7-fold increased risk of developing breast cancer, compared to those not treated with mantle field irradiation [15]. Importantly, these radiation-induced breast cancers seem to have a worse prognosis compared to sporadic breast cancers [16-18].

In this review we summarize the current literature regarding the role of ionizing radiation, used for diagnostic imaging or therapeutic purposes, in the development of a (second) primary breast cancer. Since patients with a *BRCA1/2* mutation might be more sensitive for the carcinogenic effect of both diagnostic radiation and radiotherapy because of impaired DNA repair capacity, special attention is paid to women with a *BRCA1/2* mutation. Possible implications for clinical practice and future research directions are proposed.

IONIZING RADIATION AND BREAST CANCER RISK

Ionizing radiation: general information

Ionization is the ejection of electrons from a molecule. The threshold for ionization lies in the ultraviolet region of the electromagnetic spectrum, so all X-rays used in diagnostic radiation and radiotherapy and gamma-rays (sometimes used in radiotherapy) are ionizing radiation. Ionizing radiation can cause cellular damage by indirect DNA damage via the production of free radicals but can also induce DNA damage directly. Radiation-induced single base damage and single strand DNA breaks are in general repaired rapidly and adequately through base excision repair and DNA ligation respectively, which eventually results in complete DNA repair. In contrast, repair of radiation-induced double strand DNA

breaks is much more complex and inadequate repair often leads to cell death. Repair of double strand DNA breaks is possible by so-called homologous recombination by which the breaks are repaired by using a sister chromatid or homologous chromosome as a template. However, cells with impaired homologous recombination, such as *BRCA1/2* deficient cells, make use of the less adequate non-homologous end-joining to repair the double strand DNA breaks. Using non-homologous end-joining, the two double strand DNA ends are linked together without using any information of a template and, as this is less accurate, often results in cell death [19,20].

The vulnerability of cells for ionizing radiation largely depends on the rate of cell proliferation, the total dose of radiation, the fractionation scheme and the capability of the cells to repair DNA damage. As a consequence, tumor cells, which in general have an accelerated cell division and frequently an impaired DNA repair capacity, are more prone to ionizing radiation compared to surrounding healthy tissue. For therapeutic ionizing radiation, it is therefore of great importance to maximize the therapeutic window, i.e. to increase the ratio of probability of antitumor effect over the risk of causing detrimental effects by exposing healthy tissue to radiation (Figure 1). The total radiation dose combined with the capacity of DNA damage repair in both malignant and healthy tissue will finally determine the favorable risk ratio of radiotherapy [21].

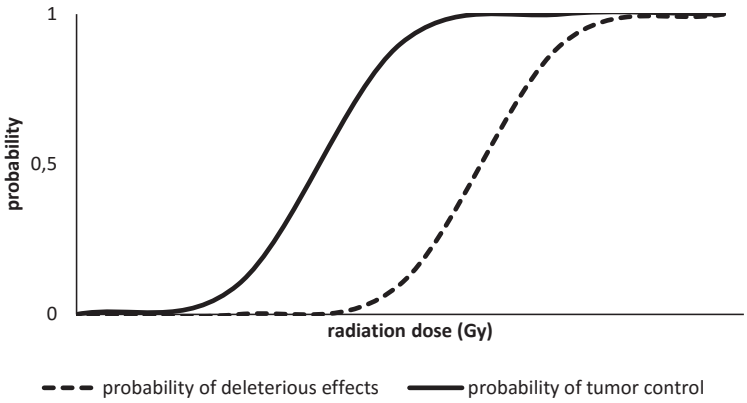

Figure 1. The vulnerability of particular malignant tumors for ionizing radiation depends to a large extent on the total dose of radiation to which the tumor cells are exposed. On the other hand (scattering) ionizing radiation to surrounding healthy tissue can cause unwanted acute and late toxicity. For therapeutic ionizing radiation, it is therefore of great importance to maximize the therapeutic window, i.e. increasing the ratio of probability of antitumor effect over the risk of causing detrimental effects.

Interestingly, the capacity to repair DNA damage might substantially differ between breast cancer patients, in particular when considering patients with or without a *BRCA1* or *BRCA2* mutation. BRCA1 and BRCA2 proteins are involved in various processes of DNA damage repair, including the repair of double strand DNA breaks by homologous recombination. Breast cancer arising in carriers of either a *BRCA1* or *BRCA2* gene mutation lacks BRCA1 or BRCA2 proteins, leading to inadequate repair of

double strand DNA breaks [22,23]. This leads to the hypothesis that radiotherapy administered for *BRCA1/2*-associated breast cancer might be more effective than for sporadic breast cancer, while, on the other hand, the surrounding healthy breast tissue might be more sensitive to the deleterious effects of radiation, among which the development of a second primary breast cancer. If true, the risk-benefit ratio of radiation differs between sporadic breast cancer patients and *BRCA1/2* mutation carriers and may have important clinical implications.

Dose-effect relation

The estimated total dose of ionizing radiation to the breast depends on the type of exposure and is reviewed in Table 1 [4,8,11,24-26]. Interestingly, studies among atomic bombing survivors and among patients exposed to diagnostic radiation (both low cumulative dose) have shown a statistically significantly increased breast cancer risk associated with increasing total radiation dose. The dose-effect relations found in these studies were linear [5,9,10] and did not show a threshold, which strongly suggests that any exposure to ionizing radiation can be carcinogenic [27]. In Hodgkin's lymphoma survivors, treated with high dose of ionizing radiation, the breast cancer risk also increased with larger radiation fields and higher cumulative radiation doses, although less sharp [11-13]. The dose-effect relation seems therefore to be best described by a linear-quadratic model (Figure 2), with a linear increasing risk for low cumulative doses, a flattening of the curve beginning at a cumulative dose of 10 Gy, and a less steep or even no further increase of the risk above a dose of 20 Gy [28].

Table 1. Estimated breast dose (Gy) by different types of ionizing radiation [4,8,11,24-26].

ionzing radiation	Dose
Chest X-ray	0.0005 Gy
Diagnostic mammography (2007)	0.004 Gy
Diagnostic mammography (1965)	0.0186 Gy
Computed tomography of the chest	0.02 Gy
Atomic bombing survivors	0.02-5 Gy
Scatter to contralateral breast	0.5-4 Gy
Scatter to contralateral breast (older studies)	up to 7 Gy
Mantle field radiotherapy (Hodgkin's lymphoma)	30-40 Gy
Adjuvant radiotherapy breast cancer	50 Gy (+ 16-20 Gy to the tumor bed region)

Duration of follow-up

Follow-up duration is another important factor to consider when interpreting studies on radiation-induced breast cancer risk. Studies among atomic bombing survivors and patients exposed to diagnostic radiation suggest a minimal latency period of 10-12 years before an increased breast cancer risk becomes apparent [4,9]. In studies of Hodgkin's lymphoma patients, the median latency period has been reported to be around 18 years (range 7-30 years) [13,29]. Thus, excluding a carcinogenic

effect of ionizing radiation is only possible when the median follow-up period after radiation exposure is long enough, e.g. at least longer than 10-15 years.

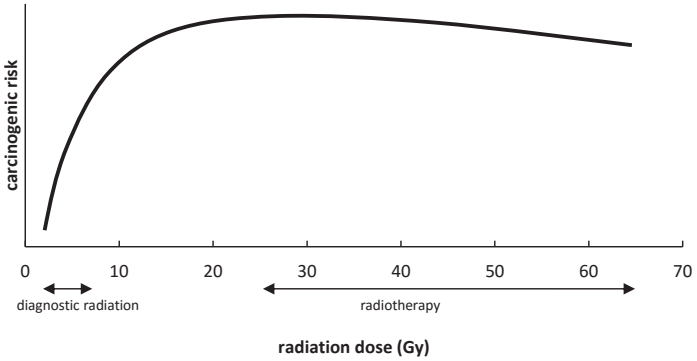

Figure 2. The dose-effect relation for the carcinogenic effects of both diagnostic and therapeutic radiation seems to be best described by a linear-quadratic model, with a linear increase of risk for low cumulative dose, flattening of the curve beginning at a cumulative dose of 10 Gy and less steep or even no evident further increase with a dose above 20 Gy.

Carcinogenic effect of adjuvant radiotherapy for primary breast cancer

Adjuvant radiotherapy after breast conserving surgery or mastectomy might be associated with an increased risk of a second primary breast cancer applying to the complete area of healthy breast tissue (ipsilateral and/or contralateral) exposed to (scattering of) ionizing radiation. Quantifying this risk, however, is quite challenging for several reasons.

Firstly, developing a true ipsilateral recurrence after breast cancer treatment is not due to the detrimental effects of adjuvant radiotherapy, but rather caused by failure of the adjuvant radiotherapy. Differentiating between a true recurrence, originating from residual cancer cells, and a second primary breast cancer, arising within residual glandular breast tissue, however, was not easy in times that molecular techniques were not yet available. Several characteristics have then been used trying to distinguish between these two entities, including time between primary breast cancer and ipsilateral relapse (<5 years: mostly true recurrence, and >5 years: most likely a second primary breast cancer), location of the first and second tumor (similar or different quadrants), and histological and immunohistochemical tumor characteristics (similar or different) [30-32]. However, since these clinical and pathological variables often failed to reliably distinguish between true recurrences and second primaries, other molecular techniques are to be preferred based on loss of heterozygosity patterns [33]. Studies in an unselected population of ipsilateral breast tumor recurrences using the latter technique found that the majority (60-76%) of recurrences after adjuvant radiotherapy were true recurrences [33-35]. Also, the chance that an ipsilateral breast tumor recurrence turned out to be a

second primary breast cancer instead of a true recurrence increased with longer disease free interval (18, 52 and 67% for breast cancer recurrence within 0-5 years, 5-10 years and >10 years respectively) [33], although the number of cases was small ($n = 57$).

Secondly, it is important to use within studies the most appropriate optimal control group. Investigating the risk of an ipsilateral second breast cancer in patients treated with breast conserving therapy including radiotherapy, the comparison should be made with patients undergoing breast conserving surgery without adjuvant radiotherapy. This question has been previously investigated by several randomized trials comparing breast conserving surgery with or without radiotherapy, and the results have been studied in a large individual patient data meta-analysis [2] of these studies (17 trials activated between 1976 and 1999; including $n = 10,801$; of whom 87% of patients had node negative breast cancer). After a median follow-up of 9.5 years, radiotherapy added to breast conserving surgery significantly reduced the locoregional recurrence rate at 10 years from 25 to 8%. While these figures are quite striking, it has to be taken into account that at the time these studies were performed, adjuvant systemic therapy was less often used and was less effective together with less optimal surgery compared to the present time. Unfortunately, these studies did not distinguish between true recurrences and second primary breast cancers [2].

Concerning the risk of contralateral breast cancer (CBC) in relation to adjuvant radiotherapy for primary breast cancer, this should be studied in patients treated with breast conserving therapy including radiotherapy or mastectomy and radiotherapy compared to patients treated with mastectomy or breast conserving surgery without adjuvant radiotherapy. Importantly, an increased CBC risk after adjuvant radiotherapy for primary breast cancer becomes apparent only after more than 10 years [1,8,26], indicating that studying this item requires a long follow-up time period. Valuable results are provided by a meta-analysis performed among patients with ductal carcinoma in situ treated with breast conserving surgery with or without radiotherapy ($n = 3,665$). A significantly increased CBC risk was found in the group treated with radiotherapy (RR 1.53; 95% confidence interval (CI) 1.05-2.24) suggesting a detrimental effect of ionizing radiation [36]. Other studies investigating the risk of CBC are reviewed in Table 2 [1,8,26,37-39]. Most of these studies found no significantly increased risk of CBC after adjuvant radiotherapy compared to no radiotherapy. The EBCTCG meta-analysis regarding the effects of locoregional treatments (surgery and/or radiotherapy) on local recurrence and survival found a RR of 1.18 ($p = 0.002$) for the development of CBC after adjuvant radiotherapy for primary breast cancer, resulting in only a small absolute risk of CBC of 4.4% in 15 years after adjuvant (versus no) radiotherapy [1]. Two of the other mentioned studies investigated the risk of CBC using an estimation of the radiation dose to the quadrant where the second primary breast cancer developed [26,39]. It was observed that the amount of (scattered) radiation dose to the contralateral breast in general is lower in case of radiotherapy after mastectomy compared to radiotherapy after lumpectomy [39]. Stovall et al. found that patients treated with radiotherapy for primary breast cancer had a significantly higher proportion of CBCs located in the inner and central quadrants of the contralateral breast, compared to the CBC locations of patients not receiving radiotherapy for primary breast cancer [26].

Hooning et al. reported a 1.5-fold increased risk of CBC for patients treated with radiotherapy after lumpectomy versus patients treated with radiotherapy after mastectomy [39].

In summary, based on the currently available data there seems to be a carcinogenic effect of scatter ionizing radiation of adjuvant radiotherapy for primary breast cancer among sporadic patients, mainly occurring with longer follow-up and taking the estimated radiation dose at the location of the recurrent breast tumor into account. Importantly, the magnitude of the increased breast cancer risk is smaller than expected given that the total amount of scatter radiation is comparable to the amount of ionizing radiation received by atomic bombing survivors (Table 1) in whom a clearly increased breast cancer risk has been found. Potential explanations for the former findings include the relatively short follow-up period in the various studies, the small part of healthy breast tissue being exposed during radiotherapy to scatter ionizing radiation, and the risk-reducing effect of administered adjuvant systemic therapy (or performed oophorectomy) on CBC. Furthermore in case of atomic bombing the radiation dose was delivered over a short time period, while in case of radiotherapy the total dose is delivered in fractions, resulting in a lower biologically equivalent dose. Finally, carcinogenic risk of ionizing radiation might be more pronounced in particular subgroups, for example in patients being younger at radiation exposure and/or *BRCA1/2* mutation carriers. We will describe these influencing factors in more detail below.

Age in relation to radiation-induced breast cancer risk

Most of the studies regarding the risk of developing CBC after radiotherapy for primary sporadic breast cancer found significantly elevated risks mainly in younger age groups, defined as age below 40 or 45 years (Table 2) [8,26,38,39]. Studies among women exposed to an even lower dose of ionizing radiation from atomic bombing or diagnostic imaging also showed an increased relative risk of developing breast cancer with decreasing age [4,5,10].

A possible explanation for the increased breast cancer risk at younger age could be the fact that a high breast cell proliferation (during puberty/adolescence and pregnancy) and thus increased DNA synthesis might render breast tissue particularly susceptible to the carcinogenic effects of radiation. Nulliparous women have been found to be more sensitive for the deleterious effect of radiation to the breast compared to parous women [40,41]. Further, it is possible that the observation of the increased risk at younger age reflects the fact that women developing breast cancer at younger age more likely to have a genetic susceptibility for developing breast cancer, in particular a *BRCA1/2* mutation. Still, the role of young age as a modifying factor in itself, independent of hereditary predisposition, is strongly supported by the finding that breast cancer risk in women treated with mantle field radiotherapy for Hodgkin's lymphoma is the highest in case of exposure around puberty and decreases with increasing age at radiotherapy [12,13,15].

Table 2. Radiotherapy for primary breast cancer and risk of contralateral breast cancer.

First author, year of publication	Study type	Population	number of patients		Relative risk of CBC after radiotherapy (95% CI or *p*-value)	
			total	CBC (%)		
Boice et al., 1992	Nested case control study 1:2 matching	time between PBC and CBC[a] >5 yr 66% follow-up ≥15 yr 30% age <45 yr	1,844	655 (35)	overall SA: age at primary BC SA: estimated radiation dose contralateral breast age at primary BC <45 yr and estimated radiation dose to the contralateral breast: 0.01-1.99 Gy 2.00-3.99 Gy ≥4.00 Gy	1.19 (0.94-1.50) n.s. n.s. 1.54 2.61 2.35 *p*-value for trend **0.003**
Storm et al., 1992	Nested case control study 1:1 matching	time between PBC and CBC[a] >8 years 40% follow-up ≥15 yr 19% age <45yr	1,058	529 (50)	overall SA: age at primary BC SA: estimated radiation dose contralateral breast	1.04 (0.74-1.46) n.s. n.s.
Stovall et al., 2008	Nested case control study 1:2 matching	WECARE study; time between PBC and CBC[a] >1 year 9% follow-up 10-14 yr 42% age <45 yr	2,107	708 (34)	overall SA: estimated radiation dose contralateral breast SA: estimated location-specific dose estimated location-specific dose ≥1.0 Gy and age at primary BC: <40 yr 40-44 yr 45-54 yr Age at and time since primary BC: <40 and <5 yr <40 and ≥5 yr ≥40 and <5 yr ≥40 and ≥5 yr	1.1 (0.9-1.3) n.s. n.s. **2.5 (1.4-4.5)** 1.0 (0.6-1.8) 1.0 (0.7-1.4) **2.3 (1.1-4.6)** **3.0 (1.1-8.1)** 1.0 (0.7-1.5) 1.0 (0.6-1.6)
Gao et al., 2003	Retrospective cohort study	SEER database PBC in 1973-1996 12% follow-up >15 yr 15% age <45yr	134,501	5,679 (4.2)	overall Time since primary BC ≥5 yr and age: <45 yr 45-55 yr ≥55 yr	1.04 (0.97-1.10) **1.32 (1.06-1.64)** 1.02 (0.83-1.24) **1.15 (1.01-1.32)**

Table 2. Radiotherapy for primary breast cancer and risk of contralateral breast cancer *(Continued)*.

First author, year of publication	Study type	Population	number of patients		Relative risk of CBC after radiotherapy (95% CI or *p*-value)	
			total	CBC (%)		
Hooning et al., 2008	Retrospective cohort study	1-year survivors of PBC. Median follow-up 13.8 year 45% follow-up >15 yr 31% age <45 yr	7,221	503 (7.0)	Overall SA: age at primary BC Age <45 yr and estimated radiation dose to contralateral breast: 0-2.2 Gy 2.2-4.1 Gy ≥4.1 Gy Age <45 yr and estimated radiation dose medial part of contralateral breast: 0-3.6 Gy 3.6-6.6 Gy ≥6.6 Gy	1.15 (0.89-1.50) n.s. 0.95 (0.49-1.84) 1.67 (0.85-3.27) **2.15 (1.04-4.43)** Risk of medial CBC: 1.23 (0.34-4.48) 2.72 (0.75-9.79) **5.26 (1.44-19.3)**
EBCTCG, 2005	Meta-analysis Above mentioned trials not included.	EBCTCG (primary question: locoregional treatments and influence on locoregional risk of recurrence).	32,800	1,451 (4.4)	Overall Age at primary BC: <50 yr ≥50 yr	**1.18 (0.002)** 1.09 (0.3) **1.25 (0.002)**
Viani et al., 2007	Meta-analysis 4 RCT	BCS with or without RT in patients with DCIS	3,365	115 (3.4)	overall	**1.53 (1.05-2.24)**

Unless otherwise stated, patients treated with radiotherapy were compared to patients not treated with radiotherapy, irrespective of type of surgery (mastectomy or breast conserving surgery (BCS) . In these studies patients were not tested for *BRCA1/2* mutation.

BC: breast cancer; CBC: contralateral breast cancer; PBC: primary breast cancer; SA: subgroup analysis; RT: radiotherapy; DCIS: ductal carcinoma in situ; RCT: randomized controlled trial; CI: confidence interval; yr: year; n.s.: not significant; SEER: Surveillance, Epidemiology, and End Results database; WECARE: Women's Environmental, Cancer, and Radiation Epidemiology study; EBCTCG: Early Breast Cancer Trialist Collaborative Group).

[a] under the assumption that the development of radiation-induced CBC takes some years, studies excluded patients who developed CBC in the first years after radiation for PBC.

IONIZING RADIATION IN *BRCA1/2* MUTATION CARRIERS

Impact of radiation in *BRCA1/2* mutation carriers: introduction

The lifetime risk of developing both a primary breast cancer and CBC is increased in female *BRCA1/2* mutation carriers as compared to the population risk [42-46]. Regarding ipsilateral second primary breast cancer no significantly increased risk has been found for *BRCA1/2* mutation carriers versus sporadic patients. Many studies, however, are limited by short follow-up periods and most studies did nog distinguish between a second primary breast cancer and an ipsilateral true recurrence. The subsequent question regarding our topic of interest is whether *BRCA1/2* mutation carriers are relatively more prone to the carcinogenic effect of ionizing radiation than non-carriers. We will first focus on the role of diagnostic radiation, and thereafter of therapeutic radiation in relation to the risks of developing a primary or second primary breast cancer, respectively.

Risk of breast cancer in *BRCA1/2* mutation carriers as a result of diagnostic imaging

The ideal study design to investigate this question would be a prospective cohort study, including healthy female *BRCA1/2* mutation carriers comparing those with and those without exposure to diagnostic ionizing radiation. This design, however, is not ethical as identified *BRCA1/2* mutation carriers nowadays are offered and enrolled in breast cancer screening programs. An acceptable alternative methodology could be a retrospective study among female *BRCA1/2* mutation carriers investigating the association between breast cancer diagnosis and the total amount of ionizing radiation exposure in the past. This methodology has been used in several studies [24,47-52] (Table 3). Importantly, three sorts of bias should be kept in mind while interpreting the data of such retrospective studies. Firstly, recall bias, i.e. the reporting of more exposure by affected women compared to non-affected women, which might lead to an overestimation of the breast cancer risks due to diagnostic radiation. This bias is probably low since several studies showed a good concordance between self-reported exposure to diagnostic radiation by questionnaires and radiation exposure obtained through data collection from the medical records [50]. Another and probably more important form of bias is introduced by the fact that those women with relevant abnormalities on screening tests will undergo additional diagnostic imaging tests increasing the total radiation dose further. Thirdly, survival bias, i.e. the fact that patients who died cannot be included in retrospective studies using patient reported exposures, might lead to an underestimation of radiation-induced breast cancer risk.

The studies of Narod et al. and Goldfrank et al., comprising 213 and 3,200 *BRCA1/2* mutation carriers respectively, did not find an increased breast cancer risk in subjects exposed to ionizing radiation by mammography [47,48]. The study by John et al. comprised 727 *BRCA1/2* mutation carriers (age <50 years) and did not find an increased breast cancer risk in subjects exposed to chest X-rays [49]. Details of these studies are mentioned in Table 3. Additional analyses among different subgroups, regarding age at exposure and age at diagnosis, did not show an increased risk either. The study by Goldfrank et al., however, was relatively small and included only a small proportion (14%) of women being younger than 30 years at exposure, precluding to draw firm conclusions [48]. Also in the study of John et al., only 7% of women were younger than 20 years at exposure [49].

Table 3. Diagnostic radiation and risk of primary breast cancer in *BRCA1/2* mutation carriers.

First author, year of publication	Study type	Population	number of patients		Diagnostic procedure	Hazard ratio/odds ratio of breast cancer (95% CI) by diagnostic radiation.	
			total	CBC (%)			
Narod et al., 2006	Nested case control study (Cases: BC; controls no BC)	Mutation carriers from 44 centers in six countries (North America, Europe, Israel)	3,200	1,600 (50%)	Mx (yes/no)	Overall SA: Age at first Mx: (≤30 vs. 31-40 vs. ≥41 yr)	OR 1.03 (0.94-1.50) n.s.
Goldfrank et al., 2006	Retrospective cohort study	Mutation carries from two centers (New York, Spain) Median follow-up: 11-12 yr	213	85 (40%)	Mx (number of Mx)	Overall SA: Age at diagnosis/questionnaire	OR 0.94 (0.88-1.00) n.s.
Andrieu et al., 2006	Retrospective cohort study	Mutation carriers from IBCCS. 2/3 of carriers are from GENEPSO, EMBRACE, HEBON	1,601	853 (53%)	Chest X-ray	Overall (any exposure) Age at diagnosis: ≤40 >40 Exposure before age 20 yr only Exposure after age 20 yr only 1-4 X-rays >4 X-rays	HR 1.54 (1.1-2.1) **HR 1.97 (1.3-2.9)** HR 1.27 (0.8-1.8) **HR 5.21 (1.6-17.5)** HR 1.91 (0.9-4.1) HR 1.76 (0.9-3.4) **HR 2.69 (1.4-5.3)**
Lecarpentier et al., 2011	Retrospective cohort study	Mutation carriers from GENEPSO	990	379 (38%)	Chest X-ray	Overall (any exposure) Before age 20 yr After age 20 yr One period with 1-4 X-rays Two periods with 1-4 X-rays At least one period with >4 X-rays	**HR 4.29 (2.09-8.81)** **HR 4.16 (2.03-8.56)** **HR 6.45 (2.86-14.6)** **HR 4.83 (1.83-12.8)** **HR 6.22 (2.94-13.1)** **HR 2.80 (1.30-6.05)**

Table 3. Diagnostic radiation and risk of primary breast cancer in *BRCA1/2* mutation carriers *(Continued)*.

First author, year of publication	Study type	Population	number of patients		Diagnostic procedure	Hazard ratio/odds ratio of breast cancer (95% CI) by diagnostic radiation.		
			total	CBC (%)				
Pijpe et al., 2012	Retrospective cohort study	Mutation carriers in GENE-RAD-RISK study	1,993	848 (43%)	Individually estimated cumulative breast dose	Overall Before age 30 yr Age 30-39 yr <0.0020 Gy ≥0.0174 Gy		HR 1.65 (1.11-2.46) **HR 1.90 (1.20-3.00)** HR 1.06 (0.66-1.71) HR 1.48 (0.94-2.33) HR 4.16 (2.01-8.62)
John et al., 2013	Retrospective cohort study	Mutation carriers from three registries (United States, Canada and Australia / New Zealand)	727	261 (36%)	Any exposure to chest X-ray		*BRCA1*	*BRCA2*
						Overall	OR 1.16 (0.64-2.11)	OR 1.22 (0362-2.42)
						Before age 20 yr	OR 0.57 (0.22-1.48)	OR 1.55 (0.54-4.47)
						Age ≥20 yr	OR 1.69 (0.76-3.76)	OR 1.06 (0.43-2.60)
						1-2 X-rays	OR 0.84 (0.33-2.13)	OR 0.80 (0.28-2.28)
						3-5 X-rays	OR 1.22 (0.35-4.21)	**OR 10.63 (1.93-58)**
						≥6 X-rays	OR 1.20 (0.37-3.96)	OR 0.89 (0.15-5.49)
Gronwald et al., 2008	Case only study	*BRCA1* mutation carriers from 18 centers in Poland	296	296 (100%) 138 *BRCA1* 158 non-*BRCA1*	Any exposure to chest X-ray before age 30	Chest X-ray below age 30 (carriers vs. non-carriers)		OR 1.8 (1.2-2.9)
						Age of first chest X-ray (carriers vs. non-carriers)		26.4 vs. 30.5 ($p = 0.01$)
						Mean number of chest X-rays before age 30		1.8 vs. 1.0 ($p = 0.002$)

SA: subgroup analysis; BC: breast cancer; Mx: mammography; OR: odds ratio; HR: hazard ratio; CI: confidence interval; yr: year; n.s.: not significant; IBCCS: International *BRCA1/2* Carrier Cohort Study; GENEPSO: French national *BRCA1/2* carrier cohort; HEBON: Dutch national *BRCA1/2* carrier cohort; EMBRACE: UK national *BRCA1/2* carrier cohort; GENE-RAD-RISK: European cohort study with carriers from GENEPSO, HEBON and EMBRACE.

Although the sample size in the study of Narod et al. was sufficiently large, several comments have to be made regarding the results interpretation [47]. Information regarding dose-response effects is not available, since exposure to ionizing radiation by mammography was used as a dichotomized variable (yes/no). Furthermore, information about time between exposure and breast cancer detection is lacking. In our opinion, it cannot be excluded that these factors might have underestimated a possible detrimental effect of diagnostic ionizing radiation in *BRCA1/2* mutation carriers.

In contrast, other retrospective studies found a positive association between diagnostic ionizing radiation and risk of developing breast cancer [24,50,52]. First, the large retrospective cohort study of Andrieu et al. including 1,601 *BRCA1/2* mutation carriers, showed that any exposure to chest X-rays versus no exposure was associated with an increased risk of breast cancer (hazard ratio (HR) 1.54 (1.1-2.1); $p = 0.007$) [50]. The study of Lecarpentier et al. including 990 *BRCA1/2* mutation carriers found a HR of 4.29 (95% CI 2.09-8.81) for the risk of breast cancer, comparing any exposure to no exposure to chest X-rays [52]. In accordance to the findings among the general female population, both Andrieu et al. and Lecarpentier et al. found evidence of both a dose-effect relation and an effect of age at exposure (Table 3). Unfortunately, data on ionizing radiation exposure in both studies were gathered from questionnaires and data on mammography (or other diagnostic imaging) exposure were not available for analyses. Furthermore it is important to mention that there is overlap in patient population between these two studies [50,52]. The most detailed study ($n = 1,993$) was performed by Pijpe et al. taking into account the cumulative ionizing radiation exposure to the breast as a result of all types of diagnostic imaging procedures [24]. In this study, a clear dose-effect relation between any exposure to diagnostic radiation before the age of 30 years (versus above 30 years) and breast cancer risk was observed (HR 1.90; 95% CI 1.20-3.00). Interestingly, within the younger age group, a positive association was also found within the group exposed to the lowest dose of ionizing radiation (below 0.0066 Gy). Ionizing radiation exposure above the age of 30 years, on the other hand, was not associated with an increased risk of breast cancer.

In a case control study, Gronwald et al. compared 138 breast cancer patients with a *BRCA1* mutation to 158 age-matched sporadic breast cancer patients not carrying a *BRCA1* mutation [51]. Among *BRCA1* mutation carriers, the mean age of first chest X-ray exposure was lower, and the total number of chest X-rays before the age of 20 years was higher compared to sporadic cases. The results of this cross-sectional study could support the hypothesis that early radiation exposure may be a risk factor for breast cancer development in *BRCA1* mutation carriers.

Taking into account the results of the study of Pijpe et al., the Dutch guidelines regarding female *BRCA1/2* mutation carriers advice to start annual breast MRI screening at age 25, and to add annual mammography as of the age of 30 years (http://www.oncoline.nl/mammacarcinoom). The United Kingdom National Institute for Health and Care Excellence (NICE) guidelines, meanwhile, advice to start annual breast MRI screening at age 30, to offer annual mammography from age 40 years onwards and to consider annual mammography from age 30 onwards. (http://www.nice.org.uk/guidance/cg164/resources/guidance-familial-breast-cancer-pdf). The United States National Comprehensive Cancer

Network (NCCN) guidelines still advice to screen *BRCA1/2* mutation carriers with annual mammogram and breast MRI starting at age 25 years (http://www.nccn.org/professionals/physician_gls/pdf/breast-screening.pdf). In our opinion the Dutch policy is supported by several computer simulation models, based on data regarding radiation induced breast cancer risk in the sporadic population, known sensitivity and specificity of the different screening methods and known risks of developing breast cancer in *BRCA1/2* mutation carriers, taking into account both beneficial and detrimental effects of various types of breast cancer screening. The simulation models incorporating MRI as screening tool, suggest that the best approach is to start screening with MRI at age 25 years, followed by the combined use of MRI alternating with mammography starting at age 30 [53-55].

Risk of breast cancer in *BRCA1/2* mutation carriers as a result of therapeutic radiation

As holds true for sporadic breast cancer patients, investigating the potential carcinogenic damage of therapeutic radiation for primary breast cancer among *BRCA1/2* mutation carriers, it is important to define the optimal control population. Furthermore it is important to realize that there are several confounding factors such as adjuvant systemic therapy (and oophorectomy) and prophylactic mastectomy.

Ipsilateral second primary breast cancer

As mentioned before, the study question on a possibly increased ipsilateral second primary breast cancer risk by adjuvant radiotherapy is challenging, since most studies did not differentiate between true recurrences and second primary tumors, while it has been shown that adjuvant radiotherapy decreases the risk of an ipsilateral true recurrence, and on the other hand possibly increases the risk of developing a second primary tumor in *BRCA1/2* mutation carriers. In our opinion, the only way to answer this question within the *BRCA1/2* mutation cohort is to compare breast cancer patients treated with breast conserving surgery followed by adjuvant radiotherapy to those not treated with radiotherapy. The previously mentioned very early studies on this issue did not provide data on the *BRCA1/2* status, and therefore cannot be used in this matter. Further, as omitting radiotherapy after lumpectomy for invasive breast cancer is not standard practice anymore, data heron are very rare. Metcalfe et al. published data on the risk of ipsilateral breast cancer in 396 women carrying a *BRCA1/2* mutation who were treated with breast conserving surgery, whereby 46 patients were not treated with adjuvant radiotherapy [56]. After a median follow-up of 10.5 years, the ipsilateral breast tumor recurrence rate was reduced with 72% in the group treated with adjuvant radiotherapy (RR 0.28; 95% CI 0.12-0.63; versus no radiotherapy). Again, this study did not distinguish between true recurrences and second primary breast cancers.

All other studies investigating the risk of an ipsilateral breast tumor recurrence (IBTR, either recurrence or second primary) after breast conserving therapy including radiotherapy compared patients with a *BRCA1/2* mutation to sporadic patients [45,57-61]. Interestingly in the study of Haffty et al., the pattern of IBTR in *BRCA1/2* mutation carriers and in sporadic patients followed a similar course for five years and then began to diverge with higher risks in *BRCA1/2* mutation carriers [45]. Seynaeve et al. also

found a higher rate of IBTR in hereditary cases compared to sporadic cases after five years only. Most recurrences in the hereditary cases occurred not in the same quadrant of the breast as the primary tumor, suggesting second primaries [57]. These findings together suggest that adjuvant radiotherapy in *BRCA1/2* mutation carriers has a beneficial effect on early ipsilateral recurrence (probably true recurrences) which is at least comparable to the effect in sporadic patients, while there might be an increased risk of late recurrence (probably second primary tumors). From the reported studies, however, it cannot be concluded that the increased risk of second primary tumor on the long term is caused by the administered radiotherapy.

Contralateral breast cancer

The available studies regarding the risk of CBC in *BRCA1/2* mutation carriers in relation to radiotherapy are reviewed in Table 4 [60,62,63]. These studies did not find an increased risk of CBC in patients treated with adjuvant radiotherapy compared to patients not treated with radiotherapy. The studies of Pierce et al. and Metcalfe et al. were multicenter, retrospective cohort studies of patients attending high-risk clinics, and reported after a median follow-up of eight years and eleven years, respectively.

Bernstein et al. performed a nested case control study within the WECARE study, a population-based study of patients with metachronous CBC (n = 603) and matched patients with unilateral breast cancer (n = 1,199). All patients were tested for *BRCA1/2* mutations and 158 *BRCA1/2* mutation carriers were identified of whom 96 had a metachronous CBC and 62 had no CBC (= controls). In these *BRCA1/2* mutation carriers no increased risk of CBC after radiotherapy for primary breast cancer was observed, even when adjusting for age at primary breast cancer, age at menarche, number of full term pregnancies, age at menopause, family history, adjuvant systemic treatment, histology and stage of the first primary [63].

In a case only study (n = 247), Broeks et al. found a higher proportion of mutations in DNA damage repair genes (*BRCA1*, *BRCA2*, *CHEK2* or *ATM*) in CBC patients being treated with adjuvant radiotherapy for the primary invasive breast cancer versus no radiotherapy. This suggests that carriers of a mutation in one of the DNA damage repair genes may be at increased risk of developing CBC after radiotherapy compared to non-carriers (odds ratio 2.18; 95% CI 1.03-4.62). The increased CBC risk was higher in younger patients, and after an interval between the primary and contralateral breast cancer of more than five years. The absolute number of *BRCA1/2* mutation carriers (n = 32) in this study was however small [64].

Based on the available data from the limited number of studies and patients, and in view of the limitations of the available literature, there are no hard data on a carcinogenic effect of scatter ionizing radiation after adjuvant radiotherapy for *BRCA1/2*-associated breast cancer patients. The situation, however, may be different for *BRCA1/2* mutation carriers at a young age. Since low dose (diagnostic) ionizing radiation seems already carcinogenic among young *BRCA1/2* mutation carriers, clinicians remain concerned regarding a carcinogenic effect of therapeutic ionizing radiation in young *BRCA1/2* mutation carriers.

Table 4. radiotherapy for primary breast cancer and risk of contralateral breast cancer in *BRCA1/2* mutation carriers.

First author, year of publication	Study type	Population	Number of patients		Relative risk of CBC by use of adjuvant radiotherapy for primary BC in *BRCA1/2* mutation carriers		
			Total	CBC (%)			
Pierce et al., 2010	Retrospective cohort study	BC patients with a known *BRCA1/2* mutation.	643 302 BCS + RT 241 Mastectomy 103 Mastectomy + RT	148 (23%)	5-year 10-year 15-year	0.98 1.29 1.15	$p = 0.44$
Metcalfe et al., 2011	Retrospective cohort study	BC patients from families with known *BRCA1/2* mutation	810 424 RT 373 no RT 396 BCS +/- RT 417 Mastectomy +/- RT	149 (18%)	Overall Age at PBC: <50 yr ≥50 yr	1.11 ($p = 0.56$) 1.11 ($p = 0.56$) 0.57 ($p = 0.35$)	
Bernstein et al., 2013	Nested case control study WECARE	Patients with a *BRCA1/2* mutation: asynchronous bilateral breast cancer, compared to unilateral breast cancer	158[a] 109 RT 49 no RT	96	overall	1.4 (95% CI 0.6-3.3)	
					Odds ratio (95% CI) of CBC by use of adjuvant radiotherapy for primary BC in patients with mutations in DDRP genes		
Broeks et al., 2007	Case only study	Women with asynchronous CBC aged <50 years at PBC	247 169 RT 78 no RT	247	overall Age <40 yr Age 40-50 yr Interval 1-5 yr Interval >5 yr	2.18 (1.03-4.62) 2.77 (0.74-10.39) 1.85 (0.73-4.65) 1.41 (0.34-5.78) **2.51 (1.03-6.10)**	

Patients treated with radiotherapy were compared to patients not treated with radiotherapy, irrespective of type of surgery (mastectomy or breast conserving surgery (BCS).

CBC: contralateral breast cancer; RT: radiotherapy; BCS: breast conserving surgery; BC: breast cancer; yr: year; WECARE: Women's Environmental, Cancer, and Radiation Epidemiology study. DDRP: DNA damage repair pathway. CI: confidence interval.

[a] no information about type of surgery.

The latter question however, cannot be definitely answered with the data from the available studies. In the two described retrospective cohort studies [60,62], only approximately 25% of the patients were younger than 35 years, and subgroup analyses regarding age at breast cancer treatment were not performed. Also in the WECARE study no specific subgroup analyses in younger patients were performed, while mean age at first breast cancer diagnosis was 46 years (range 23-55) [63].

CONCLUSIONS

Ionizing radiation (both diagnostic and therapeutic) is a well-known risk factor for the development of primary breast cancer. There is a clear positive dose-risk relation, which is modified by age, whereby young age at exposure is associated with an increased risk.

For sporadic breast cancer patients diagnosed above 45 years of age, adjuvant radiotherapy in the context of primary breast cancer treatment is associated with no or, if present, a negligibly increased risk of a second primary breast cancer and there is no reason to withhold radiotherapy in adjuvant setting. For younger patients no definite conclusion can be drawn based on the current data.

For the subgroup of *BRCA1/2* mutation carriers, data regarding a carcinogenic effect of ionizing radiation (both diagnostic and therapeutic) are scarce, which can be partly explained by the fact that it is difficult to conduct well-designed studies excluding selection bias. Nevertheless for screening purposes there seems sufficiently enough evidence to incorporate mammography in breast cancer screening programs for *BRCA1/2* mutation carriers only after the age of 30 years.

For those *BRCA1/2* mutation carriers who developed breast cancer above the age of 30 years and opting for breast conserving therapy, there are no hard data regarding a possibly increased carcinogenic effect of adjuvant radiotherapy with respect to a second primary breast cancer, either ipsilateral or contralateral. However, a carcinogenic effect of adjuvant radiotherapy on the long term in this population has certainly not been excluded. Since low dose diagnostic radiation increases the risk of primary breast cancer in very young *BRCA1/2* mutation carriers (<30 years), caution with regard to breast conserving surgery and radiotherapy seems warranted in this patient group.

Further research on the carcinogenic effects of specifically therapeutic ionizing radiation in the *BRCA1/2* population is urgently needed to answer the question whether breast conserving treatment is an appropriate option for these women, not only on the short but also on the long term. Research should focus mainly on the younger age group as in these patients the highest radiation induced risks can be expected. For future studies it is crucial to have an adequate study design, sufficiently long period of follow-up, information available on total ionizing radiation dose received (both diagnostic and therapeutic) and diagnostic plans regarding the distinction between a true recurrence or a second ipsilateral tumor. Furthermore, it is important to take other influencing factors into account, such as tumor characteristics, use of adjuvant systemic therapy, oophorectomy and/or prophylactic mastectomy. It might also be important to make a distinction between *BRCA1* and *BRCA2* mutation carriers. This requires the concentration of *BRCA1/2* mutation carriers, at least the data of them, on a national and even an international level.

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3

Adjuvant radiotherapy for primary breast cancer in *BRCA1* and *BRCA2* mutation carriers and risk of contralateral breast cancer with special attention to patients irradiated at younger age

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ABSTRACT

The purpose of this study was to estimate the influence of adjuvant radiotherapy for primary breast cancer on the risk of contralateral breast cancer (CBC) in *BRCA1* or *BRCA2* (*BRCA1/2*) mutation carriers, with special attention to patients irradiated at age younger than 40 years. Additionally, tendencies in locoregional treatments and rates of contralateral risk-reducing mastectomy over time were explored. In this retrospective cohort study, 691 *BRCA1/2*-associated breast cancer patients treated between 1980 and 2013 were followed from diagnosis until CBC or censoring event including ipsilateral breast cancer recurrence, distant metastasis, contralateral risk-reducing mastectomy, other invasive cancer diagnosis, death, or loss to follow-up. Hazard ratios (HR) for CBC associated with radiotherapy were estimated using Cox regression. Median follow-up time was 8.6 years [range 0.3-34.3 years]. No association between radiotherapy for primary breast cancer and risk of CBC was found, neither in the total population (HR 0.82; 95% confidence interval (CI) 0.45-1.49) nor in the subgroup of patients younger than 40 years at primary diagnosis (HR 1.36; 95% CI 0.60-3.09). During follow-up, the number of patients at risk decreased substantially since a large proportion of patients were censored after contralateral risk-reducing mastectomy or breast cancer recurrence. Over the years, increasing preference for mastectomy without radiotherapy compared to breast conserving surgery with radiotherapy was found ranging from less than 30% in 1995 to almost 50% after 2010. The rate of contralateral risk-reducing mastectomy increased over the years from less than 40% in 1995 to more than 60% after 2010. In this cohort of *BRCA1/2*-associated breast cancer patients, no association between radiotherapy for primary breast cancer and risk of CBC was observed in the total group, nor in the patients irradiated before the age of 40 years. The number of patients at risk after 10 and 15 years of follow-up, however, was too small to definitively exclude harmful effects of adjuvant radiotherapy.

INTRODUCTION

Both normal breast tissue and breast cancer cells are sensitive to ionizing radiation. Although adjuvant radiotherapy for early breast cancer reduces the risk of local recurrence and improves breast cancer specific survival [1,2], it also leads to a low dose scatter radiation to the surrounding healthy tissue with potentially carcinogenic effects. In sporadic breast cancer patients, adjuvant radiotherapy has been associated with an increased risk of contralateral breast cancer (CBC), although only among women younger than 45 years at primary breast cancer diagnosis and after a latency period of at least 10-15 years [3-6].

The vulnerability of cells for ionizing radiation largely depends on the rate of cell proliferation, the total dose of radiation, the fractionation scheme, and the capability of the cells to repair DNA damage [7]. Younger patients have higher breast cell proliferation (in particular during puberty, adolescence, and pregnancy) and thus increased DNA synthesis that might render breast tissue particularly susceptible to the carcinogenic effects of radiation [8,9]. The capacity to repair DNA damage might substantially differ between breast cancer patients, in particular when considering patients with or without a *BRCA1* or *BRCA2* (*BRCA1/2*) mutation.

BRCA1/2-associated breast cancer is characterized by homologous recombination deficiency, leading to inadequate repair of double strand DNA breaks [10, 11]. Ionizing radiation can cause cell damage by induction of double strand DNA breaks. This has led to the hypothesis that adjuvant radiotherapy administered for *BRCA1/2*-associated breast cancer might be more effective than radiotherapy administered for sporadic breast cancer. On the contrary, surrounding healthy breast tissue among breast cancer patients with a *BRCA1/2* mutation might be more vulnerable to the deleterious effects of adjuvant radiotherapy, including the development of a CBC, compared to those without a *BRCA1/2* mutation.

In unaffected *BRCA1/2* mutation carriers, exposure to low cumulative doses of diagnostic radiation (including screening mammography) at young age (<30 years) has been reported to be associated with an increased risk of breast cancer, with a clear dose-effect relationship compared to no exposure to diagnostic radiation [12]. The possible carcinogenic effect of scatter ionizing radiation after adjuvant radiotherapy on the contralateral breast in *BRCA1/2*-associated breast cancer patients, however, is not clear. Although a number of studies addressed this question, all these studies are compromised by a short duration of follow-up and the lack of subgroup analyses regarding young breast cancer patients [13-15]. Knowledge about the possibly increased risk of CBC by radiotherapy might be of great importance for optimal shared decision making regarding mastectomy without radiotherapy versus breast conserving surgery including radiotherapy at primary breast cancer diagnosis.

We therefore studied the impact of radiotherapy on the risk of CBC among *BRCA1/2*-associated breast cancer patients in a retrospective cohort study, with special attention to patients younger than 40 years at primary breast cancer diagnosis. Since over the years an increasing proportion of *BRCA1/2* mutation carriers after developing breast cancer seems to opt for bilateral mastectomy

instead of unilateral mastectomy or breast conserving treatment with radiotherapy [16], we also explored potential tendencies in locoregional treatments and the rates of contralateral risk-reducing mastectomy over the past decades.

METHODS

Patient selection

From the Rotterdam Family Cancer Clinic database, we extracted all female patients with early stage breast cancer (*n* = 2,268). From this population, we selected proven or obligate *BRCA1* or *BRCA2* mutation carriers, treated at the Erasmus MC Cancer Institute. Patients diagnosed from January 1, 1980, corresponding to the start of linear accelerators use for adjuvant breast radiotherapy at the Erasmus MC, to January 1, 2013 were included (*n* = 790). Time of observation ended at April 1, 2014. Patients with less than three months of follow-up were excluded (*n* = 52; see statistical analysis). Patients who were treated with breast/chest wall radiotherapy or systemic anticancer therapy because of another invasive malignancy, either prior or synchronous to the primary breast cancer, were excluded (*n* = 16). Patients who had synchronous bilateral breast cancer and received bilateral radiation therapy or mastectomy (*n* = 31) were also excluded, leaving a total of 691 patients available for the analyses.

For the eligible patients, data on primary breast cancer and CBC characteristics (type of histology, differentiation grade, estrogen receptor status, progesterone receptor status, HER2 status, and stage) and primary breast cancer therapy (surgery, radiotherapy, chemotherapy, and/or endocrine therapy) were retrieved. We also collected data on type of mutation (i.e., *BRCA1* or *BRCA2*), date of birth, primary breast cancer and CBC diagnoses, dates of and findings at contralateral risk-reducing mastectomy and salpingo-oophorectomy, and dates of disease recurrence and death or date of last follow-up if no event occurred.

Statistical analysis

The primary endpoint was the development of CBC defined as the occurrence of carcinoma in situ or invasive breast cancer in the contralateral breast at least three months after primary breast cancer diagnosis and no signs of metastatic disease. CBC diagnosis within three months was considered as synchronous bilateral breast cancer and assumed to be unrelated to the delivery of radiotherapy for the first breast cancer [3-5]. For this reason, patients with less than three months of follow-up were excluded.

For comparisons of patient, tumor, and treatment characteristics between subgroups, we used Pearson's χ^2 tests. Differences in age at primary breast cancer diagnosis and follow-up time were analyzed using the Wilcoxon rank-sum test (Mann-Whitney).

In the Cox analyses, we applied left truncation of analysis time and so considered outcome data from prospective follow-up only. Hereby, we aimed to correct for potential selection bias, possibly arising due to inclusion of patients undergoing genetic testing after primary breast cancer or CBC diagnosis

[17,18]. Censoring events were ipsilateral breast cancer recurrence for which radiotherapy or systemic therapy was applied, distant metastasis, contralateral risk-reducing mastectomy, other (non-breast) invasive cancer for which radiotherapy or systemic therapy was applied, death, and loss to follow-up. We estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for radiotherapy (after lumpectomy vs. after mastectomy vs. none), adjuvant chemotherapy (yes vs. no), adjuvant endocrine therapy (yes vs. no), salpingo-oophorectomy (treated as time-dependent variable), age at primary breast cancer, and *BRCA* mutation type (*BRCA1* vs. *BRCA2*) using Cox regression in univariate and multivariate analyses. The cumulative 5-, 10-, and 15-year risks of CBC were calculated using Kaplan-Meier analysis including only patients who underwent DNA testing for *BRCA1/2* mutation before the diagnosis of CBC, to correct for potential selection bias.

Analyses were performed for the total group and for patients younger than 40 years at primary breast cancer, as it has been previously reported that younger patients are more susceptible for radiation-induced breast cancer [3-6].

The proportion of patients undergoing different locoregional treatments over time, including breast conserving treatment and mastectomy with or without radiotherapy, was estimated with a regression line of best fit and 95% CI based on the proportion per year. The same was performed for the proportion of patients undergoing contralateral risk-reducing mastectomy over time. For statistical analysis STATA, version 13.0, was used. For computing the figures, R version 3.2.2 (released on 2015-08-14) and the package GGplot version 1.0.1. were used.

RESULTS

A total of 691 *BRCA1/2*-associated breast cancer patients, consisting of 517 *BRCA1* and 174 *BRCA2* mutation carriers, were eligible for data analysis (Tables 1 and 2). Median time of follow-up of the entire cohort was 8.6 years with a range from 0.3 to 34.3 years. A total of 439 patients were treated with radiotherapy either after lumpectomy (n = 349) or after mastectomy (n = 85). A total of 325 patients were younger than 40 years at primary breast cancer diagnosis (Table 2). Further details on patient, tumor, and treatment characteristics are presented in Tables 1 and 2.

Of all patients, 161 (23%) developed CBC, of whom 87 were younger than 40 years at breast cancer onset. The cumulative 5-, 10-, and 15-year risks of CBC for the total cohort were 8, 19, and 32%, respectively. Among the patients younger than 40 years, the cumulative 5-, 10-, and 15-year CBC risks were 11, 32, and 40%, respectively. Cumulative risks for *BRCA*-specific subgroups suggest a higher cumulative risk for *BRCA1*-associated patients compared to *BRCA2*-associated patients (Table 3). Median time interval between primary breast cancer and CBC was 4.8 years (range 0.5-29.0) for the entire cohort and 5.5 years (range 0.5-29.0 years) for patients diagnosed before the age of 40.

Left truncation was applied to correct for survival bias that may occur in studies with patient recruitment at a variable time after diagnosis (see statistical analysis). Consequently, a considerable number of patients did not contribute person time to the prospective follow-up, leaving 418 patients

Table 1. Characteristics of the patients, radiotherapy vs. no radiotherapy.

	Total (***n*** **= 691)**[a] ***n*** **(%)**	**RT after lumpectomy (*n* = 349)** ***n*** **(%)**	**No RT after mastectomy (*n* = 252)** ***n*** **(%)**	**RT after mastectomy (*n* = 85)** ***n*** **(%)**	***p*-value**
Age at primary breast cancer					
<30 years	55 (8.0)	29 (8.3)	19 (7.5)	7 (8.2)	
30-34 years	115 (16.6)	59 (16.9)	39 (15.5)	15 (17.0)	
35-39 years	155 (22.4)	78 (22.3)	57 (22.6)	20 (23.5)	
40-44 years	129 (18.7)	64 (18.3)	49 (19.4)	16 (18.8)	
45-50 years	100 (14.5)	48 (13.8	35 (13.9)	16 (18.8)	
>50 years	137 (19.8)	71 (20.3)	53 (21.0)	11 (12.9)	0.943
Mutation status					
BRCA1	517 (74.8)	277 (79.4)	186 (73.8)	50 (58.8)	
BRCA2	174 (25.2)	72 (20.6)	66 (26.2)	35 (41.2)	<0.001
Period of primary breast cancer					
1980-1989	105 (15.2)	64 (18.3)	27 (10.7)	14 (16.5)	
1990-1999	256 (37.1)	139 (39.8)	101 (35.3)	27 (31.8)	
2000-2013	330 (47.8)	146 (41.8)	164 (54.0)	44 (51.8)	0.017
Tumor stage					
Tis	26 (4.0)	14 (4.1)	12 (5.2)	0	
T1	364 (56.0)	209 (61.8)	130 (56.5)	25 (30.9)	
T2	227 (34.9)	114 (33.7)	80 (34.8)	32 (39.5)	
T3	25 (3.9)	0	7 (3.0)	18 (22.2)	
T4	8 (1.2)	1 (0.3)	1 (0.4)	6 (7.4)	
Unknown	41	11	22	4	<0.001
Nodal status					
N0	424 (64.3)	241 (71.9)	169 (70.1)	13 (16.0)	
N1-3	235 (35.7)	94 (28.1)	72 (29.9)	68 (84.0)	
Unknown	32	14	11	4	<0.001
Histological grade					
Grade 1	17 (3.3)	8 (3.1)	7 (3.6)	2 (3.0)	
Grade 2	106 (20.4)	54 (21.0)	37 (19.2)	14 (20.9)	
Grade 3	396 (76.3)	195 (75.9)	149 (77.2)	51 (76.1)	
unknown	172	92	59	18	0.988
Hormone receptor status					
Positive	227 (39.5)	108 (37.8)	80 (37.9)	39 (50.0)	
Negative	348 (60.5)	178 (62.2)	131 (62.1)	39 (50.0)	
Unknown	116	63	41	7	0.124
HER2 status					
Positive	17 (6.7)	9 (8.1)	5 (5.2)	3 (7.5)	
Negative	236 (93.3)	101 (91.8)	95 (94.8)	37 (92.5)	
Unknown	438	239	152	45	0.646
(Contralateral) risk-reducing mastectomy	424 (64.5)	243 (73.0)	127 (51.8)	54 (68.4)	
No	233 (35.5)	90 (27.0)	118 (46.2)	25 (31.7)	
Yes	34	16	7	6	<0.001
Unknown					

Table 1. Characteristics of the patients, radiotherapy vs. no radiotherapy *(Continued)*.

	Total (***n*** **= 691)**[a] ***n*** **(%)**	**RT after lumpectomy** (***n*** **= 349)** ***n*** **(%)**	**No RT after mastectomy** (***n*** **= 252)** ***n*** **(%)**	**RT after mastectomy** (***n*** **= 85)** ***n*** **(%)**	***p*-value**
Salpino-oophorectomy					
No	259 (41.2)	135 (42.5)	87 (38.2)	35 (44.3)	
Yes	370 (58.8)	183 (57.5)	141 (61.8)	44 (55.7)	
Unknown	62	31	24	6	0.499
(Neo)adjuvant chemotherapy					
No	319 (46.6)	176 (51.0)	109 (43.6)	30 (35.7)	
Yes	365 (53.4)	169 (49.0)	141 (56.4)	54 (64.3)	
Unknown	7	4	2	1	0.022
Adjuvant endocrine therapy					
No	555 (81.1)	300 (87.2)	203 (81.2)	48 (56.5)	
Yes	129 (18.9)	44 (12.8)	47 (18.9)	37 (43.5)	
Unknown	7	5	2	0	<0.001

RT: radiotherapy.
[a] Data on type of surgery (either lumpectomy or mastectomy) was missing in five patients who were treated with radiotherapy.

Table 2. Characteristics of the patients with age at primary breast cancer diagnose <40 years, radiotherapy vs. no radiotherapy.

	Total (***n*** **= 325)**[a] ***n*** **(%)**	**RT after lumpectomy** (***n*** **= 166)** ***n*** **(%)**	**No RT after mastectomy** (***n*** **= 115)** ***n*** **(%)**	**RT after mastectomy** (***n*** **= 42)** ***n*** **(%)**	***p*-value**
Age at primary breast cancer					
<30 years	55 (16.9)	29 (17.5)	19 (16.5)	7 (16.7)	
30-34 years	115 (35.4)	59 (35.5)	39 (33.9)	15 (35.7)	
35-39 years	155 (47.7)	78 (47.0)	57 (49.6)	20 (47.6)	0.996
Mutation status					
BRCA1	261 (80.3)	143 (86.1)	89 (77.4)	27 (64.3)	
BRCA2	64 (19.7)	23 (13.9)	26 (22.6)	15 (35.7)	0.004
Period of primary breast cancer					
1980-1989	43 (13.2)	33 (19.9)	5 (4.4)	5 (11.9)	
1990-1999	114 (35.1)	68 (41.0)	35 (30.4)	10 (23.8)	
2000-2013	168 (51.7)	65 (39.2)	75 (65.2)	27 (64.3)	<0.001
Tumor stage					
Tis	9 (2.9)	4 (2.6)	5 (4.5)	0	
T1	179 (58.5)	95 (60.5)	70 (63.6)	14 (35.9)	
T2	103 (33.7)	57 (36.3)	31 (28.2)	15 (38.5)	
T3	8 (2.6)	0	3 (2.7)	5 (12.8)	
T4	7 (2.3)	1 (0.6)	1 (0.9)	5 (12.8)	
Unknown	19	9	5	3	<0.001

Table 2. Characteristics of the patients with age at primary breast cancer diagnose <40 years, radiotherapy vs. no radiotherapy *(Continued)*.

	Total (***n*** **= 325)**[a] ***n*** **(%)**	**RT after lumpectomy (*n* = 166)** ***n*** **(%)**	**No RT after mastectomy (*n* = 115)** ***n*** **(%)**	**RT after mastectomy (*n* = 42)** ***n*** **(%)**	***p*-value**
Nodal status					
N0	206 (66.0)	120 (74.5)	78 (70.3)	7 (17.9)	
N1-3	106 (34.0)	41 (25.5)	33 (29.7)	32 (82.1)	
Unknown	13	5	4	3	<0.001
Histological grade					
Grade 1	6 (2.5)	2 (1.7)	2 (2.1)	2 (6.5)	
Grade 2	45 (18.4)	21 (17.7)	17 (18.1)	7 (22.6)	
Grade 3	193 (79.1)	96 (80.7)	75 (79.8)	22 (71.0)	
Unknown	81	47	21	11	0.561
Hormone receptor status					
Positive	93 (33.1)	41 (29.5)	31 (30.7)	21 (52.5)	
Negative	188 (66.9)	98 (70.5)	70 (69.3)	19 (47.5)	
Unknown	44	27	14	2	0.020
HER2 status					
Positive	10 (7.6)	4 (7.8)	3 (5.5)	3 (12.0)	
Negative	122 (92.4)	47 (92.2)	52 (94.5)	22 (88.0)	
Unknown	193	115	60	17	0.592
(Neo)adjuvant chemotherapy					
No	125 (38.9)	75 (45.7)	33 (28.9)	16 (39.0)	
Yes	196 (61.1)	89 (54.3)	81 (71.1)	25 (61.0)	
Unknown	4	2	1	1	0.019
Adjuvant endocrine therapy					
No	262 (81.4)	148 (90.2)	90 (78.9)	22 (52.4)	
Yes	60 (18.6)	16 (9.8)	24 (21.1)	20 (47.6)	
Unknown	3	0	1	0	<0.001
Contralateral risk-reducing mastectomy					
No	174 (55.8)	105 (66.0)	46 (41.1)	23 (56.1)	
Yes	138 (44.2)	54 (34.0)	66 (58.9)	18 (43.9)	
Unknown	13	7	3	1	<0.001
Salpingo-oophorectomy					
No	128 (42.8)	66 (43.7)	43 (40.6)	18 (45.0)	
Yes	171 (57.2)	85 (56.3)	63 (59.4)	22 (55.0)	
Unknown	26	15	9	2	0.825

RT: radiotherapy.

[a] Data on type of surgery (either lumpectomy or mastectomy) was missing in two patients who were treated with radiotherapy.

for the main analyses. In univariate analysis, the risk of CBC was increased in patients younger than 40 years compared to those older than 40 years at primary breast cancer (HR 2.42; 95% CI 1.34-4.38). Furthermore, mutation carriership of *BRCA1* was associated with increased risk of CBC as compared to *BRCA2* mutation carriership (HR 2.32; 95% CI 0.98-5.51). Both chemotherapy and endocrine therapy were significantly associated with a decreased risk of CBC (HR 0.45; 95% CI 0.25-0.81 and HR 0.27; 95% CI 0.08-0.86, respectively). For salpingo-oophorectomy, no association with CBC risk was found (HR 0.73; 95% CI 0.37-1.43) (Table 4).

No deleterious effect of radiotherapy for primary breast cancer, either after lumpectomy or after mastectomy, on CBC risk was found for the entire population (HR 0.84; 95% CI 0.46-1.55 and HR 0.62; 95% CI 0.17-2.23, respectively) (Table 4). Adjusting for age, adjuvant chemotherapy, adjuvant endocrine therapy, and type of *BRCA* mutation in a multivariate analysis still showed no association of radiotherapy on CBC risk (HR 0.74; 95% CI 0.40-1.37 and HR 0.96; 95% CI 0.23-3.97, respectively).

Table 3. Cumulative 5-, 10-, and 15-year risks of contralateral breast cancer.

Years after diagnosis	Overall % (*n* at risk)	*BRCA1* mutation % (*n* at risk)	*BRCA2* mutation % (*n* at risk)	Age <40 % (*n* at risk)	Age ≥40 % (*n* at risk)
5	8 (198)	9 (140)	5 (58)	11 (86)	6 (112)
10	19 (98)	21 (75)	15 (23)	32 (39)	10 (59)
15	32 (47)	35 (37)	15 (10)	40 (17)	23 (30)

Cumulative 5-, 10-, and 15-year risks of contralateral breast cancer in different subgroups of breast cancer patients (*BRCA1* mutation carriers vs. *BRCA2* mutation carriers and age at primary breast cancer <40 years vs. ≥40 years). Only those patients who underwent DNA testing for *BRCA1/2* mutation before the diagnosis of contralateral breast cancer were included.

Subgroup analyses of patient younger than 40 years at BC onset

Also in the subgroup of patients younger than 40 years at primary breast cancer diagnosis, no effect of radiotherapy for primary breast cancer, either after lumpectomy or after mastectomy, on CBC risk was found in univariate analysis (n = 211; HR 1.41; 95% CI 0.62-3.23 and HR 0.94; 95% CI 0.18-4.86, respectively), and this was maintained in multivariate analysis (HR 1.53; 95% CI 0.22-10.51 and HR 0.97; 95% CI 0.41-2.30, respectively) (Figure 1; Table 4). Median time interval between primary breast cancer and CBC diagnoses was not significantly different between those treated with radiotherapy for primary breast cancer compared to those patients not receiving radiotherapy (5.5 vs. 4.9 years, $p = 0.88$).

During follow-up, the number of patients at risk substantially decreased because a large proportion of patients were censored as they underwent a contralateral risk-reducing mastectomy, developed a breast cancer recurrence or a second non-breast malignancy. In the group younger than 40 years at breast cancer onset, 165 of 325 patients (51%) were censored in the first 10 years of follow-up because of these three reasons (Figure 2). Furthermore, since a large proportion of patients had less than 10 years of follow-up time, only 29 and 14 patients were available for the prospective analyses after 10 and 15 years of follow-up in this age group, respectively.

Table 4. Univariate and multivariate hazard ratios for risk of contralateral breast cancer associated with selected factors.

	Overall	**Age <40 years**	
	Univariate analyses **Number of patients: *n* = 418** **Person years: 1105 years**	**Univariate analyses** **Number of patients: *n* = 211** **Person years: 467 years**	**Multivariate analysis[a]** **Number of patients: *n* = 211** **Person years: 467 years**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**
Age at primary breast cancer			
<40 years	2.42 (1.34-4.38)		
≥40 years	1		
Age at primary breast cancer			
Continuous	**0.94 (0.90-0.97)**	0.93 (0.85-1.01)	0.96 (0.88-1.06)
BRCA mutation			
BRCA1	2.32 (0.98-5.51)	3.52 (0.83-14.99)	2.33 (0.51-10.73)
BRCA2	1	1	1
Chemotherapy			
No	1	1	1
Yes	**0.45 (0.25-0.81)**	0.51 (0.24-1.09)	0.52 (0.24-1.14)
Endocrine therapy			
No	1	1	1
Yes	**0.27 (0.08-0.86)**	0.24 (0.06-1.02)	0.25 (0.05-1.23)
Salpingo-oophorectomy (time dependent)			
No	1	1	
Yes	0.73 (0.37-1.43)	1.22 (0.53-2.81)	
Radiotherapy			
No radiotherapy after mastectomy	1	1	1
Radiotherapy after mastectomy	0.62 (0.17-2.23)	0.94 (0.18-4.86)	0.97 (0.41-2.30)
Radiotherapy after lumpectomy	0.84 (0.46-1.55)	1.41 (0.62-3.23)	1.53 (0.22-10.51)

HR: hazard ratio; CI: confidence interval

[a] The following variables were incorporated in the multivariate model: age at primary breast cancer (continuous variable), type of *BRCA* mutation (*BRCA1* vs. *BRCA2*), adjuvant chemotherapy (yes vs. no), adjuvant endocrine therapy (yes vs. no) and radiotherapy (no radiotherapy after mastectomy vs. radiotherapy after mastectomy and vs. radiotherapy after lumpectomy).

Treatment choices over time

Over the past decades, the proportion of patients at risk for radiation-induced CBC changed substantially as a result of an increased rate of mastectomy without radiotherapy instead of breast conserving therapy for primary breast cancer, and an increased rate of contralateral risk-reducing mastectomy (Figures 3 and 4). For example, patients aged younger than 40 years at diagnosis more often opted for mastectomy without radiotherapy instead of breast conserving therapy in 2010 (reaching 50%), compared to less than 30% in 1995. The proportion of patients receiving radiotherapy following mastectomy was relatively stable over time being around 10-15% (Figure 3). Since 2010, more than 60% of patients younger than 40 years at primary diagnosis opted for contralateral risk-reducing mastectomy, after primary breast cancer treatment, which was less than 40% in 1995 (Figure 4).

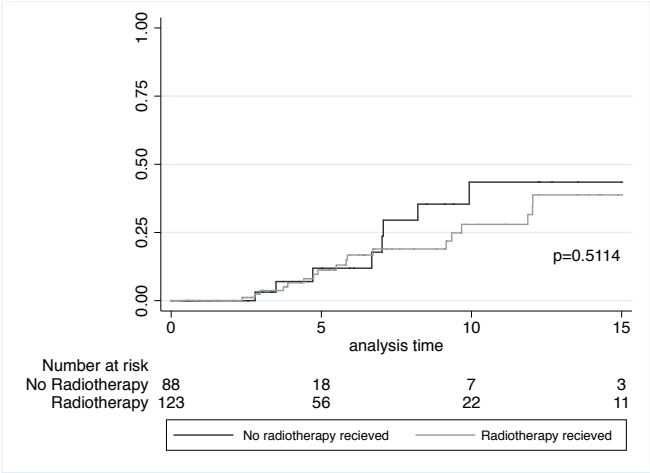

Figure 1. Kaplan-Meier estimates of the contralateral breast cancer (CBC) risk in *BRCA1/2* mutation carriers, younger than 40 years of age at primary breast cancer diagnosis. For this analysis, left truncation of analysis time at the DNA test date was applied, to correct for survival bias. Patients treated with radiotherapy (either after lumpectomy or after mastectomy) were compared to those not treated with radiotherapy at primary breast cancer diagnosis.

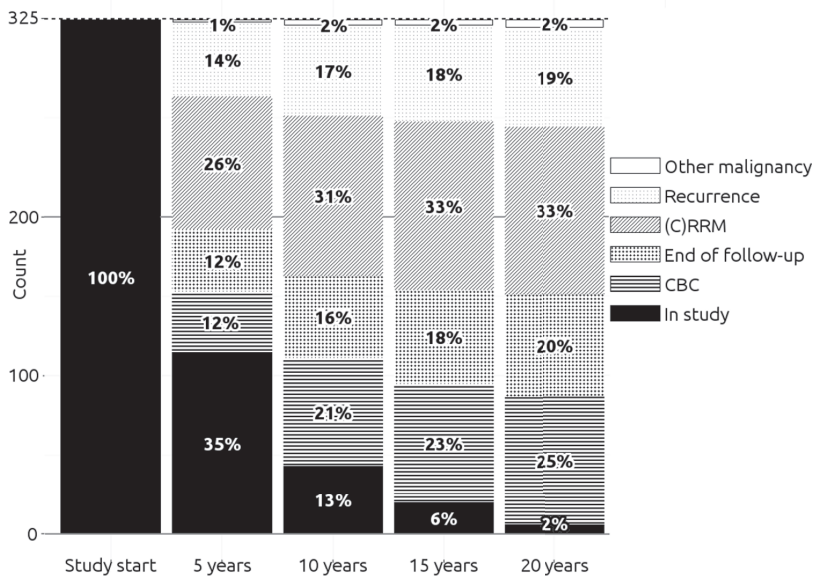

Figure 2. Cumulative frequency of contralateral breast cancer or reasons for censoring event at study start and after 5, 10, 15, and 20 years of follow-up in all included patients who were younger than 40 years of age at primary breast cancer diagnosis. Recurrence includes both ipsilateral recurrence, a second ipsilateral primary tumor, and metastatic disease. End of FU (follow-up) comprises patients who did not reach the primary endpoint or other censoring event at data cut-off or were lost to follow up.

(C)RRM: (contralateral) risk-reducing mastectomy; CBC: contralateral breast cancer.

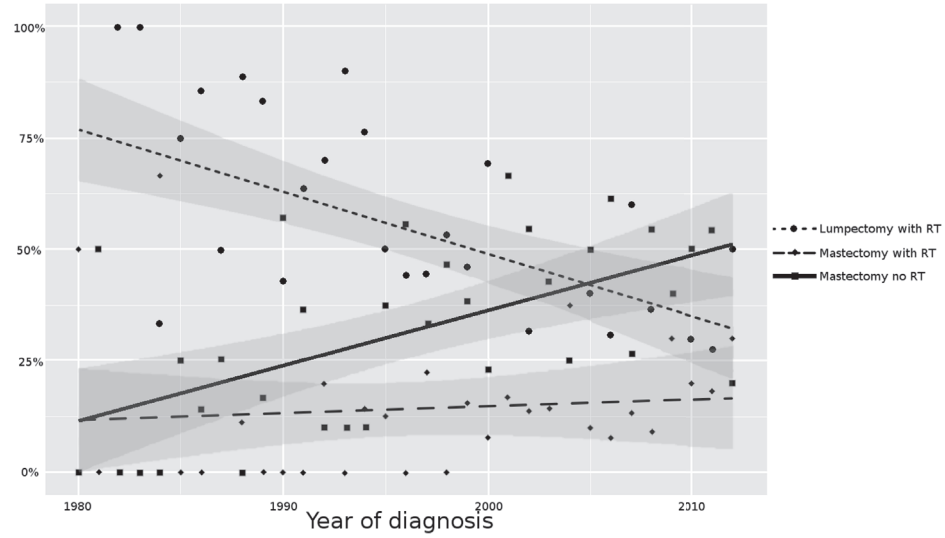

Figure 3. Distribution of the choice of local therapy at primary breast cancer diagnosis by year of diagnosis among patients younger than 40 years of age with a *BRCA1* or *BRCA2* mutation. Regression line of best fit and estimate of 95% confidence interval (gray).

RT: Radiotherapy.

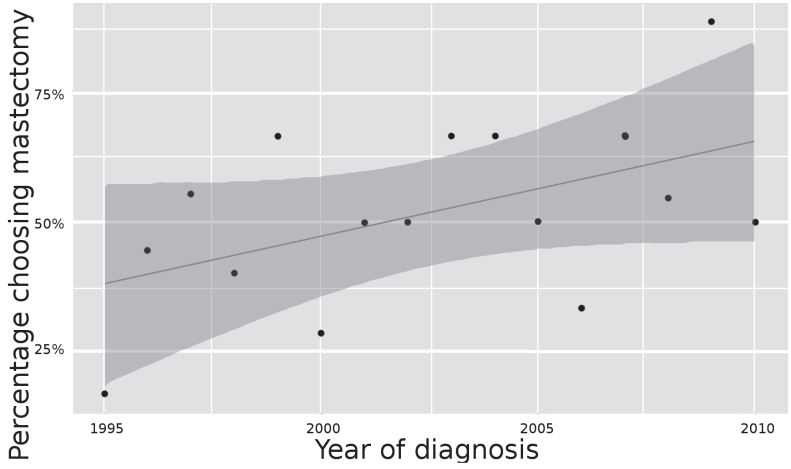

Figure 4. Proportion of patients with a *BRCA1* or *BRCA2* mutation and breast cancer diagnosis below the age of 40 opting for contralateral (or bilateral) risk-reducing mastectomy (either at primary breast cancer treatment or within the years after primary breast cancer) by year of breast cancer diagnosis. Regression line of best fit and estimate of 95% confidence interval (gray).

DISCUSSION AND CONCLUSION

The risk of CBC among breast cancer patients with a *BRCA1/2* mutation is high, especially for younger patients. An association between adjuvant radiotherapy and the development of CBC in *BRCA1/2*-associated breast cancer patients was not observed, neither in the entire cohort, nor in the subgroup of patients younger than 40 years at primary diagnosis. We found in this study that during follow-up the number of patients at risk for developing CBC substantially decreased due to either contralateral risk-reducing mastectomy or breast cancer recurrence (26 and 14%, respectively, within the first five years after primary BC among patients younger than 40 years). As a consequence, the number of patients at risk after 10 and 15 years of follow-up was too small to definitively exclude harmful effects of radiotherapy on the development of CBC among young *BRCA1/2* mutation carriers.

A few other studies also reported on CBC risk in *BRCA1/2*-associated breast cancer patients treated with adjuvant radiotherapy compared to patients not treated with radiotherapy [13-15], and did not find an increased risk of CBC associated with adjuvant radiotherapy either. In the two multicenter retrospective cohort studies of breast cancer patients attending high-risk clinics [13,14], the numbers of young *BRCA1/2* mutation carriers and follow-up periods were comparable to our study (145 out of 655 patients younger than 35 years with a median follow-up of eight years in the study of Pierce et al. [13], and 357 out of 810 patients younger than 40 years with a median follow-up of eleven years in the study of Metcalfe et al. [14]). However, subgroup analyses among these younger patients were not reported. Bernstein et al. performed a nested case control study within the WECARE study (Women's Environmental Cancer and Radiation Epidemiology Study), which is a population-based study of patients with metachronous CBC [15], but again no results of subgroup analysis in younger patients were shown.

The main limitation of our study regarding the impact of radiotherapy on the CBC risk is the small number of patients at risk for CBC after 10-15 years of follow-up, as studies including sporadic patients suggest that a minimal latency period of 10-15 years is needed to develop radiation-induced BC [19,20]. It is, however, not known whether the latency period between exposure and development of a radiation-induced malignancy is similar for *BRCA1/2* mutation carriers compared to sporadic patients. Even, if the latency period in *BRCA1/2* mutation carriers is shorter, the number of patients at risk for CBC in our study group was too small to make definitive conclusions, especially since a large proportion of patients were already censored in the first five years. Given the number of events in patients younger than 40 years at primary breast cancer diagnosis, our study had 80% power to find an HR of at least 2.8 for adjuvant radiotherapy to be associated with increased risk of CBC.

In our total cohort, the 10-year cumulative risk of CBC in *BRCA1/2* mutation carriers was 19%, while in the subgroup of patients younger than 40 years at breast cancer onset this risk was 32%. These risks are comparable to the risks reported in other studies [14,21,22]. Furthermore, the CBC risk was higher in *BRCA1* compared to *BRCA2* mutation carriers. Both the increased risk in younger patients and the increased risk in *BRCA1*- compared to *BRCA2*-associated breast cancer patients have been described

in other studies [14,21-23]. Additionally, in our cohort adjuvant systemic therapy for primary breast cancer, applying for both endocrine therapy and chemotherapy, was associated with a decreased risk of CBC. This effect, however, was only significant in the entire cohort and not in the subgroup of younger patients. Since the HRs were similar, this might be due to the lack of statistical power. The risk-reductive effect of adjuvant endocrine therapy on CBC risk in *BRCA1/2* mutation carriers has been reported in previous studies [14,24,25]. Regarding chemotherapy, three studies have investigated the association between chemotherapy and CBC [14,23,26], whereby only Reding et al. found a significant association with a relative risk of 0.5. Although this latter association is biologically not totally clear, further research is certainly warranted. We did not find any impact of salpingo-oophorectomy on CBC risk, which is in contrast with previous reports [27,28], but is in line with more recent literature [29].

In our cohort, we found a growing preference over time for mastectomy without radiotherapy instead of breast conserving therapy including radiotherapy. At the same time, the rate of contralateral risk-reducing mastectomy after primary breast cancer treatment has increased. Important reasons for the shift toward ablative breast surgery might be the improvements in and availability of (direct) breast reconstructive options, the increased awareness of the magnitude of the CBC risk and distress of screening, and the wish to avoid another treatment session for a second primary breast cancer. Finally, the important findings of Heemskerk et al. showing that contralateral risk-reducing mastectomy improves survival, mainly in younger patients and those with favorable primary tumor characteristics [30], might lead to an even larger proportion of younger patients opting for mastectomy without radiotherapy and contralateral risk-reducing mastectomy after primary breast cancer diagnosis in the nearby future.

These trends in locoregional treatments eventually decreased the proportion of patients at risk for radiation-induced CBC over the past few decades. Nevertheless, the question whether adjuvant radiotherapy has deleterious effect on CBC risk still remains clinically important for a significant number of patients, who want to conserve their (ipsilateral and) contralateral breast. Moreover, in the nearby future a larger proportion of patients potentially might opt for breast conserving treatment and abstain from contralateral risk-reducing mastectomy, due to an increased use of endocrine therapy as chemoprevention, improved diagnostic imaging techniques for screening, and improved effectiveness of adjuvant systemic therapy (for example, in combination with PARP inhibitors) [31-33]. In the current study, we could not find an association between radiotherapy for primary breast cancer and risk of CBC in (young) *BRCA1/2* mutation carriers compared to sporadic patients; however, the number of patients at risk after 10 and 15 years of follow-up was too small to definitively exclude harmful effects of adjuvant radiotherapy. An increase in the percentage of young patients with *BRCA1/2*-associated breast cancer choosing for conserving their (ipsilateral and) contralateral breast is not unlikely. Therefore, future research in larger study populations with minimal follow-up of 10 years is needed to achieve a better understanding of the true effect of radiotherapy on the CBC risk in *BRCA1/2*-associated breast cancer patients. This will only be possible by combining study populations through collaborative efforts on a national or even international level.

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4

Toxicity of (neo)adjuvant chemotherapy for *BRCA1*- and *BRCA2*-associated breast cancer

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ABSTRACT

Treatment with (neo)adjuvant chemotherapy for breast cancer, as currently given, causes cell damage by induction of double strand DNA breaks. Because BRCA1 and BRCA2 proteins play a role in the repair of DNA damage, the efficacy of (neo)adjuvant chemotherapy may be increased in *BRCA1* and *BRCA2* (*BRCA1/2*)-associated breast cancer patients. As a downside, acute chemotherapy-related toxicity may also be increased. We selected all female patients who were treated at the Erasmus MC Cancer Institute, with (neo)adjuvant chemotherapy for primary or locoregional recurrence of breast cancer (PBC/LR) between January 1, 2004 and December 31, 2014. The primary outcome was the relative total dose intensity (RTDI), calculated for anthracyclines and taxanes separately. Secondary outcomes were the occurrence of febrile neutropenia, delay in chemotherapy administration, and switch to another chemotherapy regimen due to toxicity. In total, 701 patients treated for PBC/LR were eligible for data analyses, among which 85 *BRCA1/2* mutation carriers (n = 67 *BRCA1* and n = 18 *BRCA2*). The mean RTDI for anthracyclines was not significantly different between both groups (98.7% in the *BRCA1/2* and 96.6% in the sporadic group, p = 0.27). Also the mean RTDI for taxanes was not significantly different between the groups (93.6% in the *BRCA1/2*-associated and 90.0% in the sporadic group, p = 0.12). Linear regression analysis revealed no significant effect of *BRCA1/2* mutation carriership on the RTDIs. No significant differences were found in the percentages of patients presenting with febrile neutropenia, having a delay in chemotherapy administration or switching to an altered chemotherapy regimen. Additionally, the odds ratios showed no significant effect of *BRCA1/2* mutation carriership on the secondary outcome variables. (Neo)adjuvant chemotherapy-related toxicity was not different between *BRCA1/2*-associated and sporadic breast cancer patients suggesting that the DNA damage repair mechanism of non-cancer cells with only one normal copy of either the *BRCA1* or *BRCA2* gene is sufficiently functional to handle acute chemotherapy-associated toxicity.

INTRODUCTION

Carriers of a germline *BRCA1* or *BRCA2* (*BRCA1/2*) mutation face an increased lifetime risk of developing breast cancer, estimated to range from 47 to 66% for *BRCA1* mutation carriers and from 40 to 57% for *BRCA2* mutation carriers [1,2].

Carriers of a germline *BRCA1/2* mutation, by definition, have one allele with a mutation in the *BRCA1/2* gene, while the gene on the other allele is intact. In normal cells, it seems that enough BRCA1 or BRCA2 protein is present for adequate functioning of cells in the various tissues of these women. However, *BRCA1/2*-associated breast cancers often have lost the wild type allele through somatic alterations during tumor development. As a consequence, there is no functional BRCA1 or BRCA2 protein in these tumor cells. Since BRCA1 and BRCA2 proteins are essential in the repair of double strand DNA breaks by homologous recombination [3,4], treatments which cause double strand DNA breaks might be more effective in *BRCA1/2*-associated than in sporadic breast cancer patients, which tumor cells mostly have an intact homologous recombination repair system. The platinum derivates carboplatin and cisplatin, both strong inducers of double strand DNA breaks, indeed showed higher efficacy in *BRCA1/2*-associated compared to sporadic breast cancer patients [5-7]. Although less pronounced, anthracyclines are also known to induce indirect double strand DNA breaks by inhibiting topoisomerases, causing DNA interstrand cross-links and the generation of free radicals [8]. Accordingly, several clinical studies have shown increased sensitivity for anthracycline-containing chemotherapy in *BRCA1/2* mutation carriers [9-11].

An important question is whether acute toxicity due to (neo)adjuvant chemotherapy is different in *BRCA1/2* mutation carriers treated for breast cancer when compared with sporadic breast cancer patients. Since (neo)adjuvant chemotherapy induces massive DNA damage also in normal cells, one might argue that the amount of functional BRCA1 or BRCA2 protein in mutation carriers is too low to repair all the DNA damage created, compared to sporadic breast cancer patients, resulting in more toxicity. Thus far two studies investigated the acute toxicity of (neo)adjuvant chemotherapy in *BRCA1/2*-associated, compared to sporadic breast cancer patients, with inconsistent results [12,13]. In the retrospective study of Shanley et al., comparing 62 *BRCA1/2* mutation carriers with breast cancer to 62 matched sporadic breast cancer cases, a large proportion of patients (80/124; 65%) was treated with older chemotherapy regimens without anthracyclines, while no patient was treated with taxanes. In *BRCA2* mutation carriers, less hematological toxicity and dose alterations were observed compared to both *BRCA1*-associated and sporadic breast cancer patients, while no differences were seen for *BRCA1*-associated versus sporadic patients [12]. In the study by Huszno et al., comparing 41 *BRCA1/2*-associated with 229 breast cancer patients without a *BRCA1/2* mutation, all patients were treated with an anthracycline-based regimen and also patients treated with taxanes were included [13]. It was found that the proportion of patients with neutropenia at the planned start date of the second chemotherapy cycle was significantly higher in breast cancer patients with a *BRCA1/2* mutation compared to patients without a *BRCA1/2* mutation. Twelve patients (4.5%), all in the group of patients

without a *BRCA1/2* mutation, required early termination of treatment due to chemotherapy toxicity, mostly because of grade 3-4 neutropenia. Nausea and vomiting were seen more often in patients without a *BRCA1/2* mutation. There were no differences in the other investigated variables (anemia, diarrhea, and mucositis).

Nowadays, standard (neo)adjuvant chemotherapy regimens for breast cancer contain both anthracycline (either epirubicin or doxorubicin) and taxanes (either paclitaxel or docetaxel). In view of the sparse available data on toxicity of taxanes and currently used chemotherapy regimens in *BRCA1/2* mutation carriers, we performed a larger single center retrospective cohort study to examine potential differences in (neo)adjuvant chemotherapy-associated toxicity between *BRCA1/2*-associated and sporadic breast cancer patients.

PATIENTS AND METHODS

Patient population

For this retrospective cohort study, we selected from the hospital pharmacy prescription registry all female patients who were treated at the Erasmus MC Cancer Institute, Rotterdam, The Netherlands, with adjuvant or neoadjuvant chemotherapy for primary breast cancer or local/locoregional recurrence (PBC/LR). Further eligibility criteria concerned: chemotherapy regimen consisting of anthracyclines and/or taxanes and chemotherapy treatment started between January 1, 2004 and December 31, 2014. Patients who were previously treated with chemotherapy for either breast or another invasive cancer were not excluded, but subgroup analyses were performed with the exclusion of these patients, since pre-treated patient might have increased hematological toxicity. Patients treated with (neo)adjuvant chemotherapy twice in the time period of the study were included for both episodes of chemotherapy treatment. Eleven PBC/LRs were excluded because of missing data concerning chemotherapy administration, leaving a total of 704 PBC/LRs (in 701 patients) eligible for the primary analysis (Figure 1).

For eligible patients, data on tumor characteristics (type of histology, differentiation grade, estrogen receptor status, progesterone receptor status, HER2 status and stage) and treatment details (surgery, radiotherapy, and/or chemotherapy) were retrieved. We also collected specific data on chemotherapy treatment (treatment regimen, dosing, delays, alterations, and complications). Data on mutation status were collected from the institutional database of the family cancer clinic. Patients not tested for a *BRCA1/2* mutation were considered as sporadic breast cancer patients.

Chemotherapy regimens

During the time period of the study, the chemotherapy regimens were not different for *BRCA1/2* mutation carriers and sporadic patients. Patients were treated with systemic therapy based on the national guidelines. For patients with HER2-negative breast cancer, the standard regimens at start of the study contained anthracyclines but no taxanes. From July 2008 till the end of the study, standard

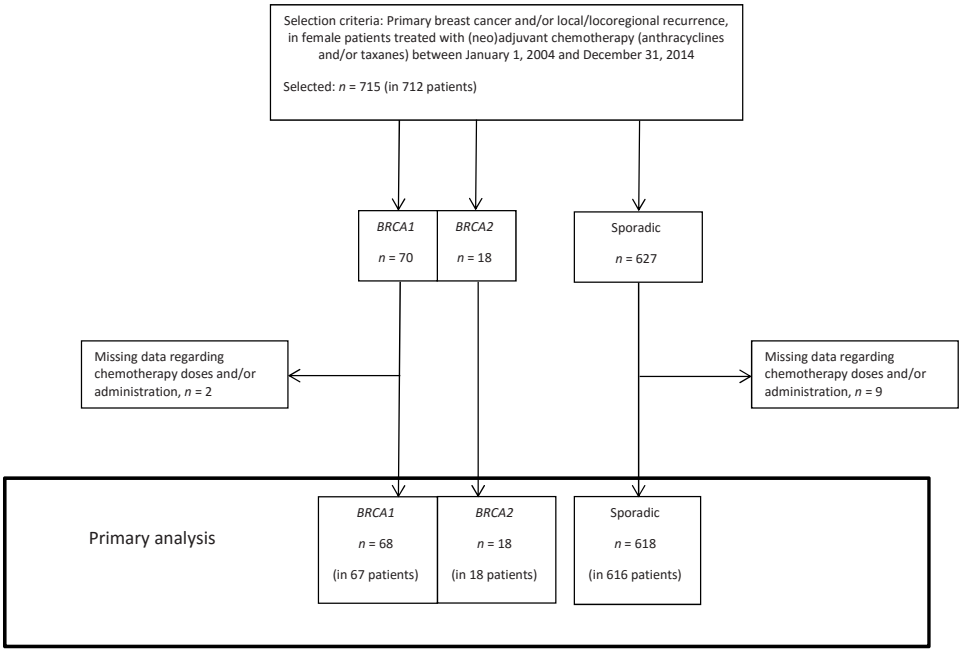

Figure 1. Study population.

regimen for node positive patients included taxanes (three-weekly docetaxel), while for node negative patients, taxanes (three-weekly docetaxel) were included in the standard regimen from October 2011 onwards. Patients with HER2-positive breast cancer were treated with anthracyclines and no taxanes till August 2006. Trastuzumab was added to this regimen from September 2005 onwards. From August 2006 till the end of the study, the standard regimen contained anthracyclines and taxanes (weekly paclitaxel) in combination with trastuzumab.

Some patients were treated with other schemes because of participation in a clinical trial, prior chemotherapy treatment, or comorbidities. Standard G-CSF (granulocyte colony-stimulating factor) prophylaxis was only used for six cycles of TAC (docetaxel, doxorubicin, and cyclophosphamide) and dose-dense regimens (AC, doxorubicin/cyclophosphamide, given every two weeks). In case of febrile neutropenia or persisting neutropenia at planned start of next chemotherapy cycle, G-CSF was added to the next treatment cycle. In case of febrile neutropenia or persisting neutropenia at planned start of next chemotherapy cycle in patients treated with G-CSF, dose reduction was considered. Furthermore, dose reduction and/or dose delay were considered based on the severity of hematological and non-hematological toxicities.

Toxicity outcomes

Primary outcome was the relative total dose intensity (RTDI), a measure of delivered (actual) total dose intensity (ATDI; i.e. administered dose over the total time course of treatment), relative to the planned total dose intensity (PTDI). The RTDI, therefore, expresses the effect of reductions, delays, as well as premature discontinuations of a treatment. The RTDI was calculated separately for anthracyclines and taxanes.

RTDI was calculated based on an adaptation of the formula described by Loibl et al. [14], and defined as the ratio of the ATDI and the PTDI, expressed as a percentage:

$$\text{RTDI (\%)} = \frac{\text{ATDI}}{\text{PTDI}} \times 100$$

The ATDI was defined as the actual total dose intensity over the real treatment duration, expressed as percentage/day. In case of permanent treatment discontinuation, the remaining cycles were calculated with the planned length and zero dose:

$$\text{ATDI (\%/day)} = \frac{\text{sum of \% of delivered dose per cycle}}{\text{duration of therapy (days)}}$$

The PTDI was defined as the planned total dose intensity over the entire treatment duration, expressed as %/day:

$$\text{PTDI (\%/day)} = \frac{\text{100\% x number of planned treatment cycles}}{\text{planned duration of therapy (days)}}$$

The secondary outcomes were the occurrence of one or more episodes of febrile neutropenia, of one or more delays in chemotherapy administration (either due to anthracycline-related toxicity or taxane-related toxicity) and of switch to another chemotherapy regimen.

Statistical analyses

We evaluated characteristics of patients, tumors and chemotherapy regimens, as well as outcome variables by comparing patients with proven *BRCA1/2*-associated breast cancer (*BRCA1/2* group) with those with sporadic breast cancer (sporadic group). For categorical variables, Pearson's χ^2 square test was used to test for significant differences between the two groups, and the two-sample Wilcoxon rank-sum (Mann-Whitney) test was used for differences between continuous variables.

To quantify the effect of carrying a *BRCA1/2* gene mutation on the RTDI of anthracyclines and taxanes, we performed univariate linear regression analyses. To estimate the effect of mutation carriership on the other endpoints (i.e. a delay in administration of chemotherapy, febrile neutropenia, and an alteration of the chemotherapy scheme due to toxicity), we used logistic regression models to obtain odds ratios (ORs) and accompanying 95% confidence intervals (CIs), using treatments for PBC/LRs in sporadic patients as the reference group. To adjust for other variables, we fitted multivariate regression models. We considered age at start of chemotherapy, previous chemotherapy, radiotherapy before chemotherapy, neoadjuvant chemotherapy, and number of administered chemotherapy cycles as potential confounders. We incorporated a variable in a regression model if (1) there was a significant difference in the median or in the distribution of the respective variable between the *BRCA1/2*-

associated and the sporadic group and (2)—for linear regression models—univariate analysis of the respective variable showed a significant association with the outcome, or—for logistic regression models—the likelihood ratio test showed that the model including the respective variable was significantly different from the model without the variable.

All *p* values were two-sided, and a significance level $\alpha = 0.05$ was used. Analyses were performed with STATA (version 13.1; StataCorp, College Station, TX, USA).

RESULTS

In total, 701 patients were eligible for data analyses, of whom one *BRCA1* mutation carrier and two sporadic patients were treated with two separate episodes of (neo)adjuvant chemotherapy for a PBC/LR during the study period. Tables 1 and 2 depict the patient and tumor characteristics, and the treatment features, respectively. Eighty-five patients (12%) were *BRCA1/2* mutation carriers (*n* = 67 *BRCA1* and *n* = 18 *BRCA2*). The median age at start of chemotherapy was significantly lower in the *BRCA1/2* group compared to the sporadic group (38 years [range 21-64] vs. 51 years [range 23-77], respectively, *p* <0.001). PBC/LRs in *BRCA1/2* mutation carriers more often showed high differentiation grade (Bloom and Richardson grade 3), triple-negative tumors and negative lymph node status compared to PBC/LRs in sporadic patients. For a total of 492 PBC/LRs (70%), treatment with both anthracycline- and taxane-containing chemotherapy was applied, while chemotherapy consisted of anthracyclines with no taxanes for 193 PBC/LRs (27%) and for 19 PBC/LRs (3%) of taxanes with no anthracyclines. In the *BRCA1/2* group, more patients were previously treated with chemotherapy for breast cancer or for another invasive malignancy (13 vs. 5% in the sporadic group, *p* = 0.004; Table 2).

Table 1. Patient and tumor characteristics.

	***BRCA1/2* mutation carriers (*n* = 85)**	**Sporadic patients (*n* = 616)**	***p*-value**
Year of birth, median (range)	1971 (1942-1990)	1957 (1936-1987)	<0.001
Year of birth, *n* (%)			
1930-1939	0 (0)	8 (1)	<0.001
1940-1949	4 (5)	130 (21)	
1950-1959	15 (18)	205 (33)	
1960-1969	18 (21)	188 (31)	
1970-1979	30 (35)	66 (11)	
1980-1989	17 (20)	19 (3)	
1990-1999	1 (1)	0 (0)	
Ethnicity, *n* (%)			
East Asian	0 (0)	17 (3)	0.33
Black	6 (7)	36 (6)	
White	79 (93)	557 (90)	
Other	0 (0)	6 (1)	
BRCA mutation, *n* (%)			
BRCA1	67 (79)	-	-
BRCA2	18 (21)	-	-

Table 1. Patient and tumor characteristics *(Continued)*.

	PBC/LRs in *BRCA1/2* mutation carriers (*n* = 86)	**PBC/LRs in sporadic patients (*n* = 618)**	***p*-value**
Age at start chemotherapy, median (range)	38 (21-64)	51 (23-77)	<0.001
Age at start chemotherapy, *n* (%)			
20-29 years	12 (14)	15 (2)	<0.001
30-39 years	34 (40)	61 (10)	
40-49 years	23 (27)	179 (29)	
50-59 years	12 (14)	221 (36)	
60-69 years	5 (6)	136 (22)	
70-79 years	0 (0)	6 (1)	
Body Surface Area (m^2), median (range)	1.8 (1.4-2.3)	1.8 (1.4-2.6)	0.41
Histologic subtype, *n* (%)			
Ductal	74 (88)	521 (86)	0.005
Lobular	1 (1)	58 (10)	
Other	9 (11)	30 (5)	
Unknown	2	9	
Histologic grade (Bloom and Richardson), *n* (%)			
1	4 (5)	48 (8)	<0.001
2	13 (16)	265 (46)	
3	64 (79)	269 (46)	
unknown	5	36	
Receptor status, *n* (%)			
Triple negative	51 (60)	101 (17)	<0.001
Estrogen receptor positive	32 (37)	466 (75)	<0.001
HER2 positive	4 (5)	135 (22)	<0.001
Lymph node status, *n* (%)			
N0	55 (65)	228 (38)	<0.001
N1	21 (25)	254 (43)	
N2	4 (5)	78 (13)	
N3	4(5)	37 (6)	
Unknown	2	21	

PBC/LR: primary breast cancer or local/locoregional recurrence.

Primary outcome

The mean RTDI for anthracyclines was high, without significant differences between the *BRCA1/2* and the sporadic groups (98.7 and 96.6%, respectively, $p = 0.27$; Table 3). The mean RTDI for taxanes was slightly lower than for anthracyclines, but again without significant differences between the two groups (93.6% in the *BRCA1/2* group and 90.0% in the sporadic group, $p = 0.12$; Table 3). As illustrated in Figure 2, *BRCA1/2* mutation carriers showed less variability in the RTDI than sporadic patients. As shown in Table 4, the linear regression models revealed no significant effect of *BRCA1/2* mutation carriership on the RTDIs.

Table 2. Features of (neo)adjuvant chemotherapy and other treatments.

	PBC/LRs in *BRCA1/2* mutation carriers (*n* = 86)	**PBC/LRs in sporadic patients (*n* = 618)**	***p*-value**
Planned chemotherapy regimen, *n* (%)			
- containing both anthracyclines and taxanes	49 (57)	443 (72)	<0.001
3xFE100C/3xD	46 (53)	290 (47)	
4xAC/12xP	1 (1)	103 (17)	
6xTAC	1 (1)	40 (6)	
Other	1 (1)	10 (2)	
- containing anthracyclines and no taxanes	30 (35)	163 (26)	
5xFE90C	19 (22)	86 (14)	
6xFE90C	7 (8)	60 (10)	
4xAC	1 (1)	13 (2)	
Other	3 (3)	4 (1)	
- containing taxanes and no anthracyclines	7 (8)	12 (2)	
Dose dense regimens, *n* (%)	3 (3)	1 (0.2)	<0.001
Regimens with standard G-CSF prophylaxis, *n* (%)	4 (5)	41 (7)	0.48
Regimens with weekly chemotherapy, *n* (%)	1 (1)	104 (17)	<0.001
Number of three-weekly chemotherapy cycles, median (range)	6 (3-10)	6 (1-8)	0.14
Previous chemotherapy, *n* (%)	11 (13)	31 (5)	<0.01
Adjuvant radiotherapy before chemotherapy, *n* (%)	2 (2)	87 (14)	<0.01
Neoadjuvant chemotherapy, *n* (%)	10 (12)	81 (13)	0.70

PBC/LR: primary breast cancer or local/locoregional recurrence; 3xFE100C/3xD: three cycles of three-weekly fluorouracil 500mg/m^2, epirubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2, followed by three cycles of docetaxel 100 mg/m^2; 4xAC/12xP: four cycles of three-weekly doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2, followed by twelve cycles of weekly paclitaxel 80 mg/m^2; 6xTAC: six cycles of three-weekly docetaxel 75 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2; 5xFE90C: five cycles of three-weekly fluorouracil 500mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 500 mg/m^2; 6xFE90C: six cycles of three-weekly fluorouracil 500 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 500 mg/m^2; 4xAC: four cycles of three-weekly doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2; G-CSF: granulocyte colony stimulating factor.

Table 3. Primary and secondary outcome variables.

	PBC/LRs in *BRCA1/2* mutation carriers (*n* = 86)	**PBC/LRs in sporadic patients (*n* = 618)**	***p*-value**
Mean relative total dose intensity, % (SD)			
Anthracyclines	98.7 (3.7)	96.6 (10.5)	0.27
Taxanes	93.6 (17.6)	90.0 (19.9)	0.12
Febrile neutropenia, *n* (%)	18 (21)	107 (17)	0.42
Delay of chemotherapy administration, *n* (%)			
Because of anthracyclines	12 (15)	90 (15)	0.97
Because of taxanes	2 (4)	46 (10)	0.13
Alteration of chemotherapy scheme, *n* (%)	8 (9)	65 (11)	0.73

PBC/LR: primary breast cancer or local/locoregional recurrence; SD: standard deviation.

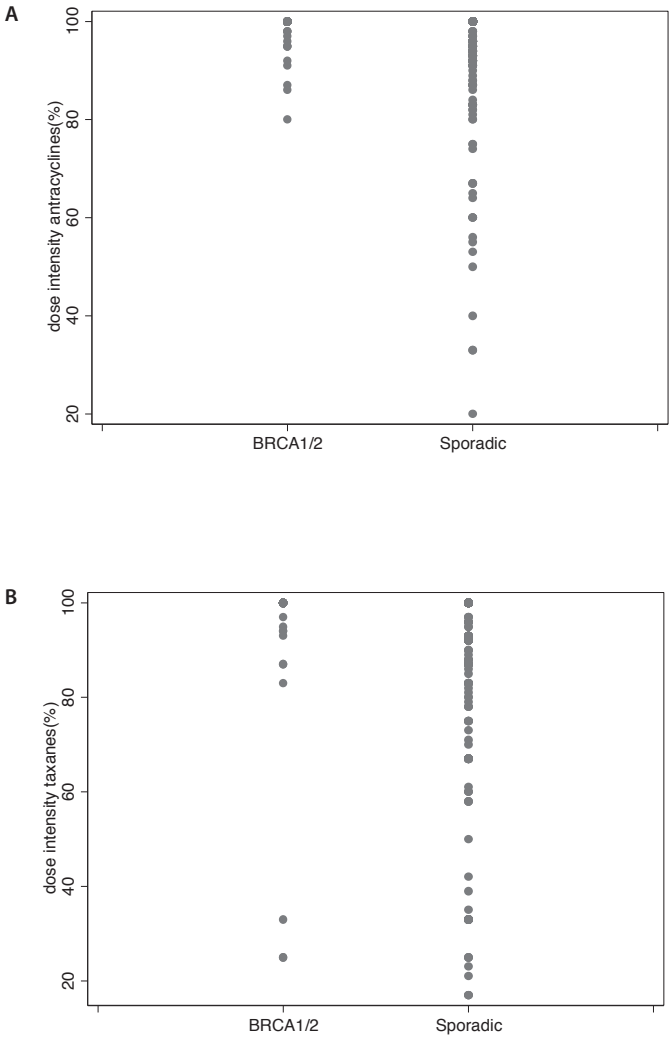

Figure 2. Relative total dose intensity (%) for (A) anthracyclines and (B) taxanes, separately for *BRCA1/2*-associated and sporadic breast cancer patients.

Secondary outcomes

As shown in Table 3, no significant differences between the *BRCA1/2*-associated and sporadic groups were found in the percentage of patients presenting with febrile neutropenia (21 and 17%, respectively, $p = 0.42$), having a delay in chemotherapy administration due to chemotherapy toxicity (for anthracyclines: 15% in both groups, $p = 0.97$; for taxanes: 4% in the *BRCA1/2*-associated and 10% in the sporadic group, $p = 0.13$) or switching to an altered chemotherapy regimen due to chemotherapy toxicity (9% in the *BRCA1/2*-associated and 11% in the sporadic group, $p = 0.73$). Additionally, the ORs yielded by logistic regression showed no significant effect of *BRCA1/2* mutation carriership on the secondary outcome variables (Table 5).

Table 4. Linear regression analyses for mean relative total dose intensity.

	Univariate model Coefficient (SE)	*p*-value	Multivariate model Coefficient (SE)	*p*-value
Mean RTDI anthracyclines (%)				
BRCA1/2 versus sporadic	1.69 (1.08)	0.12	Not applicable[c]	
Age at start chemotherapy	-0.05 (0.03)	0.12		
Previous chemotherapy[a]	-1.09 (1.84)	0.55		
Radiotherapy before chemotherapy[b]	-1.16 (1.00)	0.25		
Mean RTDI taxanes (%)				
BRCA1/2 versus sporadic	3.94 (2.98)	0.19	3.33 (2.97)	0.26
Age at start chemotherapy	-0.10 (0.08)	0.22	-	-
Previous chemotherapy[a]	6.71 (4.17)	0.11	-	-
Radiotherapy before chemotherapy[b]	-6.77 (2.75)	0.01	-6.50 (2.76)	0.02

RTDI: relative total dose intensity; SE: standard error.
[a] versus no previous chemotherapy.
[b] versus no radiotherapy before chemotherapy.
[c] none of the variables were associated with the outcome variable.

Table 5. logistic regression analyses for secondary outcome variables.

	Univariate model Odds ratio (95% CI)	Multivariate model Odds ratio (95% CI)
Febrile neutropenia	1.27 (0.71-2.27)	1.11 (0.59-2.07)[a]
Delay of chemotherapy administration		
Because of anthracyclines	0.99 (0.50-1.97)	Not applicable[b]
Because of taxanes	0.36 (0.08-1.54)	Not applicable[b]
Alteration of chemotherapy scheme	0.80 (0.33-1.93)	Not applicable[b]

Sporadic breast cancer patients as references vs. breast cancer patients with a *BRCA1/2* mutation.
CI: confidence interval.
[a] adjusted for age at start chemotherapy. The other variables did not meet the criteria for incorporation in the multivariate model;
[b] no variables did meet the criteria for incorporation in the multivariate model as described in the methods section.

Subgroup analyses

To exclude effect modification by differences in treatment regimens between the two groups on the outcome variables, we performed analyses with exclusion of certain chemotherapy regimens. Exclusion of the patients being treated with regimens administered with standard G-CSF prophylaxis ($n = 4$ treated with dose-dense regimens; $n = 41$ treated with TAC), with regimens consisting of weekly chemotherapy administration ($n = 105$) or with regimens containing taxanes with no anthracyclines ($n = 19$) did not significantly influence the results of both primary and secondary outcome variables (data not shown). Febrile neutropenia was then found in 25% of the *BRCA1/2*-associated and in 20% of the sporadic group, $p = 0.57$. Excluding the patients who were previously treated with adjuvant chemotherapy for breast cancer or for another invasive cancer ($n = 42$) also did not significantly influence the results of both primary and secondary outcome variables (data not shown). When taking the *BRCA1/2*-associated and the sporadic group together, the RTDI was not significantly different between patients previously versus not previously treated with chemotherapy (for anthracyclines RTDI: 96.8% in both groups, $p = 0.80$; for taxanes RTDI: 95.8 vs. 90.1%, $p = 0.20$).

DISCUSSION

In this single center retrospective cohort study, we found no differences in RTDI of (neo)adjuvant chemotherapy (both for anthracyclines and taxanes) between *BRCA1/2*-associated and sporadic breast cancer patients. Furthermore, we found no differences in the occurrence of febrile neutropenia, in delay of chemotherapy administration or in alteration of the chemotherapy regimen due to toxicity between the two groups. Our observations on the absence of increased acute toxicity due to (neo)adjuvant chemotherapy in *BRCA1/2* mutation carriers, compared to sporadic breast cancer patients, suggest that the DNA damage repair mechanism of non-cancer cells with only one normal copy of either the *BRCA1* or *BRCA2* gene is sufficiently functional to handle acute chemotherapy-associated toxicity.

Our results have to be interpreted in the light of the two previously published studies on chemotherapy-associated toxicity in *BRCA1/2* mutation carriers. Huszno et al. found more neutropenia at the planned date of the second chemotherapy cycle in *BRCA1/2*-associated ($n = 41$) than in sporadic breast cancer patients ($n = 229$) [13]. It is unclear what the clinical relevance of this finding is, since they did not mention the proportion of patients needing dose reductions, experiencing delay in chemotherapy administration and febrile neutropenia. We choose to use more clinically relevant outcome measures such as dose intensity which is likely to be associated with efficacy [15] and febrile neutropenia that might have consequences for the subsequent cycle. The data of the study of Shanley et al., not finding increased chemotherapy-associated toxicity in *BRCA1/2*-associated ($n = 62$) compared to sporadic breast cancer patients ($n = 62$) [12], are hardly comparable to our study observations, since a large part of their patients were treated with older chemotherapy regimens.

To the best of our knowledge, our study is the largest published on this topic so far. We did not

find any differences in clinically relevant toxicity measures after treatment with anthracyclines and/or taxanes between *BRCA1/2*-associated and sporadic breast cancer patients. In both previous studies, as well as in our study, age at the start of chemotherapy was significantly lower in the *BRCA1/2* group than in the sporadic group. Although increased risk of myelosuppression at increased age of administration has been previously reported [16], in our study no difference was seen in mean RTDI comparing *BRCA1/2* mutation carriers aged >50 years to BRCA1/2 mutation carriers younger than 50 years (data not shown).

In the *BRCA1/2* group more patients were previously treated with adjuvant chemotherapy than in the sporadic group, mainly for an earlier primary breast cancer. Since there is a maximum cumulative dose for anthracyclines, a relevant proportion of these patients did receive a non-anthracycline-containing regimen. One might expect increased toxicity when patients are treated for a second time with chemotherapy. Leaving out all pre-treated patients, however, did not influence the results, and comparing previously treated patients with non-previously treated patients (irrespective of *BRCA1/2* mutation status) showed no significant differences in the RTDI, suggesting that previous treatment with chemotherapy does not increase acute chemotherapy-related toxicity. In the *BRCA1/2*-associated group, fewer patients were treated with weekly chemotherapy regimens and with regimens containing standard G-CSF prophylaxis. However, exclusion of patients treated with these regimens did not significantly influence the results. The percentage of patients presenting with febrile neutropenia in the sporadic group increased in the subgroup analyses, compared to the percentage found in the primary analysis, which might be explained by the fact that in the sporadic group a larger proportion of patients were treated with regimens containing standard G-CSF prophylaxis.

We are aware of a number of shortcomings to be mentioned. Despite the fact that our study is the largest published on this topic with inclusion of 86 PBC/LRs in *BRCA1/2*-associated patients, being 12% of the total study group, this number is still quite low. The numbers were too small to perform useful analyses for *BRCA1* and *BRCA2* mutation carriers separately, which would be of interest since *BRCA1* and *BRCA2* proteins have different roles in the DNA repair mechanism and the cell cycle. Nevertheless, it is unlikely that a clinically relevant difference will be found with higher numbers of patients, since the RTDI, especially for anthracycline is very high. In contrast to the study of Huszno et al., not all our patients were tested for a *BRCA1/2* mutation, but we expect, if any, only a small proportion of *BRCA1/2* mutation carriers in the sporadic subgroup, since at our institution (and in The Netherlands) patients are already tested with a low suspicion of *BRCA1/2* mutation carriership. Further, in the current study, we did not include non-hematological toxicity as an outcome, since it is well known that these outcome variables are more prone to inter-observer variability and are less clinically relevant when they do not lead to dose delay or dose reduction [17]. For the same reason, we did not include hematological laboratory values measured at planned start of a new cycle, since these are only relevant when they lead to dose reduction, delay in chemotherapy administration, or alteration of chemotherapy regimen. It could have been of scientific interest to compare neutrophil nadir levels

between *BRCA1/2* mutation carriers and sporadic patients. Unfortunately, due to the retrospective nature of our data, these data are lacking.

Recent data showed increased efficacy of platinum derivates in patients with triple-negative breast cancer and/or a *BRCA1/2* mutation, leading to incorporation of carboplatin in standard (neo)adjuvant chemotherapy regimens in this population [5-7]. These studies did not report on differences in toxicity between *BRCA1/2* mutation carriers and sporadic breast cancer patients. In our study, the number of patients treated with carboplatin was very low and no conclusions can be drawn hereon. Poly ADP-ribose polymerase (PARP) inhibitors are an important new class of targeted anticancer drugs which induce double strand DNA breaks in tumors with homologous recombination deficiency due to, for example, a mutation in one of the *BRCA* genes. Recently, the first PARP inhibitor has been approved for the treatment of *BRCA1/2*-associated ovarian cancer, while trials in early and metastatic breast cancer are ongoing. Lederman et al. compared toxicity of the PARP inhibitor olaparib in *BRCA1/2* mutation carriers and sporadic patients with ovarian cancer and found no differences in toxicity [18]. Both platinum derivates and PARP inhibitors have a much higher capacity to induce double strand DNA breaks, compared to anthracyclines. Therefore, further research on the toxicity of these regimens in *BRCA1/2* mutation carriers compared to sporadic patients is warranted, especially since these drugs will be increasingly used in the treatment of *BRCA1/2*-associated breast cancer.

CONCLUSION

In conclusion, there seems no clinically relevant difference in toxicity of anthracycline- and taxane-containing (neo)adjuvant chemotherapy regimens for *BRCA1/2*-associated compared to sporadic breast cancer patients, which suggests that the DNA damage repair mechanism of non-cancer cells with only one normal copy of either the *BRCA1* or *BRCA2* gene is sufficiently functional to handle acute chemotherapy-associated toxicity.

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5

Neutrophil-guided dosing of anthracycline-cyclophosphamide-containing chemotherapy in patients with breast cancer: a feasibility study

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ABSTRACT

The aim of this study was to investigate whether neutrophil-guided dose escalation of anthracycline-cyclophosphamide-containing chemotherapy (ACC) for breast cancer is feasible, in order to optimize outcome. Breast cancer patients planned for three-weekly ACC were enrolled in this study. The first treatment cycle was administered in a standard body surface are (BSA)-adjusted dose. The absolute neutrophil count was measured at baseline and at day 8, 11 and 15 after administration of ACC. For patients with none or mild (CTC grade 0-2) neutropenia and no other dose limiting toxicity, we performed a 10-25% dose escalation of the second cycle with the opportunity to a further 10-25% dose escalation of the third cycle. Thirty patients were treated in the adjuvant setting with either FE100C (n = 23) or AC (n = 4), or in the palliative setting with FAC (n = 3). Two out of 23 patients (9%) treated with FEC did not develop grade 3-4 neutropenia after the first treatment cycle. Dose escalation was performed in these two patients (30% in one and 15% in the other patient). During dose escalation, there were no complications like febrile neutropenia. No patients treated with FAC or AC could be escalated, since all of them developed grade 3-4 neutropenia. We conclude that asymptomatic grade 3-4 neutropenia is likely to be achieved in the majority of patients with breast cancer treated with ACC according to presently advocated BSA-based dose levels. Escalation of currently advocated ACC doses without G-CSF, with a target of grade 3-4 neutropenia, is feasible, but only possible in a small proportion of patients.

INTRODUCTION

Both anthracyclines and cyclophosphamide are highly effective drugs in the treatment of breast cancer [1,2]. According to international guidelines, anthracycline-cyclophosphamide-containing chemotherapy (ACC) is part of (neo)adjuvant treatment schedules for early stage or locally advanced breast cancer [3]. Furthermore, in the setting of metastatic disease, ACC is often used as palliative treatment [4,5].

Although highly effective, not all patients benefit from ACC. The cumulative dose of anthracyclines administered is important. From randomized controlled trials, it is clear that higher 'standard dose' of anthracyclines for early breast cancer improves patient survival compared to lower 'standard dose' [6]. On the other hand, a reason for differences in efficacy among patients who have had a similar dose of anthracyclines administered could be the large inter-individual (between patients) as well as the intra-individual (within patients) variability in pharmacokinetic parameters [7]. Interestingly, hematological toxicity is strongly associated with the absolute dose of anthracycline and might be useful as a surrogate measure of the anthracycline dose [6]. In accordance, some retrospective studies indeed have shown that breast cancer patients given adjuvant chemotherapy but not attaining at least moderate hematological toxicity have a worse prognosis compared to those with more toxicity [8-11].

The current standard of dosing ACC is guided by body surface area (BSA) with an a posteriori dose reduction of all component drugs in case of excessive toxicity (e.g. febrile neutropenia). Dose escalation among patients without toxicity is, however, no standard of care. The administration of an inappropriately low dose of chemotherapy is therefore not recognized, leaving patients that might benefit from an increased dose unidentified. The percentage of breast cancer patients receiving a suboptimal dose of ACC is unknown, as well as the amount of underdosing in these individuals.

In the present study, we addressed the feasibility of a simple tool for neutrophil-guided dose adaptation of ACC (without primary G-CSF support), among female breast cancer patients treated with ACC for either palliative or curative intention. The aim was to reach nadir absolute neutrophil count (ANC) of ≤1.0 x 10e9/L with recovery to ≥1.5 x 10e9/L at the time of the planned next treatment cycle, without excessive hematological or non-hematological toxicity. In case successful dose escalation is possible in a substantial number of patients, this method is valid and should be further developed and refined to be ultimately tested on treatment efficacy in a prospective randomized trial of neutrophil-guided versus standard BSA-adjusted dosing.

PATIENTS AND METHODS

Participants

Chemotherapy naive female breast cancer patients aged ≥18 years and planned for treatment with at least three cycles of ACC were identified at the Department of Medical Oncology, Ikazia Hospital, Rotterdam, The Netherlands. Both patients treated with curative as well as patients treated with palliative intention were eligible. Patients with the following chemotherapy regimens were eligible: FEC (fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2), AC (doxorubicin 60 mg/m^2, cyclophosphamide 600 mg/m^2) or FAC (fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, cyclophosphamide 500 mg/m^2). Additionally, patients should have a WHO performance status 0-1, life expectancy >3 months, adequate peripheral blood cell counts (leukocytes ≥4.0 x 10e9/L and ANC ≥2.0 x 10e9/L and platelet count ≥150 x 10e9/L), adequate renal function (defined as normal serum creatinine concentration and/or estimated creatinine clearance ≥60 mL/min), adequate liver function (defined as normal serum bilirubin concentration (≤17 μmol/L) and serum ASAT and ALAT ≤3 times the upper limit of normal (≤5 times the upper limit of normal in case of hepatic metastases)), normal serum albumin concentration (35-50 g/L) and given written informed consent. Women were excluded from participation if they had been treated with chemotherapy previously, were unable to consent with weekly follow-up for blood cell counts and toxicity assessment, had symptomatic brain metastasis, had a history of cardiac dysfunction, had uncontrolled arterial hypertension (blood pressure systolic ≥180 mmHg and/or diastolic ≥110 mmHg) and/or unstable angina pectoris. Ethical approval for this study was obtained through the Institutional Review Boards, and all women signed the informed consent. The study was conducted in full accordance with the principles of the Declaration of Helsinki and local regulations. The trial adhered to the guidelines for good clinical practice and the European Union Clinical Trial Directive.

Study design

This study was a prospective single center feasibility study. The first treatment cycle was given using standard BSA-adjusted dosing. Following the administration of ACC, ANC was evaluated in peripheral venous blood samples obtained at days 8 (±1), 11 (±1) and 15 (±1), day 1 being the day of chemotherapy administration. Hematological and non-hematological toxicities were assessed weekly according to the common toxicity criteria (CTC), version 3. Subsequent cycles of ACC were given at intervals of three weeks provided that the patient had sufficiently recovered from hematological and non-hematological toxicity. Sufficient recovery of hematological toxicity was defined as an ANC of ≥1.5 x 10e9/L and a platelet count of ≥100 x 10e9/L, whereas sufficient recovery of non-hematological toxicity was defined as CTC grade ≤1 (with the exception of alopecia). In patients with nadir ANC ≥1.0 x 10e9/L and maximum non-hematological toxicity CTC grade ≤2 during the first cycle of ACC, the dose of cyclophosphamide and the anthracycline (doxorubicin or epirubicin) was increased with 10, 15 or 25% according to a predefined schedule based on ANC on day 8 and day 15. In patients treated

with chemotherapy schedules including fluorouracil (FAC or FEC), the dose of fluorouracil was not escalated due to its negligible contribution to hematological toxicity in these combination regimens [7]. Patients undergoing dose escalation of the second cycle of ACC were candidates for a further (and final) dose escalation of the third cycle of ACC following the same principles and according to the same predefined schedule. Patients experiencing excessive toxicity (i.e. febrile neutropenia, symptomatic thrombocytopenia and/or grade 3-4 non-hematological toxicity with the exception of nausea and vomiting) without previous dose escalation were treated according to standard clinical practice. In case of excessive toxicity after dose escalation, patients had to be retreated with standard BSA-adjusted dose during subsequent treatment cycles. Finally, all patients treated with ≥4 cycles of ACC received standard BSA-adjusted dosing from the fourth cycle onward. The study design is also outlined in Figure 1.

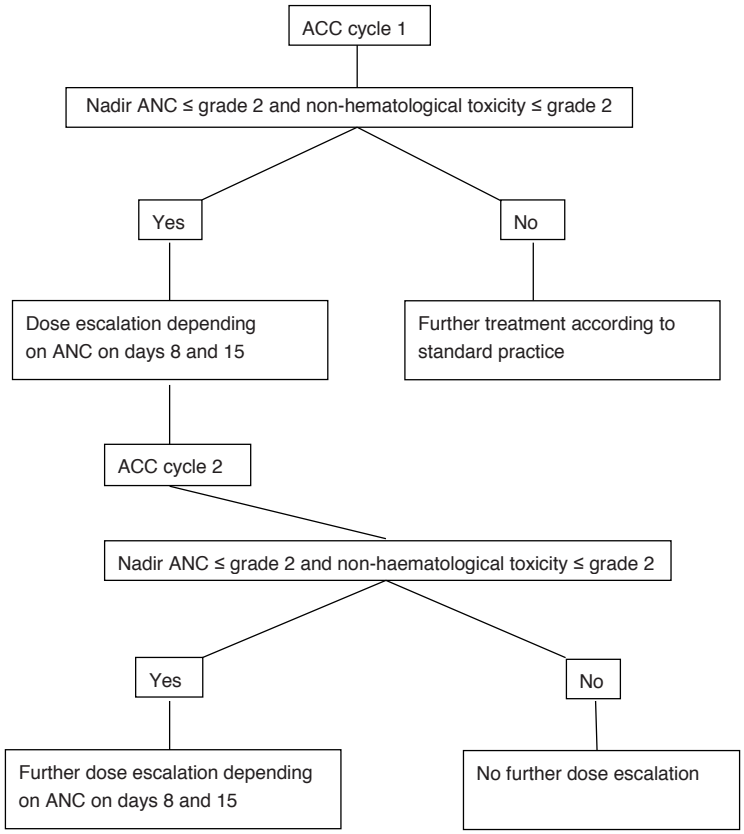

Figure 1. Study design.

ACC: anthracycline-cyclophosphamide-containing chemotherapy; ANC: absolute neutrophil count.

Statistical analysis

This study was designed as a pilot feasibility study. Therefore, a useful sample size calculation was not appropriate. We aimed to enter 30 patients. Successful dose escalation of chemotherapy was our primary goal and was rather arbitrarily defined as a ≥15% increase in anthracycline-cyclophosphamide dose without excessive hematological (febrile neutropenia, grade 3-4 thrombocytopenia) or non-hematological (grade 3-4) toxicity. We stated that if successful dose escalation was possible in a significant proportion of patients (at least 3 out of 30 patients), our experimental method of neutrophil-guided dose escalation could be feasible in daily clinical practice and should be further developed and refined to be ultimately tested on treatment efficacy in a prospective randomized trial of neutrophil-guided versus standard BSA-adjusted dosing. If successful dose escalation turned out to be possible in less than 3 out of 30 patients, it is unlikely that this method of dose escalation will have significant impact on treatment efficacy, and this method should not be further explored.

Furthermore inter-individual variation in ANC after administration of the chemotherapy was assessed as coefficient of variation (CV) for nadir ANC and for cumulative neutrophil count (expressed as the sum of CTC grades of neutropenia (0-4) on day 8, 11 and 15), addressing the duration of neutropenia.

RESULTS

A total of 30 patients were entered in this study between November 2010 and December 2013. Baseline characteristics are outlined in Table 1. Median age was 55 years (range 37-74 years). The majority of the patients were treated for early breast cancer with either FEC (77%) or AC (13%). Three patients (10%) were treated with first line palliative chemotherapy in the form of FAC.

Table 1. Baseline characteristics.

Median age, years (range)	55 (36-74)
Chemotherapy regime, *n* (%)	
FEC	23 (77)
FAC	3 (10)
AC	4 (13)
Tumor stage, *n* (%)	
Early	27 (90)
Metastatic	3 (10)
WHO performance score, *n* (%)	
WHO 0	25 (83)
WHO 1	5 (17)
Median height, cm (range)	170 (155-184)
Median weight, kg (range)	75 (53-100)
Median body surface are, m^2 (range)	1.9 (1.5-2.1)

Dose escalation was feasible in two patients. Both patients were treated with FEC for early breast cancer. So, 2 out of 23 (9%) of patients treated with FEC could be escalated, while no patients treated with FAC or AC could be escalated. Both of the escalated patients developed only grade 2 neutropenia (ANC 1.00-1.49 x 10e9/L) at day 15 of the first cycle and were escalated with 15% during the second cycle. One of these patients reached grade 3 neutropenia (ANC 0.50-0.99 x 10e9/L) in the second cycle, and no further escalation was performed. The other patient developed only grade 2 neutropenia after the second cycle and was further escalated with another 15% in cycle 3 (Figure 2; Table 2). During dose escalation, there were no complications like febrile neutropenia, grade 3-4 thrombocytopenia or increase in non-hematological toxicity. There were no relevant differences in baseline characteristics between escalated and non-escalated patients (Table 2).

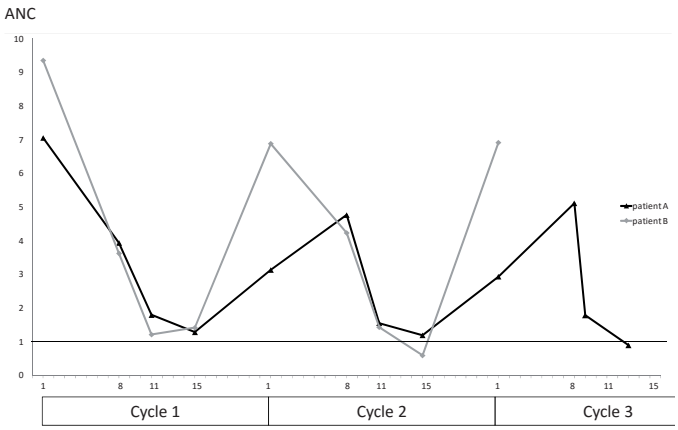

Figure 2. Absolute neutrophil counts (ANC) over time in the two escalated patients.

For the whole group of patients, mean ANC at baseline was 5.32 x 10e9/L (range 2.66-10.52), on day 8 was 3.72 x 10e9/L (range 2.02-9.60), on day 11 was 0.80 x 10e9/L (range 0.05-2.11) and on day 15 was 0.53 x 10e9/L (range 0.03-3.94). Nadir mean was 0.41 x 10e9/L (range 0.03-1.28) and was reached on day 11 in 7 patients and on day 15 in 23 patients. Coefficient of variation (CV) for mean ANC nadir was 0.77. Grade 3 neutropenia (ANC 0.50-0.99 x 10e9/L) or grade 4 neutropenia (ANC 0.50 x 10e9/L) after the first treatment cycle was observed in 28 of the 30 patients. Two of them had febrile neutropenia and were hospitalized. All patients had recovery of ANC to ≥1.5 x 10e9/L at the time of the planned next treatment cycle. Duration of neutropenia expressed as mean cumulative neutrophil count (the sum of CTC grades of neutropenia on day 8, 11 and 15) was 6.4 with a CV of 0.24 (Table 3).

Incidence of grade 3-4 neutropenia was lower after the second and third cycle of chemotherapy compared to after the first cycle (proportion of grade 3-4 neutropenia in first, second and third cycle respectively, 94, 77 and 75%, non-significant, Table 4). In these figures, patients who underwent dose escalation or used secondary G-CSF prophylaxis were excluded.

Table 2. details of escalated patients.

	Patient A	**Patient B**
Escalations	15% in first cycle 15% in second cycle	15% in first cycle No further escalation in second cycle
Age (years)	47	55
Chemotherapy regime	FEC	FEC
Body surface are, m^2	1.9	1.8
Weight (kg)	75	75
Cycle 1 ANC baseline	7.05	9.35
Cycle 1 ANC nadir	1.28	1.21
Cycle 1 ANC nadir, day reached	15	11
Cycle 2 ANC baseline, x 10e9/L	3.13	6.88
Cycle 2 ANC nadir, x 10e9/L	1.19	0.59
Cycle 2 ANC nadir, day reached	15	15

ANC: absolute neutrophil count.

Table 3. laboratory values, first cycle, all patients.

Mean ANC	
baseline, x 10e9/L (range)	5.32 (2.66-10.52)
day 8, x 10e9/L (range)	3.72 (2.02-9.60)
day 11, x 10e9/L (range)	0.80 (0.05-2.11)
day 15, x 10e9/L (range)	0.53 (0.03-3.94)
Mean ANC nadir, x 10e9/L (range)	0.41 (0.03-1.28)
Coefficient of variation	0.77
ANC nadir, *n* (%)	
day 8	0 (0)
day 11	7 (23)
day 15	23 (77)
Neutropenia nadir, *n* (%)	
CTC grade 0	0 (0)
CTC grade 1	0 (0)
CTC grade 2	2 (7)
CTC grade 3	8 (27)
CTC grade 4	20 (67)
Mean cumulative neutrophil count[a]	6.4
Coefficient of variation	0.24
Febrile neutropenia, *n* (%)	2 (7)

ANC: absolute neutrophil count.
[a] expressed as the sum of CTC grades of neutropenia (0-4) on day 8, 11 and 15.

Table 4. ANC nadir in first three cycles of ACC, all patients.

	Cycle 1 (*n* = 30)	**Cycle 2 (*n* = 21[a])**	**Cycle 3 (*n* = 16[b])**
Mean ANC nadir, x 10e9/L (range)	0.41 (0.03-1.28)	0.66 (0.09-1.50)	0.63 (0.06-1.57)
ANC nadir, *n* (%)			
day 8	0 (0)	0 (0)	0 (0)
day 11	7 (23)	2 (10)	5 (31)
day 15	23 (77)	19 (90)	11 (69)
Neutropenia nadir, *n* (%)			
CTC grade 0	0 (0)	0 (0)	0
CTC grade 1	0 (0)	0 (0)	1 (6)
CTC grade 2	2 (7)	5 (24)	3 (19)
CTC grade 3	8 (27)	10 (48)	4 (25)
CTC grade 4	20 (67)	6 (29)	8 (50)

ANC: absolute neutrophil count.

[a] exclusion of escalated patients (*n* = 2), patients with febrile neutropenia after the first cycle (*n* = 2) and patients with missing data (*n* = 5). Besides the escalated patients, there were no patients with dose alterations in the second cycle.

[b] exclusion of escalated patients (*n* = 2), patients with febrile neutropenia after the first cycle (*n* = 2) and patients with missing data (*n* = 10). There were no patients with febrile neutropenia after the second cycle. Besides the escalated patients, there were no patients with dose alterations in the third cycle.

DISCUSSION

The ANCHOR study was designed based on the improved survival found in a number of retrospective studies among patients treated with adjuvant chemotherapy for early breast cancer, who achieved a higher degree of hematological toxicity [6,9-11]. In this pilot feasibility study among breast cancer patients treated with currently advocated doses of ACC, neutrophil-guided dose escalation was feasible. Dose escalation was possible in 2 out of 23 patients (9%) treated with FEC, while dose escalation was possible in none of the patients treated with FAC or AC. Asymptomatic grade 3-4 neutropenia was achieved in the majority of patients after the first cycle of ACC. It seems therefore not useful to proceed with a large randomized controlled trial on neutrophil-guided dose escalation among patients with currently advocated doses of ACC.

Previously, two other studies have also investigated the feasibility of dose escalation of ACC, although they are hardly comparable with our current study. In the first study of tailored fluorouracil, epirubicin and cyclophosphamide (FEC) with primary granulocyte colony-stimulating factor (G-CSF) support, the dose of epiriubicin and cyclophosphamide could be escalated by 50% or more in more than half of the patients. Starting dose in this study was fluorouracil 600 mg/m^2, epirubicin 75 mg/m^2 and cyclophosphamide 900 mg/m^2. Treatment with nine cycles of tailored FEC with G-CSF support (median cumulative dose of epirubicin was 780 mg/m^2) was, however, associated with an increased

risk of acute myeloid leukemia and myelodysplastic syndrome. There were also more cardiac side effects in the tailored FEC group. Tailored FEC with G-CSF support can therefore not be advocated for clinical practice [12]. In our study, the two patients in whom escalation of ACC was feasible had a cumulative dose of epirubicin of 347 and 330 mg/m^2, respectively. In the second study by Edlund et al., the study design was comparable with our study; however, the 'standard dose' of epirubicin used in this study was substantially lower than in our study (60 mg/m^2 vs. 100 mg/m^2, respectively). In this study (n = 1,535), patients who did not reach leukopenia CTC grade 3 or 4 after a first cycle of standard FEC (in this study fluorouracil 600 mg/m^2, epirubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2) were randomized to a total of six courses of standard dosed FE(60)C (n = 526) or a total of six cycles of FEC with doses tailored to achieve grade 3 leukopenia (n = 521). The relative dose intensity (defined as the given dose delivered in the originally expected time/the expected dose in the expected time) was increased by a factor of 1.31. Median cumulative dose of epirubicin in the tailored dose group was 520 mg/m^2. There was no excess of acute non-hematological toxicity [13].

It is important to mention that both these studies used lower 'standard doses' of epirubicin compared to our study (75 and 60 mg/m^2, respectively). It can be concluded based on these and our study that neutrophil-guided dose escalation might be feasible in older regimens with lower 'standard dose' of epirubicin. With currently advocated doses (epirubicin 100 mg/m^2 and doxorubicin 50-60 mg/m^2), it is, however, not feasible to escalate a relevant proportion of patients.

Interestingly, the two patients, who were escalated, were both treated with epirubicin, while none of the patients treated with doxorubicin could be escalated. This might be due to chance. A real difference in hematological toxicity between these two anthracyclines can, however, not be excluded. When taking only the patients treated with epiriubicin into account, dose escalation was possible in 9% of patients, not reaching the predefined 10% which was considered worthwhile enough for further exploration.

Furthermore, a trend was seen in a decreased proportion of patient with grade 3-4 neutropenia over the subsequent cycles. In our study, dose escalation was only permitted when no grade 3-4 neutropenia was seen after the first cycle. When we also had allowed patients to escalate based on ANC nadir after the second cycle, five more patients could have been escalated in the third cycle. One of the currently advocated (neo)adjuvant chemotherapy regimens consists of three cycles FEC (5FU 500 mg/m^2; epiriubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2), followed by three cycles docetaxel 100 mg/m^2. In this regimen, cumulative anthracycline dose is relatively low, with a low risk of cardiotoxicity [14]. Since only three cycles of ACC are given, it is of utmost importance to dose these cycles as high as possible without unacceptable side effects. Further research should therefore focus mainly on patients treated in (neo)adjuvant setting and allow escalation also in subsequent cycles.

For most classical anticancer drugs, BSA-guided dosing is still standard practice in clinical oncology. BSA-based dosing of chemotherapy has largely resulted from its use in the extrapolation of drug doses used in experimental animals to those considered safe as starting doses for phase 1 clinical trials. However, a proper scientific rationale for BSA-based dosing of anticancer drugs in human

adult cancer patients is lacking [15-17]. Furthermore, the use of BSA does not reduce inter-individual variability in pharmacokinetic parameters for the majority of investigated anticancer drugs [18]. For irinotecan, it has been shown that flat-fixed dosing does not result in increased pharmacokinetic/pharmacodynamic variability and could be safely used [19]. Furthermore, for carboplatin, glomerular filtration rate-adjusted dosing has been widely accepted as standard [20]. Although knowledge on pharmacogenetics has rapidly been expanding, this had not led to many practically applicable dosing algorithms for classical anticancer drugs, while exposure to chemotherapy is influenced by many other interacting factors [21,22]. For fluoropyrimidines, it has been suggested to adjust the dose based on dihydropyrimidine dehydrogenase (DPYD) genotype tests [23]. The method of dose adjustment guided by plasma drug concentrations (therapeutic drug monitoring, TDM) has not been used as standard practice, which is largely due to the obscure relationship between plasma drug concentrations and treatment effects [24,25]. However, for the vast majority of classical anticancer agents, BSA-guided dosing remains still standard practice. For most (oral) targeted agents, flat-fixed dosing and a posteriori dose reduction in case of severe toxicity is a standard practice. For these agents, the relation between dose and outcome (both efficacy and safety) is even less clear, compared to classical chemotherapy, due to both differences in molecular characteristics of the tumor as well as in environmental and genetic factors.

CONCLUSION

In conclusion, inter-individual variability in hematological toxicity with currently advocated doses of ACC in breast cancer patients is limited. Escalation of currently advocated ACC doses without G-CSF, with a target of grade 3-4 neutropenia, is feasible, but only possible in a relatively small proportion of patients. Since no other dosing algorithms are available for ACC, BSA-guided dosing remains standard practice at this moment.

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6

Development and validation of an UPLC-MS/MS method for the quantification of tamoxifen and its main metabolites in human scalp hair

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ABSTRACT

The aim of this study was to validate an earlier developed high-performance highly sensitive ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method for quantification of tamoxifen and its three main metabolites (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen) in scalp hair. This non-invasive method might, by segmental analysis of hair, be useful in the determination of the concentration of drugs and its metabolites over time, which can be used to study a wide variety of clinical relevant questions. Hair samples (150-300 hair strands, cut as close to the scalp as possible from the posterior vertex region of the head) were collected from female patients taking tamoxifen 20 mg daily (n = 19). The analytes were extracted using a liquid-liquid extraction procedure with carbonate buffer at pH 8.8 and a mixture of n-hexane/isopropranol method, followed by UPLC-MS/MS chromatography, based on an earlier validated method. The calibration curves were linear in the range of 1.00-200 pmol for tamoxifen and N-desmethyl-tamoxifen, with lower limit of quantitation (LLQ) of 1.00 pmol and 0.100-20.0 pmol with LLQ of 0.100 pmol for endoxifen and 4-hydroxy-tamoxifen. Assay performance was fair with a within-run and between-run variability less than 9.24 at the three quality control samples and less than 15.7 for the LLQ. Importantly, a steep linear decline was observed from distal to proximal hair segments. Probably, this is due to ultraviolet (UV) exposure as we showed degradation of tamoxifen and its metabolites after exposure to UV light. Furthermore, higher concentrations of tamoxifen were found in black hair samples compared to blond and brown hair samples. We conclude that measurement of the concentration of tamoxifen and its main metabolites in hair is possible, with the selective, sensitive, accurate and precise UPLC-MS/MS method. However, for tamoxifen, it seems not possible to determine exposure over time with segmental analysis of hair, probably largely due to the effect of UV irradiation. Further research should therefore focus on quantification of other anticancer drugs, in segmented scalp hair, that are less sensitive to UV irradiation.

INTRODUCTION

Tamoxifen, a selective estrogen receptor modulator, is an important drug in the treatment of hormone receptor positive breast cancer. In early stage breast cancer, tamoxifen reduces the risk of recurrence, the risk of mortality and the risk of contralateral breast cancer [1-3]. In advanced hormone receptor positive breast cancer, tamoxifen improves progression free and overall survival over observation [4,5]. Tamoxifen also significantly reduces the risk of breast cancer in women at high risk to develop breast cancer [6]. However, not all women benefit from tamoxifen therapy, while treatment-related adverse reactions also vary greatly between patients. Inter-individual variability in metabolism of tamoxifen, which is influenced by both genetic and environmental factors, contributes to the differences in efficacy and toxicity of tamoxifen [7-9]. Since tamoxifen often has to be used for a longer period of time, therapy adherence, change in environmental factors and possibly resistance mechanisms might influence the systemic exposure over time [10,11].

Tamoxifen is a pro-drug and undergoes biotransformation into several active metabolites, including N-desmethyl-tamoxifen, which is the most abundant metabolite, and its potent metabolites 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) [12]. Chemical structures of tamoxifen and its main metabolites are shown in Figure 1. Endoxifen and 4-hydroxytamoxifen have equivalent anti-estrogenic potencies and are 30-100 times more active as anti-estrogens than tamoxifen and N-desmethyltamoxifen. Because of the higher plasma concentrations of endoxifen compared to 4-hydroxytamoxifen, endoxifen is believed to be the principal active metabolite [13-15]. Even low concentrations of tamoxifen and its main metabolites can be quantified in plasma with highly sensitive, selective, accurate and precise UPLC-MS/MS methods [16-19].

One option to monitor drug exposure and to adjust treatments would be by implementing therapeutic drug monitoring (TDM) as a dosing strategy. For most anticancer drugs, however, TDM is still not common practice due to undefined thresholds, and practical problems incorporating this in clinical practice due to logistical constraints. Additionally, since plasma concentrations only give information about the systemic concentration at a particular time point, several samples over time should be taken. As a result, most reliable TDM can be achieved with serial measurements of plasma concentrations of drugs and/or metabolites, which will increase the complexity and the costs of TDM [20].

Methods, which give (in retrospect) information about the course of concentrations of anticancer drugs and/or its metabolites over time would therefore be of great interest. A potential method, which is also feasible for clinical practice, could be to measure these concentrations in scalp hair. Hair is a strong and stable tissue and has the major advantage over traditional matrices (e.g. blood or urine) of giving the opportunity to study long term exposure to drugs, clearly depending on the length of hair collected. Scalp hair grows with an average rate of one centimeter per month, therefore, one centimeter of scalp hair could represent drug levels of one month [21]. So segmental analysis of hair allows the determination of the historic pattern of drug concentrations making serial venous blood collections unnecessary.

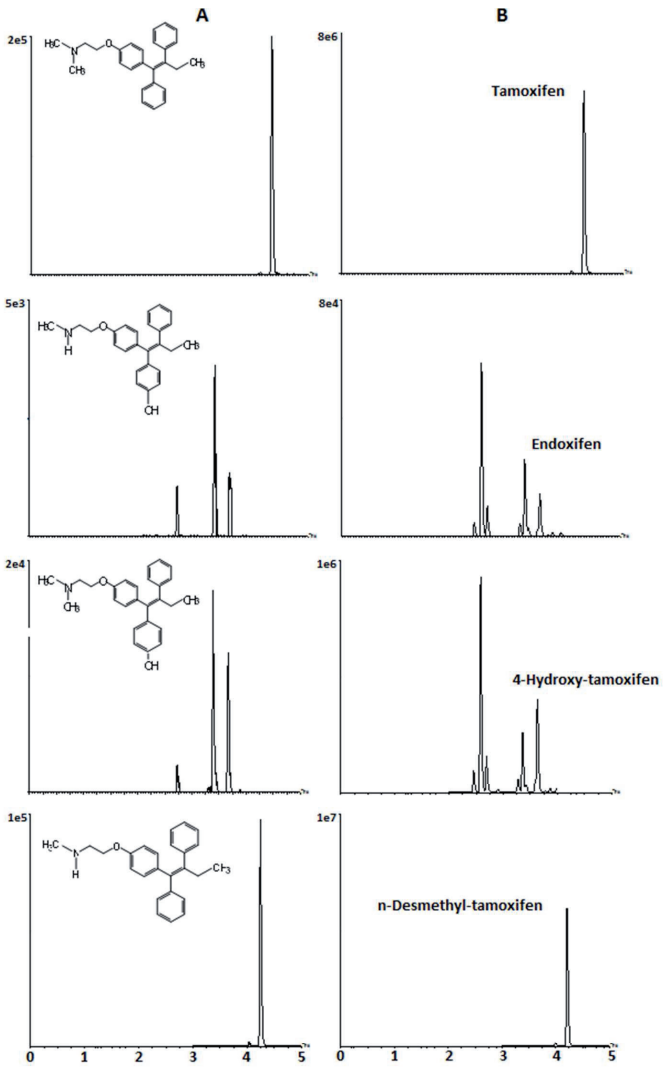

Figure 1. Chemical structures and multiple reaction monitoring (MRM) chromatograms of hair samples spiked at the concentration of the lower limit of quantitation (A) and a human scalp hair sample of a patient collected after 20 mg daily tamoxifen administration containing 9.44 pmol/mg tamoxifen, 0.694 pmol/mg endoxifen, 0.231 pmol/mg 4-hydroxy-tamoxifen and 14.2 pmol/mg N-desmethyl-tamoxifen (B).

Early experience with measurement of drugs in (scalp) hair was build up in toxicology and forensic medicine [22]. A validation of a method for screening and quantification of 96 drugs from different pharmacologic groups in hair by UPLC-MS/MS was performed by Montesano et al. [23]. Few clinical studies have been performed using measurement of drug concentrations in hair. In HIV infected

patients on antiretroviral therapy, it has been shown that concentration of the antiretroviral drug atazanavir in hair is one of the best predictors of suppression of viral load, even when adjusting for the other known important predictors [24]. Furthermore, there is increasing experience with measurement of the steroid hormone cortisol in scalp hair. Concentrations of cortisol in hair segments correspond with clinical course in patients with (cyclic) Cushing syndrome and can be used for monitoring of therapy [25,26]. So far, no studies have been performed with measuring of anticancer drugs or its metabolites in hair. Obviously, when measurement of concentrations of (metabolites of) anticancer drugs in hair is feasible this could open an important new way to study a wide variety of clinical relevant questions in patients suffering from cancer. We have here developed and validated an UPLC-MS/MS assay for measurement of tamoxifen and its metabolites in scalp hair. Furthermore, we investigated whether or not segmental analysis of hair can be used to determine the historic pattern of drug concentrations.

MATERIALS AND METHODS

Chemicals

Pure Z (cis)-isomers of tamoxifen, N-desmethyl-tamoxifen and 4-hydroxy-tamoxifen, the stable labeled deuterated internal standards tamoxifen-d5, N-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and a racemic mixture of the Z- and E-isomers (1:1) of 4-hydroxy-N-desmethyl-tamoxifen-d5 and 4'-hydroxy-tamoxifen, the stable labeled deuterated internal standard 4'-hydroxy-tamoxifen-d6 and N-desmethyl-4'-hydroxy-tamoxifenwere obtained from Toronto Research Chemicals (North York, ON, Canada). The pure Z (cis)-isomer of 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) was kindly provided by Jina Pharmaceuticals Inc. (Libertyville, IL). All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were from Bio-solve BV (Valkenswaard, The Netherlands). Dimethylsulphoxide (DMSO), ammonium carbonate and 2-propanol were purchased from Merck (Darmstadt, Germany), n-hexane was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), and formic acid from J.T. Baker (Deventer, The Netherlands). Blank human scalp hair was obtained from healthy volunteers.

Preparation of stock solutions, calibration standards and quality control samples

Stock solutions containing 1.00 mM free base of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen, 4´-hydroxy-tamoxifen, N-desmethyl-4´-hydroxy-tamoxifen and endoxifen in DMSO were prepared individually and stored at T <-70°C. Individual stock solutions of tamoxifen and its metabolites were used for the preparation of a working stock solution, containing 200 µM tamoxifen, 200 µM N-desmethyl-tamoxifen, 20 µM4-hydroxy-tamoxifen, 20 µM 4´-hydroxy-tamoxifen, 20 µM N-desmethyl-4´-hydroxy-tamoxifen and 20 µM endoxifen in DMSO. The working stock solution was divided into 150 µL aliquots, which were used for the construction of calibration curve standards

during the validation. Separate stock solutions of tamoxifen and its metabolites were used for the preparation of the pools of quality control (QC) samples. The variation between the stock solutions of tamoxifen and its metabolites used for the construction of the calibration standards and QC samples was in all cases <5%.

Deuterated internal standards were dissolved in DMSO separately, to obtain internal standard stock solutions at a concentration of 1 mg/mL free base, which subsequently were aliquotted and stored at T <-70°C. Aliquots of 10 µL of the individual stock solutions were concurrently 10,000-fold diluted in acetonitrile, resulting in an internal standard working solution containing 100 ng/mL tamoxifen-d5, N-desmethyl-tamoxifen-d5,4-hydroxy-tamoxifen-d5, 4-hydroxy-N-desmethyl-tamoxifen-d5 and 4′-hydroxy-tamoxifen, which was stored at T <8°C for a maximum of three months.

Calibration curve standards were freshly prepared for each run, by addition of 10 µL aliquots of appropriate dilutions of the working stock solution in acetonitrile/DMSO (1:1, v/v) to 190 µL aliquots of 10 mg overnight extracted scalp hair in methanol at the following amounts: 1.00, 2.00, 10.0, 20.0, 50.0, 100, 180, and 200 pmol for tamoxifen and N-desmethyl-tamoxifen and 0.100, 0.200, 1.00, 2.00, 5.00, 10.0, 18.0, and 20.0 pmol for 4-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen, N-desmethyl-4′-hydroxy-tamoxifen and endoxifen.

A total of four pools of quality control (QC) samples were prepared by spiking appropriate dilutions of stock solutions of tamoxifen and its metabolites to blank extracted scalp hair at amounts of 1.00 pmol (lower limit of quantification, LLQ), 3.00 pmol (QC-Low), 80.0 pmol (QC-Middle) and160 pmol (QC-High) for tamoxifen and N-desmethyl-tamoxifen and at 0.100 pmol (LLQ), 0.300 pmol (QC-Low), 8.00 pmol (QC-Middle) and 16.0 pmol (QC-High) for 4-hydroxy-tamoxifen, 4′-hydroxy-tamoxifen, N-desmethyl-4′-hydroxy-tamoxifen and endoxifen. Pools of QC samples were aliquotted and stored at T <-70°C until analysis.

Aliquots of 50 µL of internal standard working solution and 750 µL methanol was added to 200 µL of calibration curve standards and QC samples in 2-mL micro centrifuge tubes and shaken for 24h at ambient temperature. Hereafter, the organic phase was transferred to a clean 2-mL micro centrifuge tube and evaporated to dryness under nitrogen at T = 60°C. The residues were reconstituted in 200 µL aliquots of sodium carbonate buffer (pH 8.8) and 1 mL aliquots of hexane/2-propanol (95:5, v/v). The samples were vortexed and centrifuged at 18,000 × *g* at ambient temperature for ten minutes. Aliquots of 800 µL of the organic phase were transferred into 4.5 mL glass tubes and evaporated to dryness under nitrogen at T = 60°C. Hereafter the residues were reconstituted in 100 µL aliquots of acetonitrile/water/formic acid (40:60:0.1, v/v/v) and centrifuged for five minutes at 4,000 × *g*. The supernatants were transferred into 350 µL 96-well plates, which were placed into a chilled (T = 10°C) autosampler, from which aliquots of 5 µL were injected onto the UPLC column.

Hair sample preparation

Root ends of hair samples (150-300 hair strands) were divided into sections of 1 cm and subsequently cut into pieces of approximately 1 mm in length. Aliquots of 50 µL of internal standard working

solution and 950 µL of methanol were added to 10-30 mg of hair samples in 2 mL micro centrifuge tubes and shaken for 24h at ambient temperature, protected from light. Subsequently, the organic phase was transferred to a clean 2 mL micro centrifuge tube and evaporated to dryness under nitrogen at T = 60°C. The residues were reconstituted in 200 µL aliquots of sodium carbonate buffer (pH 8.8) and 1 mL aliquots of hexane/2-propanol (95:5, v/v). Hereafter, the samples were vortexed and centrifuged at 18,000 × *g* at ambient temperature for ten minutes. Aliquots of 800 µL of the organic phase were transferred into 4.5 mL glass tubes and evaporated to dryness under nitrogen at T = 60°C. Hereafter, the residues were reconstituted in 100 µL aliquots of acetonitrile/water/formic acid(40:60:0.1, v/v/v) and centrifuged for five minutes at 4,000 × *g*. The supernatants were transferred into 350 µL 96-well plates, which were placed into a chilled (T = 10°C) autosampler, from which aliquots of 5 µL were injected onto the UPLC column.

Equipment

The UPLC-MS/MS system was composed of a Waters Aquity UPLC Sample Manager coupled to a Waters TQ Detector (Waters, Etten-Leur, The Netherlands). The MassLynx V4.1 SCN627 software package was used for the acquisition and processing of data. Quantification was performed using QuanLynx as implemented in the MassLynx software.

Chromatographic conditions

Analytes including 4′-hydroxy-tamoxifen and N-desmethyl-4′-hydroxy-tamoxifen were separated on an Aquity UPLC®BEHC 18 column 1.7 µm, 100 mm × 2.1 mm, (Waters, Etten-Leur, The Netherlands) and thermostatted at T = 50°C. Aqueous ammonium formate (0.2 mM) and acetonitrile, both acidified with 0.1% formic acid, were used as mobile phase A and mobile phase B, respectively. A linear gradient at a flow-rate of 0.300 mL/min was achieved with 30-80% of mobile phase B from 0 to 6 min, then 80-30% of mobile phase B over 2 minutes, which was held for 2 minutes for re-equilibration of the system. An autosampler (at 10°C) injected volumes of 5 µL onto the UPLC column. The overall run time was ten minutes. The needle of the autosampler was washed using a strong needle wash solvent (acetonitrile/methanol/2-propanol/water/formic acid, 25:25:25:25:0.1 v/v/v/v/v) and a weak needle wash solvent (30% acetonitrile in water). The column effluent was introduced to the mass spectrometer and monitored.

Mass spectrometry

Tandem mass spectrometry was performed in the positive ion electrospray ionization mode. Mass transitions of m/z were optimized for tamoxifen, its metabolites and the deuterated internal standards of tamoxifen and its metabolites by infusion of the respective analytes in acetonitrile/water/0.1% formic acid (40:60:0.1, v/v/v) via combined infusion. Optimal MS settings were adjusted manually. The desolvation gas was set at 800 L/h, the cone gas at 25 L/h (nitrogen) and the ionspray voltage was kept at 1.50 kV. We used a source temperature of T = 150°C and desolvation temperature of T

= 350°C. The dwell times were set at 20 ms and the inter-channel delay at 10 ms. Multiple reaction monitoring (MRM) mode was applied for the quantitation with the parameters as presented in Table 1. The collision cell pirani pressure was set at ~5e^{-3} mbar (argon).

Table 1. MS/MS settings.

Analyte	Scan window (minutes)	Parent (m/z)	Daughter (m/z)	Collision (V)	Cone voltage (V)
Tamoxifen	3.00-5.50	372	72	25	45
Tamoxifen-d5	3.00-5.50	377	72	25	45
N-desmethyl-tamoxifen	3.00-5.50	358	58	21	42
N-desmethyl-tamoxifen-d5	3.00-5.50	363	58	21	42
N-Desm.-4'hydroxy-tamoxifen	2.50-5.00	374	58	23	45
4-OH-tamoxifen	2.00-5.00	388	72	25	47
4-OH-tamoxifen-d5	2.00-5.00	393	72	25	47
4'OH-tamoxifen	2.50-5.00	388	72	25	47
4'OH-tamoxifen-d6	2.50-5.00	394	78	25	47

Quantitation

Calibration curves were constructed by plotting the peak area ratios of the components to internal standards versus the known amount with a weight factor of 1/concentration^2.

Patients and hair sampling

Hair samples from 19 female breast cancer patients, aged from 33 to 73 years were collected for the study. Hair samples (150-300 hair strands) were cut, as close to the scalp as possible, from the posterior vertex region of the head, since this region of the scalp is associated with least variation in growth rate. All patients received 20 mg tamoxifen daily for a period of six months up to five years, at the moment of participation in this study. All patients reported that they were compliant with the medication. The hair samples were cut into segments of one centimeter length starting from the hair root which corresponds to one month growth. The study was approved by the local ethical board (METC Erasmus MC, study number MEC14-346).

Method validation

The UPLC-MS/MS method was validated for tamoxifen and the three main metabolites, 4-hydroxy-tamoxifen, endoxifen and N-desmethyl-tamoxifen in agreement with the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA [27]. Blank human hair samples of ten different healthy volunteers were analyzed to determine the potential presence of endogenous contaminating compounds that may interfere with the assay.

For the determination of the LLQ, blank human hair samples of ten healthy volunteers were spiked at

an amount of 1.00 pmol for tamoxifen and N-desmethyl-tamoxifen and 0.100 pmol for endoxifen and 4-hydroxy-tamoxifen and analyzed during one run. Accuracy (ACC), within-run precision (WRP) and the between-run precision (BRP) were determined by analyzing five replicates of pools of LLQ and QC samples independently over a three-run period. The ACC, WRP and BRP at the level of the LLQ and QC samples were calculated by one-way analysis of variance, using the run as the variable as described earlier [28]. Reproducibility of the method was performed by measuring one hair segment of one patient on five separate occasions.

Matrix effect and recovery

Recovery was determined by comparing the areas of the peak from spiked hair before extraction to spiked hair after extraction. The matrix effect was measured by comparing the areas of the peak from spiked hair after extraction to spiked methanol at the same concentration. Concentrations of 5.00 pmol and 16.0 pmol for recovery and matrix effect were used for tamoxifen and N-desmethyl-tamoxifen and 0.500 pmol and 1.60 pmol for endoxifen and 4-hydroxy-tamoxifen.

Stability of tamoxifen and its metabolites in hair to ultraviolet (UV) irradiation

Hair samples obtained from patients receiving 20 mg tamoxifen daily were used to investigate the influence of UV irradiation from an UV lamp (254 nm). One group of hair samples was protected from light, while the second group was exposed to UV light (254 nm) for one hour. The two groups of hair samples were analyzed by UPLC-MS/MS using the conditions described in the experimental sections.

Relationship between hair concentration and pigmentation

To investigate the relationship between hair pigmentation and tamoxifen concentration in root hair samples, hair samples with different hair color were analyzed according to the protocol. Patients who had bleached their hair less than one month before were excluded for this analysis. A total of seven hair samples were divided into three groups of natural hair color, i.e. two hair samples were classified as blond, three hair samples were classified as brown and two hair samples were classified as black. One of these patients had permed her hair more than two months before collection of the hair sample while five patients painted their hair less than three months before collection.

RESULTS AND DISCUSSION

UPLC-MS/MS conditions and method development

The presented method for the determination of tamoxifen and its main metabolites in human scalp hair is based on the validated method described by Binkhorst et al. [16]. The selected product ions, cone voltages and collision energies of tamoxifen, its metabolites and their respective deuterated internal standards are presented in Table 1. The validation of 4'-hydroxy-tamoxifen and N-desmethyl-4'-hydroxy-tamoxifen failed for accuracy described in the Guidance for Industry, Bio-analytical

Method Validation, as specified by the FDA. Good baseline separation and separation from early eluting hydrophilic, potentially interfering matrix components was achieved for all compounds while maintaining a relative short injection to injection time of ten minutes with elution times of 2.6 minutes for endoxifen, 2.7 minutes for 4-hydroxy-tamoxifen, 2.9 minutes for N-desmethyl-4'-hydroxy-tamoxifen, 3.0 minutes for 4'-hydroxy-tamoxifen, 3.6 minutes for N-desmethyl-tamoxifen and 3.7 minutes for tamoxifen (Figure 1).

Adequate management of the scalp hair samples before performing the analyses is of great importance in the development of an analytical method. Remaining endogenous compounds may cause ion-suppression or ion-inducement and can negatively affect the sensitivity of the assay. A variety of extraction procedures have been described for drug analysis in human scalp hair depending on the chemical structure of the compounds of interest. The first step in hair analysis is the removal of the drug from the hair. Several methods have been described and include alkaline digestion, acidic extraction, enzymatic digestion and simple extraction with methanol. The use of methanol for the removal of tamoxifen and its metabolites from the hair showed to be effective. Almost all drug was extracted within 24h of incubation of cutted hair with methanol at ambient temperature. No significant increase of drug was observed between 24h and 30h incubation of cutted hair with methanol at ambient temperature. Extraction of hair with methanol alone leads to extracts with potential interfering endogenous products. A variety of methods have been described that lead to more purified extracts. This includes several solid phase extractions and liquid-liquid extraction. Solid phase extraction has, if not automated, disadvantages including poor reproducibility and is, compared to liquid-liquid extraction, relatively laborious [29-33].

In this validated method, a liquid-liquid extraction procedure was applied with carbonate buffer at pH 8.8 and a mixture of n-hexane/isopropanol, which resulted in clean extracts. Reproducibility was investigated by analyzing one segment of one hair sample on five separate occasions. On one occasion, the concentration of tamoxifen and its main metabolites was substantial higher than the mean concentration of the measurements on the other four occasions (>400%). This measurement was excluded for further calculations. The mean concentration of tamoxifen, endoxifen, 4-hydroxy-tamoxifen and N-desmethyl-tamoxifen was 1.15 ± 0.0980 pmol/mg, 0.0931 ± 0.00604 pmol/mg, 0.0471 ± 0.00694 pmol/mg and 1.46 ± 0.113 pmol/mg, respectively. The concentration of tamoxifen and its metabolites in the scalp hair sample measured on five executive days are presented in Table 2.

Assay performance

Each validation run includes calibration curves prepared induplicate containing eight standards per calibration curve. Weighted (1/concentration^2) linear regression analysis of peak area ratios of analytes and Internal Standard, versus concentration of analytes were used for the quantitation. The method results were linear (r^2 ≥0.9979) in the range of 1.00-200 pmol for tamoxifen and N-desmethyl-tamoxifen and of 0.100-20.0 pmol for 4-hydroxy-tamoxifen and endoxifen in human scalp hair and none of the blank scalp hair samples showed potential interference for tamoxifen, N-desmethyl-

Table 2. Measurements of tamoxifen and its metabolites in a scalp hair sample during five occasions.

Day	Tamoxifen (pmol/mg)	Endoxifen (pmol/mg)	N-desmethyl-tamoxifen (pmol/mg)	4-OH-tamoxifen (pmol/mg)
1	1.20	0.0874	1.46	0.0412
2	5.16[a]	0.564[a]	5.73[a]	0.542[a]
3	1.24	0.0965	1.47	0.0451
4	1.01	0.100	1.31	0.0449
5	1.17	0.0886	1.59	0.0571
Mean	1.15	0.0931	1.46	0.0471
SD	0.0980	0.00604	0.113	0.00694
CV	8.5%	6.5%	7.7%	14.7%

SD: standard deviation; CV: coefficient of variation.
[a] Excluded from calculation.

tamoxifen, 4-hydroxy-tamoxifen, endoxifen or any of the deuterated internal standards.

The LLQ was validated at 0.100 pmol for 4-hydroxy-tamoxifen and endoxifen and at 1.00 pmol for tamoxifen and N-desmethyl-tamoxifen. The LLQ has been validated in separate runs. In one validation run, analytes were spiked to ten different lots of human scalp hair. In three other runs, a pool of LLQ samples was processed as QC-samples. For tamoxifen, the amount in all independently spiked scalp hair samples fell within the acceptable range of accuracy of 80-120%, with an average measured concentration of 1.00 ± 0.0378 pmol. The measured amount of N-desmethyl-tamoxifen for all ten independent human scalp hair samples fell within the acceptable range of accuracy, with an average observed concentration of 0.995 ± 0.0499 pmol. The average amount for 4-hydroxy-tamoxifen in the ten independent samples (nine in acceptable range) was 0.0941 ± 0.0120. For endoxifen, measured amount in all independent samples fell within the acceptable range of accuracy, with an average concentration of 0.100 ± 0.0131 pmol.

The within-run and between-run precisions and the accuracies at five tested concentrations, including at the level of the LLQ, are summarized in Table 3, and all fell within the accepted ranges as specified by the FDA [34].

The extraction recovery (RE) and matrix effect (ME) were determined in six different lots of human blank hair samples, spiked with tamoxifen and its metabolites in a concentrations of 5 pmol and 16 pmol for tamoxifen and N-desmethyl-tamoxifen and 0.500 pmol and 1.6 pmol for endoxifen and 4-hydroxy-tamoxifen. The mean measured extraction efficiencies and matrix effects are presented in Table 4. The average recovery of tamoxifen was 93% at 5.0 pmol and 82% at 16 pmol. The recovery for the metabolites ranged from 69% for endoxifen at 0.500 pmol to 99% for 4-hydroxy-tamoxifen. Matrix effect was near 100% but a slight enhancement was observed for endoxifen.

Table 3. Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples[a].

	Sample spiked (pmol)	GM (pmol)	ACC (%)	WRP (%)	BRP (%)	*n*[c]
Tamoxifen						
LLQ	1	0.882	88.2	6.65	#[b]	13 of 15
Low	3	2.82	94	5.03	#[b]	15 of 15
Middle	80	75.8	94.8	3.74	#[b]	15 of 15
High	160	152	94.8	3.07	0.923	15 of 15
N-desmethyl-tamoxifen						
LLQ	1	0.996	99.6	5.42	4.34	15 of 15
Low	3	3.04	101.3	4.07	3.78	15 of 15
Middle	80	83	103.8	2.79	3.11	15 of 15
High	160	156	97.8	3.78	#[b]	15 of 15
4-OH-tamoxifen						
LLQ	0.1	0.094	94	8.56	3.73	14 of 15
Low	0.3	0.3	100	5.51	0.822	15 of 15
Middle	8	7.84	98	3	#[b]	15 of 15
High	16	16	100	4.16	#[b]	15 of 15
Endoxifen						
LLQ	0.1	0.101	101.4	15.7	8.08	13 of 15
Low	0.3	0.306	102	9.24	3.96	12 of 15
Middle	8	8.08	101	4.46	3.16	15 of 15
High	16	16.4	102.8	3.96	0.891	15 of 15

LLQ: lower limit of quantitation; QC: quality control; GM: grand mean; WRP: within-run precision; BRP: between-run precision; ACC: average accuracy.

[a] $n = 5$ in four separate runs (three runs at the LLQ).

[b] No additional variation observed by performing the assay in different runs.

[c] Number of individual samples falling within acceptable range of accuracy of 85-115% (80-120% at LLQ).

Table 4. Extraction recovery (RE) and matrix effect (ME) in hair from six different lots spiked with tamoxifen and N-desmethyl-tamoxifen at a concentration of 5.00 pmol and 16.0 pmol and 0.500 pmol and 1.60 pmol for endoxifen and 4-hydroxy-tamoxifen.

Analyte	5.00/0.500 pmol		16.0/1.60 pmol	
	ME (%)	RE (%)	ME (%)	RE (%)
Tamoxifen	86 ± 3.6	93 ± 3.6	84 ± 2.2	82 ± 17.6
N-Desmethyl-tamoxifen	91 ± 5.9	79 ± 8.7	86 ± 3.2	76 ± 16.6
4-OH-tamoxifen	101 ± 6.0	99 ± 11.1	98 ± 3.0	99 ± 7.7
Endoxifen	114 ± 9.5	69 ± 14.3	111 ± 4.3	82 ± 13.2

Data presented as mean ± standard deviation ($n = 6$).

Stability of tamoxifen and its metabolites in hair along the hair shaft

As shown in Figure 2, reduction on concentrations of tamoxifen and its metabolites was shown from the hair segment starting from the hair root to the following segments. The decline of hair tamoxifen and its metabolites along hair shaft was probably attributed to hair washing and other treatments. Also exposure to sunlight could contribute to the decline of tamoxifen and its metabolites in hair. An average linear decline rate of 0.0203 pmol/segment, 0.0111 pmol/segment, 0.00370 pmol/segment and 0.371 pmol/segment was observed for tamoxifen, endoxifen, 4-hydroxy-tamoxifen and N-desmethyl-tamoxifen, respectively.

Stability of tamoxifen and its metabolites in hair to UV irradiation

It is well known that tamoxifen and its metabolites are light sensitive and exposure to UV light leads to degradation [16]. To investigate if exposure of hair to UV light leads to degradation of tamoxifen and its metabolites, the extent of degradation of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen under UV light was determined. Hair samples from patients receiving 20 mg of tamoxifen daily were divided into two groups. One group of hair samples was protected from light, the second group was exposed for 1h to UV light (254 nm). Tamoxifen and its metabolites were very light sensitive under UV light (254 nm). No degradation of tamoxifen or its metabolites was observed when the samples were protected from light (Figure 3). As hair is always exposed to the environmental light, including UV, photo-degradation of tamoxifen and its metabolites seems the main limiting factor in the determination of the historic pattern of drug concentrations by segmental hair analysis.

Relationship between hair concentration and pigmentation

Higher concentrations of tamoxifen were found in black hair than in blond and brown hair samples. Mean concentration (range) in, respectively, black, blond and brown hair was 6.95 (4.47-9.44) pmol/mg, 2.23 (1.02-2.77) pmol/mg and 0.726 (0.663-0.789) pmol/mg. The decline of the concentration of tamoxifen and its metabolites along the hair shaft was independent of hair pigmentation. Several publications indicate that there is a good relation between drug levels and that of melanin in hair [35,36]. Hair color complicates the interpretation of hair testing in humans.

Analysis of tamoxifen and its metabolites in patients scalp hair samples

To demonstrate the applicability of the assay, segmented scalp hair samples of eight patients receiving 20 mg tamoxifen daily for at least six months were analyzed. As a result of the long term use of tamoxifen, the patients were at steady-state. The results are shown in Table 5 and clearly show the decrease of concentration of tamoxifen and its metabolites in scalp hair segments of one centimeter from root to distal end. Decline in concentration is independent of hair color. This method is sensitive and accurate enough for measuring tamoxifen and metabolites in human scalp hair.

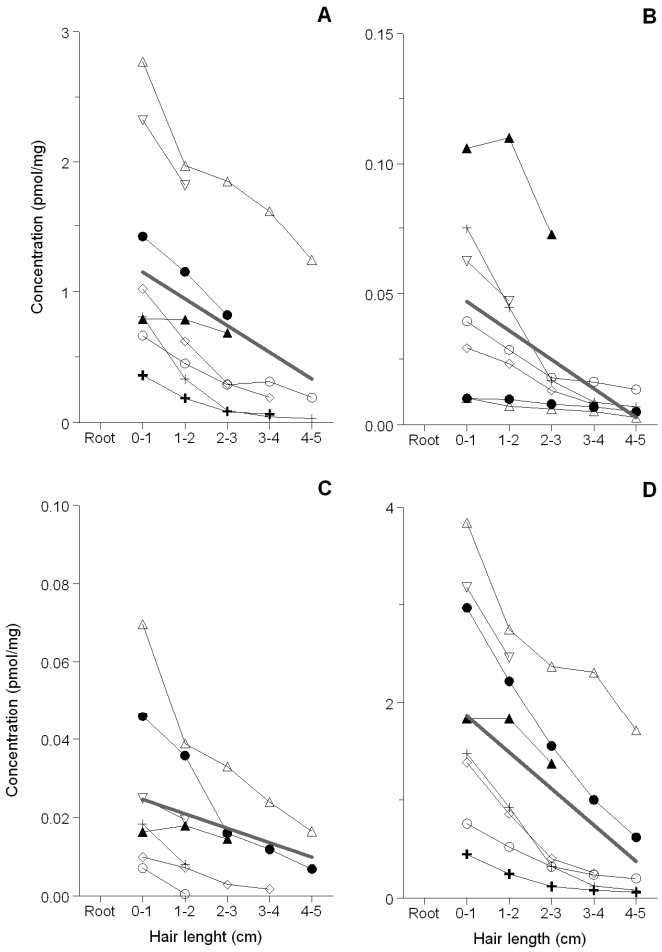

Figure 2. Concentration of tamoxifen (A), endoxifen (B), 4-hydroxy tamoxifen (C) and N-desmethyl-tamoxifen (D) in scalp hair, showing reduction on concentrations of tamoxifen and its metabolites from the hair segment starting from the hair root to the following segments, represented on the x-axis with distances in centimeter (cm). Gray line represent the mean profile of all hair samples (n = 8).

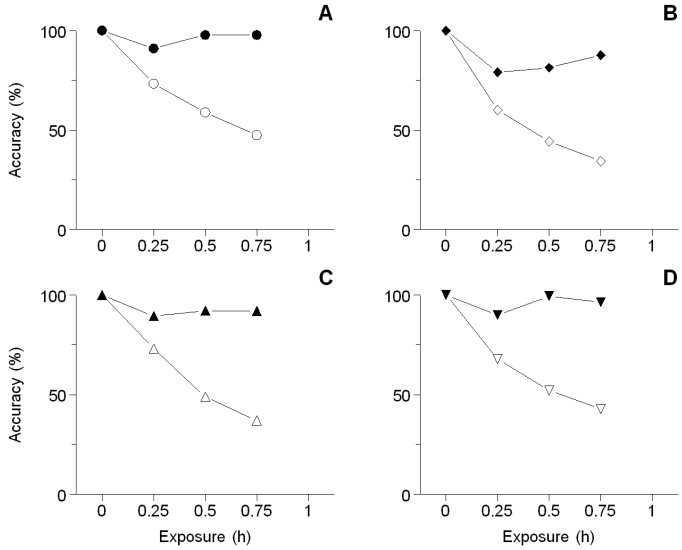

Figure 3. Accuracy of tamoxifen (A), endoxifen (B), 4-hydroxy-tamoxifen (C) and N-desmethyl-tamoxifen (D) after hair exposure to UV light source (-○-) and hair protected from light (-●-).

CONCLUSION

There are currently no published LC-MS/MS methods for quantification of any anticancer drug in hair. In this study, we are the first to show the UPLC-MS/MS method to be a selective, sensitive, accurate and precise method for the simultaneous analysis of tamoxifen and its three main phase I metabolites, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, in human scalp hair. One of the potential advantages for measuring drug concentrations in scalp hair samples is the possibility to perform segmental analysis of hair, allowing the determination of the historic pattern of drug concentrations. However, for tamoxifen and its main metabolites, a steep linear decline was observed from distal to proximal hair segments probably mainly due to the effect of UV irradiation. Further research should therefore focus on quantification of other anticancer drugs in segmented scalp hair, that are less sensitive to UV irradiation, with taking into account also influencing factors like the role of hair pigmentation.

Table 5. Concentration of tamoxifen and its metabolites in segmented human scalp hair of eight patients receiving 20 mg tamoxifen daily for at least six months. Segments of one centimeter were used and segments were numbered from root (segment 1) to distal end.

			Concentration (pmol/mg)					
			Segment					
Subject	**Age**	**Color**	**1**	**2**	**3**	**4**	**5**	**6**
Tamoxifen								
1	38	Blond	0.807	0.333	0.0904	0.0443	0.0310	0.0913
2	69	Brown	2.77	1.97	1.85	1.62	1.24	
3	70	Blond	0.789	0.785	0.684			
4	60	Blond	0.663	0.451	0.29	0.213	0.191	
5	46	Brown	1.02	0.621	0.296	0.191		
6	70	Blond	0.362	0.186	0.0855	0.0643		
7	53	Gray	1.42	1.15	0.820			
8	52	Black	9.44	6.40				
Endoxifen								
1	38	Blond	0.0750	0.0447	0.0169	0.00856	0.00685	0.00495
2	69	Brown	0.0102	0.00705	0.00593	0.00504	0.00277	
3	70	Blond	0.106	0.110	0.0725			
4	60	Blond	0.0394	0.0285	0.0179	0.0163	0.0134	
5	46	Brown	0.0292	0.0233	0.0131	0.00784		
6	70	Blond	NQ	NQ	NQ	NQ		
7	53	Gray	0.0684	0.0562	0.0389			
8	52	Black	0.694	0.556				
4-Hydroxy-tamoxifen								
1	38	Blond	0.0184	0.00806				
2	69	Brown	0.0696	0.0389	0.033	0.024	0.0165	
3	70	Blond	0.0163	0.0179	0.0145			
4	60	Blond	0.0071	0.0047	NQ	NQ	NQ	
5	46	Brown	0.00994	0.00719	0.00287	0.00176		
6	70	Blond	NQ	NQ	NQ	NQ		
7	53	Gray	0.0327	0.0341	0.0196			
8	52	Black	0.231	0.178				
N-desmethyl-tamoxifen								
1	38	Blond	1.47	0.925	0.320	0.123	0.078	0.0717
2	69	Brown	3.84	2.75	2.37	2.31	1.71	
3	70	Blond	1.83	1.83	1.37			
4	60	Blond	0.758	0.521	0.318	0.297	0.199	
5	46	Brown	1.38	0.858	0.402	0.254		
6	70	Blond	0.445	0.246	0.118	0.0794	0.0577	0.0472
7	53	Gray	1.26	1.12	0.909			
8	52	Black	14.2	11.0				

NQ: not quantifiable.

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7

A randomized phase 2 study exploring the role of bevacizumab and a chemotherapy-free approach in HER2-positive metastatic breast cancer: the HAT study (BOOG 2008-03), a Dutch Breast Cancer Research Group trial

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ABSTRACT

To explore the role of bevacizumab and a chemotherapy-free approach, the authors evaluated the combination of bevacizumab, trastuzumab, and paclitaxel (HAT) and the regimen of trastuzumab and bevacizumab (HA) with the addition of paclitaxel after progression (HA-HAT) as first line treatment for patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer. In a non-comparative phase 2 trial, patients were randomized between HAT and HA-HAT. The primary endpoint was the progression free rate at 1 year (1-year PFR). In the HA-HAT group, progression free survival (PFS) was separately established for HA (PFS1) and HAT (PFS2). Eighty-four patients received HAT (n = 39) or HA-HAT (n = 45). The 1-year PFR was 74.4% (95% confidence interval [CI], 61.8-89.4%) and 62.2% (95% CI 49.6-89.4%) in the HAT and HA-HAT arms, respectively. The median PFS was 19.8 months (95% CI 14.9-25.6 months) in the HAT arm and 19.6 months (95% CI 12.0-32.0 months) in the HA-HAT arm. In the HA-HAT arm, the median PFS1 was 10.4 months (95% CI 6.2-15.0 months), and the median PFS2 was 8.2 months (95% CI 7.0-12.6 months). The number and severity of adverse events were comparable between the arms. Both HAT and HA-HAT have promising activity in patients with HER2-positive metastatic breast cancer. In particular, starting with only targeted agents and delaying chemotherapy is worth further exploration.

INTRODUCTION

Patients with metastatic breast cancer overexpressing human epidermal growth factor receptor 2 (HER2) have poor outcomes in the absence of HER2-targeted treatments [1,2]. The advent of HER2-targeting agents, such as trastuzumab and lapatinib, and, more recently, pertuzumab and trastuzumab-emtansine, has substantially improved outcomes [3-9]. Trastuzumab as a single agent yielded a progression free survival (PFS) of 3.5 to 3.9 months [10,11], but, particularly when combined with taxanes, trastuzumab-based regimens exert considerable antitumor activity. Consequently, until recently, the combination of trastuzumab with a taxane was standard first line therapy for these patients [3,4].

Despite these improvements, not all patients benefit from first line trastuzumab combined with chemotherapy and, eventually, most if not all patients experience resistance. In addition, the combination of trastuzumab and taxanes is accompanied by chemotherapy-induced toxicities, such as bone marrow suppression and neurotoxicity, impairing quality of life. For these reasons, new treatment options are needed to improve outcomes for this patient category. One strategy to do so is to enhance antitumor activity by adding novel agents. Another approach is to reduce the toxicity from treatment by exploring strategies in which chemotherapy is being withheld while maintaining efficacy.

Based on both preclinical and clinical data, bevacizumab might be a potentially useful drug to be added to trastuzumab and chemotherapy. Bevacizumab is a fully humanized monoclonal antibody against vascular endothelial growth factor (VEGF). In addition to playing a key role in angiogenesis, it is also thought that VEGF confers chemotherapy resistance [12]. In HER2-overexpressing breast cancer cell lines, VEGF production was enhanced, and results suggested that it was causally related to the more aggressive phenotype compared with HER2-negative tumors [12,13]. It is noteworthy that targeting both VEGF and HER2 inhibited tumor growth more than either agent alone in HER2-overexpressing preclinical models [13]. In a single arm phase 2 study, the combination of trastuzumab and bevacizumab revealed a median time to progression of 9.2 months (95% confidence interval [CI], 5.4-20.4 months), which compared favorably with the time to progression achieved with the combination of trastuzumab and paclitaxel [3,14].

In addition to trastuzumab, bevacizumab also enhances the antitumor effect of chemotherapy, as demonstrated in several tumor types; and there is mounting evidence that the added value of bevacizumab depends on the schedule and chemotherapeutic agents with which it is concomitantly received [15]. This may also be true for metastatic breast cancer, in which bevacizumab seems to enhance the activity of weekly paclitaxel to a greater extent than when combined with docetaxel every three weeks [16,17].

To investigate the potential value of bevacizumab in patients with HER2-positive metastatic breast cancer and to explore the feasibility of an upfront chemotherapy-free approach, we performed a randomized, non-comparative phase 2 trial. This design gave us the opportunity to test two experimental treatment arms in parallel, which is advantageous when several interesting regimens exist that warrant screening for antitumor activity in a phase 2 setting.

Randomization was used to reduce various types of bias, including patient selection and controlling for possible baseline imbalances across the treatment arms. This facilitates the choice which regimen emerging from a phase 2 study should warrant further investigation and be pursued in a phase 3 study [18,19]. We randomized patients between the combination of bevacizumab, trastuzumab, and weekly paclitaxel and a regimen consisting of a chemotherapy- free approach with upfront trastuzumab and bevacizumab treatment with the addition of paclitaxel at the time of disease progression.

PATIENTS AND METHODS

Study design

The HAT Study was an open-label, randomized, non-comparative phase 2 trial of concomitant trastuzumab, bevacizumab, and paclitaxel (HAT) versus trastuzumab and bevacizumab (HA) followed by the combination of trastuzumab, bevacizumab, and paclitaxel at progression (HA-HAT) as first line treatment in patients with metastatic breast cancer with HER2 overexpression (Figure 1). The primary objective was to establish whether HAT and/or HA-HAT show sufficient efficacy to be studied further. The primary endpoint was the progression free rate at 1 year after randomization (1-year PFR). Secondary endpoints included PFS, overall survival (OS), the best overall response, the objective response rate (ORR) (i.e. partial responses [PRs] and complete responses [CRs]), the clinical benefit rate (CBR) (i.e. CRs, PRs, and stable disease for >6 months), duration of response, safety, and tolerability.

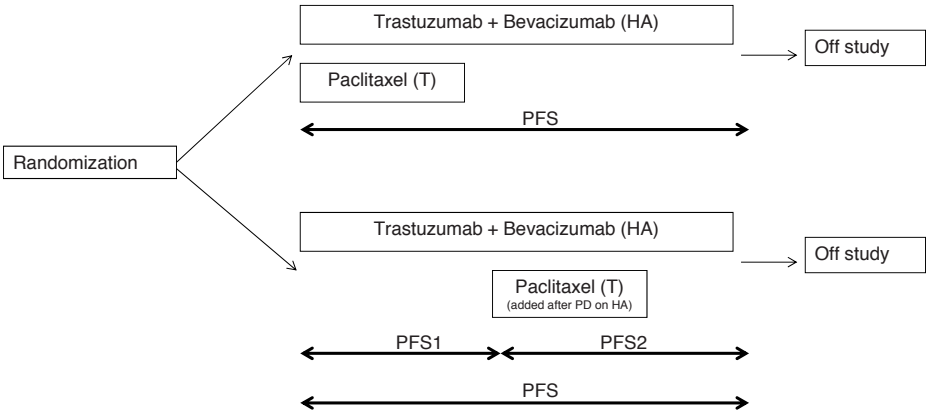

Figure 1. The study design is illustrated. Patients were randomized at a 1:1 ratio between combined bevacizumab, trastuzumab, and paclitaxel (HAT) (trastuzumab 6 mg/kg [the first dose was 8 mg/kg] plus bevacizumab 15 mg/kg every three weeks, both until progressive disease developed; and paclitaxel 90 mg/m^2 on days 1, 8, and 15 every four weeks for a maximum of six cycles) versus trastuzumab and bevacizumab (HA) plus HAT (HA-HAT) (doses of trastuzumab and bevacizumab were the same those in the HAT regimen with paclitaxel for a maximum of six cycles added at the time of disease progression). Progression-free survival (PFS) is illustrated separately for HA (PFS1) and HAT (PFS2).

The protocol was approved by an accredited medical research ethical committee (Netherlands Cancer Institute, Amsterdam). The trial was registered upfront in the Netherlands Trial Register (NTR1349) and was conducted in full accordance with the principles of the Declaration of Helsinki and local regulations. All participants provided written informed consent.

Patient Population

Patients who had measurable or evaluable locally recurrent or metastatic, HER2-positive breast cancer (LR/MBC) were eligible whenever they required first line chemotherapy. Prior endocrine therapy for metastatic disease was allowed. Positive HER2 status was defined as score of 3+ assessed by immunohistochemistry and/or gene amplification using in situ hybridization of the primary tumor or a metastatic lesion. Eligible patients had an Eastern Cooperative Oncology Group performance status 0 or 1; were aged ≥18 years; had adequate cardiac, hematologic, hepatic, and renal function; and had no known central nervous system metastases. Patients who had received trastuzumab in the adjuvant setting were eligible only when they had received at least ten months of therapy with trastuzumab and ≥6 months had elapsed since their last adjuvant administration of trastuzumab. Patients who had received (neo)adjuvant chemotherapy were only eligible if they had received their last dose ≥6months before randomization and their taxane-associated toxicities had resolved to less than grade 2.

Random Assignment and Treatment

Patients were randomized by the trial office of the Dutch Breast Cancer Research Group and were stratified by participating center at a 1:1 ratio between the HAT arm (trastuzumab 6 mg/kg [the first dose was 8 mg/kg] plus bevacizumab 15 mg/kg every three weeks and paclitaxel 90 mg/m^2 on days 1, 8, and 15 every four weeks) and the HA-HAT arm (with doses of trastuzumab and bevacizumab the same as in the HAT regimen and paclitaxel added at the time of disease progression). Paclitaxel was planned for six cycles, unless unacceptable toxicity or disease progression mandated earlier discontinuation. Both trastuzumab and bevacizumab were administered until disease progression (in the HA-HAT arm, this was defined as progression after the addition of paclitaxel), unacceptable toxicity, or withdrawal of consent. Adding endocrine therapy was not allowed for the duration of the study.

Study Assessments

Metastatic lesions were assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 at baseline and every twelve weeks thereafter, at the onset of clinical signs suggesting progression, or in case of premature discontinuation of study treatment. Local investigators performed tumor response assessments. Adverse events (AEs) were recorded at every cycle and for 30 days after the last receipt of any of the study drugs. AEs were graded according the National Cancer Institute-Common Toxicity Criteria version 3. Quality of life was assessed using the European Organization for

Research and Treatment of Cancer (EORTC) quality-of-life questionnaire (EORTC QLQ-C30, version 3) at baseline, every twelve weeks thereafter, and at the time of disease progression.

Statistical design

For the 1-year PFR, all patients in the HAT arm who were without progression within 1 year after randomization, all those in the HA-HAT arm who were without progression within 1 year after randomization and had received treatment with trastuzumab and bevacizumab, and all those who progressed during HA and started treatment with HAT but did not experience disease progression during this triple combination were considered "progression free" with respect to the primary endpoint. The rationale for the definition of 1-year PFR in the HAHAT group was that the objective of this study was to test whether it was possible to delay the initiation of chemotherapy without losing efficacy.

Secondary endpoints included PFS, measured as the time from randomization to the first documented disease progression or death from any cause in the HAT group. For the HA-HAT group, PFS was measured as the time from randomization to either the first documented disease progression after paclitaxel treatment was added to bevacizumab and trastuzumab or death from any cause. This means that documented disease progression during treatment with trastuzumab and bevacizumab (without paclitaxel) was ignored as an event if treatment with paclitaxel was started. In addition, for the HA-HAT group, a PFS1 and PFS2 were established, with PFS1 defined as the time from randomization to first documented disease progression or death from any cause and PFS2 defined as the time from starting treatment with HAT to subsequent documented disease progression or death from any cause. OS was measured as the time from randomization to either death from any cause or the date of last follow-up. For this study, a regimen that yielded a 1-year PFR of approximately 40% was deemed to be worth further exploration (p1). A Fleming one stage design was applied to both arms, with $p0 = 20\%$, $\alpha = 0.05$, and $\beta = 0.10$, indicating that, if a treatment yielded >14 of 42 patients were progression free 1 year after randomization, then the treatment was considered worth further exploration. For that reason, a total sample size of 84 patients (42 in both arms) was chosen. PFS, PFS1, PFS2, and OS were estimated using the Kaplan-Meier method for both arms. The estimated 1-year PFR and the median PFS, PFS1, PFS2, and OS values were calculated with their 95% CIs.

RESULTS

Patient population

Between April 2009 and September 2013, 84 patients were enrolled at 16 centers in the Netherlands and were randomly assigned to HAT or HA-HAT (Figure 2). A slight imbalance occurred in the treatment arm allocation (39 patients in the HAT arm and 45 patients in the HA-HAT arm) because of stratification for participating centers. One patient allocated to the HA-HAT group did not begin treatment because brain metastases became symptomatic before treatment was started. The cutoff

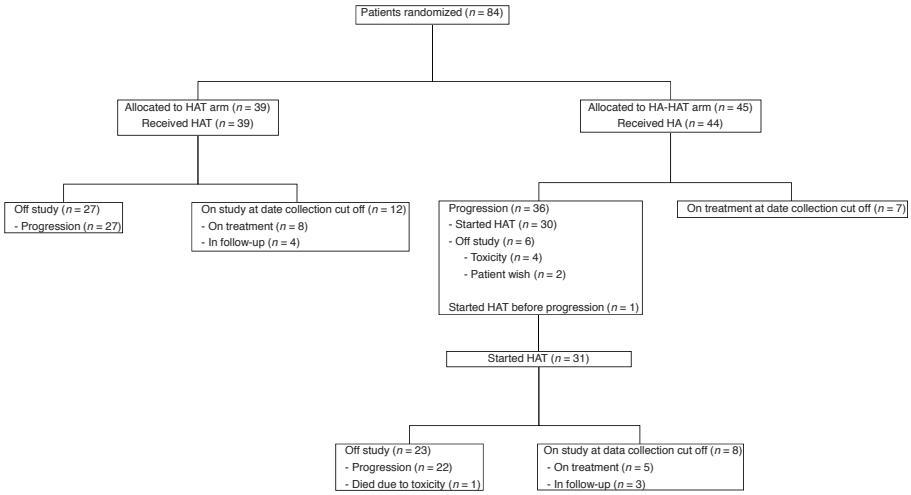

Figure 2. This is a Consolidated Standards of Reporting Trials (CONSORT) diagram of enrollment, intervention allocation, and follow-up in the current study.

HA: trastuzumab and bevacizumab; HAT: bevacizumab, trastuzumab, and paclitaxel.

date for data collection for the current analysis was one year after the last patient had been enrolled. The median follow-up for the entire group was 42 months. Patients' baseline characteristics were well balanced between the treatment arms (Table 1, [20]).

Efficacy

The 1-year PFR was 74.4% (95% CI 61.8-89.4%) in the HAT arm and 62.2% (95% CI 49.6-89.4%) in the HA-HAT arm. The median PFS for the HAT arm was 19.8 months (95% CI 14.9-25.6 months), and the median overall PFS in the HA-HAT arm was 19.6 months (95% CI 12.0-32.0 months) (Figure 3A). In the HA-HAT arm, the median PFS1 was 10.4 months (95% CI 6.2-15.0 months) (Figure 3B), and the median PFS2 was 8.2 months (95% CI 7.0-12.6 months). The median OS was 36.8 months (95% CI 29.1 months to not applicable [NA]) in the HAT group and 54.0 months (95% CI 37.4 months to NA) in the HA-HAT group (Figure 3C).

In the HAT arm, 39 patients started with HAT. The median number of paclitaxel cycles was six (range, 1-7 cycles; mean, 5.26 cycles). 29 of 39 patients completed all six cycles of paclitaxel, and six patients progressed during paclitaxel-based therapy.

In the HA-HAT arm, among those who experienced disease progression while receiving HA, paclitaxel was not added in six patients (17%) because of toxicity ($n = 4$; proteinuria, decrease of left ventricular ejection fraction, neuropathy, and/or fatigue) or patient request ($n = 2$). One patient had already started HAT based on growth of liver metastases but did not formally meet the criteria for progression

Table 1. Patient demographic and baseline characteristics.

	HAT (*n* = 39)	**HA-HAT (*n* = 45)**	**Total (*n* = 84)**
Age, median (range)	57 (35-76)	55 (29-80)	55 (29-80)
ECOG Performance status			
0, *n* (%)	27 (69)	33 (73)	60 (71)
1, *n* (%)	12 (31)	12 (27)	24 (29)
Hormone receptor status			
ER positive, *n* (%)	24 (62)	27 (60)	51 (61)
ER negative, *n* (%)	15 (38)	18 (40)	33 (39)
PR positive, *n* (%)	13 (33)	21 (47)	34 (40)
PR negative, *n* (%)	25 (64)	23 (51)	48 (57)
ER or PR positive, *n* (%)	25 (64)	28 (62)	53 (63)
ER and PR negative, *n* (%)	13 (33)	16 (36)	30 (36)
Involved tumor site			
Lung, *n* (%)	12 (31)	16 (36)	28 (33)
Liver, *n* (%)	16 (41)	22 (49)	38 (45)
Bone, *n* (%)	26 (67)	32 (71)	58 (69)
Number of different metastatic sites[a]			
1, *n* (%)	10 (26)	8 (18)	18 (21)
2, *n* (%)	15 (38)	19 (42)	34 (40)
3, *n* (%)	12 (31)	14 (31)	26 (31)
4, *n* (%)	2 (5)	4 (9)	6 (7)
Disease-free interval ≥12 months[b], *n* (%)	22 (56)	33 (73)	55 (56)
Primary metastatic disease, *n* (%)	16 (41)	12 (27)	28 (33)
Prior endocrine therapy			
Adjuvant, *n* (%)	12 (31)	16 (36)	28 (33)
Metastatic, *n* (%)	9 (23)	12 (27)	21 (25)
Prior adjuvant chemotherapy, *n* (%)	12 (31)	17 (38)	29 (35)
Prior adjuvant trastuzumab[c], *n* (%)	6 (15)	8 (18)	14 (17)

ECOG: Eastern Cooperative Oncology Group; ER: estrogen receptor; PR: progesterone receptor.
[a] Different metastatic sites included lung, liver, bone and other.
[b] The disease-free interval was defined as the time from histologic diagnosis of primary breast cancer to the diagnosis of locally recurrent or metastatic disease.
[c] The receipt of prior adjuvant trastuzumab was based on the assumption that all patients who received with adjuvant treatment for human epidermal growth factor 2-positive disease after August 2005 also received with adjuvant trastuzumab (see De Munck et al, 2011 [20]).

according to RECIST. In total, 31 patients started with HAT. The median number of paclitaxel cycles was six (range, 1-6 cycles; mean 4.68 cycles). 22 of 31 patients completed all six cycles of paclitaxel, and three patients progressed during paclitaxel-based therapy.

The investigator-assessed ORR (i.e. CRs plus PRs) was 58% in the HAT arm and 44% in the HA-HAT arm during HA. Among patients in the HA-HAT group who experienced disease progression while receiving HA, the addition of paclitaxel induced objective responses in 50%. The CBR was 87% in

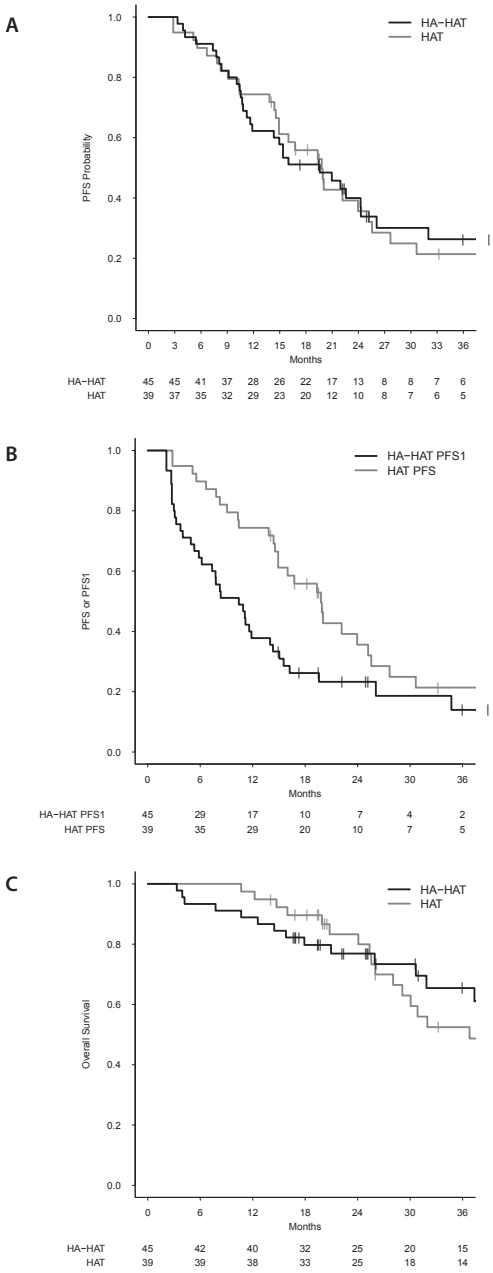

Figure 3. Kaplan-Meier survival estimates are shown. (A) Estimated progression free survival (PFS) is illustrated in patients who received concomitant trastuzumab, bevacizumab, and paclitaxel (HAT arm) versus those who received trastuzumab and bevacizumab (HA) followed by HAT at progression (HA-HAT arm). (B) Estimated PFS is compared between patients who received only HA in the HA-HAT arm (PFS1) versus those who received concomitant HAT. (C) Estimated overall survival is illustrated for patients in the HAT arm versus those who received HA followed by HAT at progression (HA-HAT arm).

the HAT arm and 58% in the HA-HAT arm during HA. Among patients in the HA-HAT group who had disease progression during HA, adding paclitaxel led to a clinical benefit in 62% of patients. In the HAT arm, among patients who had an objective response, the median response duration was 22.2 months (95% CI 16.0 months to NA). In the HA-HAT arm, among those who had an objective response who received only trastuzumab and bevacizumab ($n = 19$), the median response duration was 26.1 months (95% CI 22.6 months to NA). Baseline characteristics were comparable between patients with a PFS1 <24 months ($n = 38$) and patients with a PFS1 >24 months ($n = 7$) (data not shown).

Toxicity

The safety population, defined as patients who had received at least one administration of study drug(s), included 83 patients. Grade ≥3 AEs were reported in 84% of patients. Most grade ≥3 AEs were attributable to bevacizumab, with grade ≥3 hypertension and proteinuria in 36 and 13% of patients, respectively, and with no differences between treatment arms. The toxicity profiles of the two regimens during the whole period of study treatment were comparable (Table 2). Neutropenia, febrile neutropenia, infection, peripheral neuropathy, stomatitis, and diarrhea were reported less frequently during HA treatment than during HAT treatment. One patient in the HA-HAT arm who had been receiving treatment with bevacizumab for four months died from a perforation of a sigmoid diverticulum shortly after starting paclitaxel.

Quality of life

Quality-of-life questionnaires were collected every twelve weeks until the end of treatment. Responses to the EORTC QLC-C30 questionnaire were available from 73 patients (87%) at baseline, from 61 at twelve weeks, and from 23 after first progression. There were no relevant differences in reported quality of life when HAT treatment in the HAT arm was compared with HA treatment in the HA-HAT arm (data not shown).

DISCUSSION

In this randomized, non-comparative phase 2 study in which two experimental treatments were tested in parallel, both HAT and HA-HAT were active first line treatment regimens in patients with HER2-positive LR/MBC, yielding a 1-year PFR (the primary endpoint of the study) of 74.4 and 62.2%, respectively. The median PFS was 19.8 months in the HAT arm and 19.6 months in the HA-HAT arm, with durable OS in both arms. This implies that both treatments have met the predefined criteria for further study. On the basis of comparison with the landmark trial of Slamon et al, data from the current study suggest that bevacizumab strongly enhances the antitumor activity of combined paclitaxel and trastuzumab [3].

Our results from the addition of bevacizumab to combined paclitaxel and trastuzumab have to be compared with those from the 'Study of Avastin [Bevacizumab] in Combination With Herceptin [Trastuzumab]/Docetaxel in Patients With HER2-Positive Metastatic Breast Cancer' (AVEREL) trial, a

Table 2. Summary of main adverse events.

	All grades			**grade 3 and higher**		
	HAT arm	**HA-HAT arm**		**HAT arm**	**HA-HAT arm**	
	during HAT (*n* = 39)	**during HA (*n* = 44)**	**during HAT (*n* = 31)**	**during HAT (*n* = 39)**	**during HA (*n* = 44)**	**during HAT (*n* = 31)**
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
Nausea	18 (46.2)	18 (40.9)	13 (41.9)	-	-	1 (3.2)
Vomiting	7 (17.9)	10 (22.7)	5 (16.2)	1 (2.6)	-	1 (3.2)
Stomatitis	12 (30.8)	9 (20.5)	10 (32.3)	2 (5.1)	-	-
Diarrhea	19 (48.7)	12 (27.2)	15 (48.4)	3 (7.7)	-	3 (9.7))
Edema	12 (30.8)	10 (22.7)	4 (12.9)	-	-	-
Infection	26 (66.7)	13 (29.5)	14 (45.2)	6 (15.4)	2 (4.5)	6 (19.4)
Fatigue	32 (82.1)	27 (61.4)	23 (74.2)	2 (5.1)	-	3 (9.7)
Neutropenia	12 (30.8)	1 (2.3)	13 (41.9)	8 (20.5)	-	5 (16.1)
Febrile neutropenia	1 (2.6)	-	-	1 (2.6)	-	-
Peripheral neuropathy	28 (71.8)	10 (22.7)	17 (54.8)	1 (2.6)	1 (2.3)	-
Hypertension	29 (74.4)	25 (56.8)	11 (35.5)	16 (41.0)	13 (29.5)	5 (16.1)
Proteinuria	13 (33.3)	12 (27.2)	7 (22.6)	4 (10.3)	6 (13.6)	4 (12.9)

phase 3 study in patients with HER2-positive LR/MBC who received docetaxel and trastuzumab with or without bevacizumab in which no significant difference in median PFS was observed between treatment arms (PFS 16.5 vs. 13.7 months, respectively) [21]. It is noteworthy that docetaxel every three weeks was used in that study, whereas preclinical evidence indicates that not every chemotherapeutic drug will have its efficacy enhanced by the addition of a VEGF-targeting drug. Paclitaxel is one of the drugs which antitumor activity is augmented when combined with bevacizumab [15]. Accordingly, in patients with HER2-negative LR/MBC, the absolute PFS benefit from bevacizumab added to weekly paclitaxel over paclitaxel alone in the Eastern Cooperative Oncology Group 2100 study (PFS 11.8 vs. 5.9 months; hazard ratio 0.60; p <0.001) was greater than the gain achieved from bevacizumab added to docetaxel versus docetaxel alone, as reported in the 'Avastin and Docetaxel' (AVADO) study (PFS 10.1 vs. 8.2 months; hazard ratio 0.77; 95% CI 0.64-0.93) [16,17], suggesting greater activity of bevacizumab when added to weekly paclitaxel than when added to docetaxel every three weeks.

Along with the AVEREL study, two other (although much smaller), single-arm phase 2 trials have investigated the efficacy and safety of adding bevacizumab to combinations of trastuzumab and chemotherapeutic agents in the first line treatment of patients with advanced, HER2-positive breast cancer [22,23]. Those two studies combined trastuzumab and bevacizumab with vinorelbine and

capecitabine, respectively, and demonstrated a lower median PFS compared with that in our study (PFS 9.9 and 14.4 months, respectively). The study on vinorelbine was closed early because of toxicity. The study on capecitabine met the primary endpoint of that study, although the combination with capecitabine is not standard first line treatment for LR/MBC.

In the adjuvant setting, the 'Bevacizumab and Trastuzumab Adjuvant Therapy' (BETH) trial, a phase 3 study of the addition of bevacizumab to a standard regimen with docetaxel, carboplatin, and trastuzumab, did not demonstrate a benefit from the addition of bevacizumab [24]. Recently, phase 2 neoadjuvant trials demonstrated high pathologic complete response rates (range 54-64%) when bevacizumab was added to standard neoadjuvant regimens [25,26]. However, the results from those perioperative studies cannot be extrapolated to the treatment of metastatic disease.

Another way to improve outcome is by reducing treatment-induced toxicity. Therefore, we also explored the option of delaying the start of chemotherapy by beginning with trastuzumab and bevacizumab and adding paclitaxel only at the time of disease progression. In that analysis, we demonstrated that a significant proportion of patients who received treatment with monoclonal antibodies alone could achieve a durable PFS, i.e. a median PFS1 of 10.4 months and a median response duration of 26.1 months among those who had an objective response to treatment with trastuzumab and bevacizumab only. The median PFS1 is in line with the previously mentioned phase 2 study of combined trastuzumab and bevacizumab, which demonstrated a median time to progression of 9.2 months (95% CI 5.4-20.4 months) [14]. By comparison, studies on trastuzumab monotherapy as first line treatment for patients with HER2-positive LR/MBC reported a median PFS of only 3.5 to 3.9 months [10,11], stressing that bevacizumab probably adds to the activity of trastuzumab in an advanced setting. To the best of our knowledge, no other studies have been published on a chemotherapy-free approach with the combination of trastuzumab and bevacizumab. Several non-first line phase 2 studies in the metastatic setting have been performed on dual HER2 blockade using trastuzumab in combination with either pertuzumab or lapatinib, all of which demonstrated a median PFS of ≥6 months [27-30].

It is noteworthy that, although ORR, CBR, and PFS values appeared to be lower when we started treatment with trastuzumab and bevacizumab versus combined trastuzumab, bevacizumab, and paclitaxel, adding chemotherapy at the time of disease progression in the HA-HAT arm yielded a similar total PFS compared with that in the HAT arm. Furthermore, in a substantial number of patients who progressed while receiving trastuzumab and bevacizumab, adding paclitaxel re-induced objective responses. Currently, follow-up duration is too short to draw conclusions on OS results.

The toxicity profiles of both regimens seem to be in favor of beginning treatment with bevacizumab and trastuzumab only and delaying the start of cytotoxic chemotherapy, thereby postponing chemotherapy-associated toxicities. The large majority of grade ≥3 AEs in this study were attributable to treatment with bevacizumab and mainly consisted of hypertension and proteinuria, which, overall, were easily manageable. Bevacizumab-related toxicities, as demonstrated in other studies, do not result in decreased quality of life for most patients [31]. One patient died from a spontaneous

bowel perforation, which is a known complication of bevacizumab, although its reported incidence in bevacizumab-treated patients with advanced breast cancer is <1% [32]. We did not observe any suggestion of a difference in quality of life between the two groups. Less therapy-associated toxicity during HA compared with HAT might have led to decreased quality of life during HAT. Conversely, the response rates under HA were lower than under HAT, which might lead to decreased quality of life during HA. These compensatory explanations might have outweighed each other. Furthermore, the number of returned questionnaires was quite low for unknown reasons. This hampers our ability to draw firm conclusions.

Adding endocrine therapy to the combination of trastuzumab and bevacizumab was not allowed in our study. The results might have been even better if endocrine therapy had been added for patients with hormone receptor positive disease.

With publication of the 'Clinical Evaluation of Pertuzumab and Trastuzumab' (CLEOPATRA) trial indicating a median overall survival benefit of 15.7 months for the combination of trastuzumab, pertuzumab, and docetaxel compared with combined trastuzumab and docetaxel, this regimen has become the standard first line therapy for patients with HER2-positive metastatic breast cancer [9]. When taking into account the solid phase 3 data from the CLEOPATRA trial and the favorable toxicity profile of pertuzumab, compared with bevacizumab, it is unlikely that further phase 3 trials on the combination of bevacizumab, trastuzumab, and chemotherapy will be performed. The results from our study, however, indicate that the concept of starting with a chemotherapy-free approach using only biologics is an approach that deserves further study. New trials of chemotherapy-free treatments should incorporate newer HER2-targeted agents, such as pertuzumab, trastuzumab-emtansine, novel agents, and also endocrine therapy in hormone receptor positive patients. Preclinical evidence suggests that the combination of trastuzumab, pertuzumab, and bevacizumab is very active [33]. Obviously, randomized controlled trials investigating combinations of targeted agents (with and without bevacizumab) and endocrine therapy (in hormone receptor positive patients) followed by the addition of chemotherapy at the time of progression as first line treatment for patients with HER2-positive metastatic breast cancer would be interesting. Results on the efficacy of combined trastuzumab and pertuzumab without chemotherapy will follow from the ongoing PERNETTA trial (a randomized phase 2 trial of pertuzumab in combination with trastuzumab with or without chemotherapy, both followed by trastuzumab emtansine, in patients with HER2-positive MBC; clinicaltrials.gov no. NCT01835236) and GCC 1303 trial (trastuzumab and pertuzumab with hormone therapy or chemotherapy in women aged ≥60; clinicaltrials.gov no. NCT02000596).

CONCLUSION

In summary, both the HAT combination and the sequential treatment starting with a chemotherapy-free approach using HA followed by adding paclitaxel at progression seem to be active regimens in patients who have HER2-positive metastatic breast cancer. The results from this study strongly suggest that bevacizumab adds clinically relevant antitumor activity to certain trastuzumab-based regimens. In particular, a sequential approach with bevacizumab and trastuzumab and delaying the start of cytotoxic chemotherapy is worth further exploration in studies that incorporate newer HER2-targeted agents along with endocrine therapy in patients who have hormone receptor positive disease.

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Denosumab in breast cancer treatment

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ABSTRACT

The bone is the most common site to which breast cancer metastasises. Recently, denosumab, a fully human monoclonal antibody that binds to receptor activator of nuclear factor kappa-B ligand (RANKL) has been developed as a new targeted bone therapy. In a large randomized phase 3 study with a head-to-head comparison of denosumab to zoledronic acid in patients with bone metastases of breast cancer, denosumab significantly delayed the time to first skeletal related event. In the adjuvant setting denosumab significantly increased bone mineral density compared to placebo in a phase 3 study in patients treated with aromatase inhibitors. Preclinical data suggest an effect of denosumab on tumor growth and even on carcinogenesis. This review describes the current indications for denosumab in the various settings of breast cancer treatment, with special attention for efficacy, short and long term toxicity and other relevant issues for clinical practice. Furthermore possible and necessary future research questions are proposed.

INTRODUCTION

The bone is the most common site to which breast cancer metastasises (up to 70%) [1]. Although bone metastases can be asymptomatic for years, the occurrence of skeletal related events (SREs), which are defined as pathological fractures, necessity for radiation or surgery due to (threaten) spinal cord compression or hypercalcemia due to bone metastases, has a negative impact on patients' quality of life and generally portends a worse prognosis [2,3]. Since the life expectancy of metastatic breast cancer patients has increased over the years, preventing SREs have become a major challenge in optimizing the care for these patients.

For years, bisphosphonates have been the cornerstone for the prevention and management of SREs, especially in patients with metastatic breast cancer. Recently, denosumab, a fully human monoclonal antibody that binds with high affinity and high specificity to receptor activator of nuclear factor kappa-B ligand (RANKL), has been developed as a new targeted bone therapy [4]. In this review we will describe denosumab within the breast cancer population in more detail, with special attention for efficacy, short and long term toxicities and other issues relevant for clinical practice. Furthermore, possible and necessary research questions are proposed.

The RANK-RANKL system in bone metastases

In physiological circumstances, the bone undergoes constant remodeling, resulting from the tightly controlled balance between osteoblast and osteoclast activity. The ratio between RANKL and its physiological antagonist osteoprotegerin is crucial in maintaining this balance and thereby controls bone health. RANKL is a member of the tumor necrosis factor (TNF) superfamily and is produced by the osteoblasts. After releasing into the bone micro-environment, RANKL binds to its receptor RANK, which is expressed by immature osteoclasts and initiates maturation to activating osteoclasts by signaling through the nuclear factor kappa-B and Jun N-terminal kinase pathways [4]. As a consequence of RANKL production, bone resorption will occur. On the contrary, excessive bone resorption is prevented by the decoy receptor osteoprotegerin, also produced by osteoblasts, that binds to RANKL thereby preventing its binding to RANK [5]. The extent of bone resorption is thus regulated via osteoclast formation and activity through the coordinated expression of RANKL and osteoprotegerin.

In patients with a solid tumor and bone metastases, increased expression of both RANKL and osteoprotegerin can occur [6]. This increased expression of RANKL or osteoprotegerin is induced by osteotropic factors, produced by cancer cells, such as parathyroid hormone, parathyroid hormone related peptide, 1,25-dihydroxyvitamin D3, and prostaglandins. The local balance between RANKL and osteoprotegerin determines whether bone metastatic lesions have a lytic or blastic appearance on radiographic images [6]. A disproportional high level of RANKL will lead to lytic lesions, while a disproportional high level of osteoprotegerin will lead to blastic lesions.

Bone metastases in breast cancer are predominantly osteolytic [7]. The reason for that is that

parathyroid hormone related peptide is probably the most important factor produced by breast cancer cells, which leads to increased RANKL expression that activates osteoclasts causing enhanced bone resorption [8]. Besides bone resorption, activated osteoclasts also activate release of growth factors from the bone matrix that stimulate tumor growth and bone destruction [9]. This reciprocal interaction between breast cancer cells and the bone micro-environment results in a vicious circle that increases both bone destruction and the tumor burden (Figure 1).

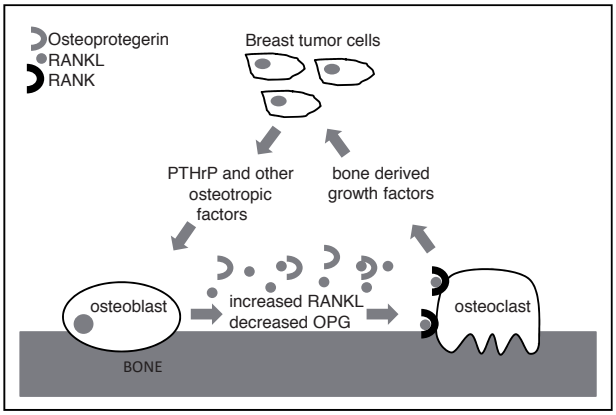

Figure 1. The vicious circle of bone metastases in breast cancer. Breast cancer cells (BC) produce parathyroid hormone related peptide (PTHrP) and other osteotropic factors which lead to increased expression of RANKL and decreased expression of the decoy receptor osteoprotegerin (OPG) by osteoblasts, resulting in increased binding of RANKL to the RANK receptor, which is expressed by immature osteoclasts. Increased RANK-RANKL interaction leads to increased activation of osteoclasts which has a direct effect on bone resorption (lytic lesions), but also activates production and release of bone derived growth factors, which stimulate tumor growth.

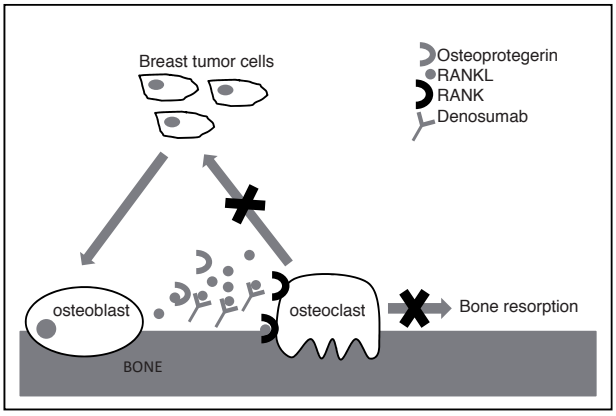

Figure 2. Working mechanism of denosumab. Denosumab specifically binds to RANKL. Like osteoprotegerin, this prevents binding of RANKL to RANK on osteoclasts and inhibits the downstream signaling that induces osteoclast activation, leading to reduction of bone resorption and disruption of the vicious circle of bone metastases.

Denosumab

Denosumab is a fully human monoclonal antibody (IgG2) that specifically binds to human RANKL. Like osteoprotegerin, this prevents activation of the receptor RANK on osteoclasts and inhibits the downstream signaling that induces osteoclast activation, migration and differentiation and so reduces bone resorption [4] (Figure 2).

Denosumab has been developed for subcutaneous (sc) administration, and shows a non-linear, dose-dependent pharmacokinetic, so that the drug is detected in peripheral blood samples within one hour and up to nine months after one single dose [10,11]. This leads to a mean elimination half-life of denosumab of 28 days and a steady state which is reached by six months (Amgen Inc. Denosumab: full prescribing information. http://pi.amgen.com/united_states/xgeva/xgeva_pi.pdf (accessed 22 June 2012)).

Compared to denosumab, bisphosphonates differ greatly in terms of the administration route as well as the pharmacokinetics. Although oral administration of bisphosphonates is possible, the poor absorption makes intravenous administration necessary to ensure sufficiently high dose of bisphosphonates to prevent SREs in the advanced setting. Once in the circulation, bisphosphonates are directly cleared from the blood by binding to hydroxyapetite in the bone matrix. As a consequence, 24h after a single dose of zoledronic acid, less than 1% can be measured in the blood.

DENOSUMAB IN ADVANCED BREAST CANCER

Efficacy and tolerability of denosumab in advanced breast cancer patients

Denosumab was originally approved for the treatment of postmenopausal osteoporosis as well as bone loss in patients receiving adjuvant aromatase inhibitor therapy for early breast cancer or androgen deprivation therapy for non-metastatic prostate cancer. Recently, three large randomized phase 3 studies with a head-to-head comparison of denosumab to zoledronic acid in patients with bone metastases of breast, prostate and all other solid tumors (and multiple myeloma), showed denosumab to be non-inferior to zoledronic acid for preventing first on-study SREs [12-14]. In the study on breast cancer patients with bone metastases (n = 2,046), denosumab (120 mg every four weeks) significantly delayed time to first on-study SRE by 18% (HR 0.82; 95% CI 0.71-0.95, p = 0.01 (superiority)) compared to zoledronic acid (4 mg every four weeks). The median time to first on study SRE was 26.4 months for the zoledronic acid group and had not yet been reached for the denosumab group. Denosumab was also superior compared with zoledronic acid in delaying time to first and subsequent on-study SREs (HR 0.77; 95% CI 0.66-0.89, p = 0.001) [14]. No differences in overall survival or disease progression were observed between the two study groups. Although the study was initially designed as a non-inferiority study with time to first on-study SRE as primary endpoint, superiority criteria were prespecified in the protocol [14]. In general the tolerability of denosumab was acceptable and quite well comparable with the side effects of zoledronic acid. Acute-phase reactions characterized by fever, myalgia and bone pain within the first three days of treatment were observed in a slightly lower percentage of patients treated with

denosumab (8.7%) compared to those treated with zoledronic acid (20.0%) [12-14]. Hypocalcemia was more frequent with denosumab than with zoledronic acid (9.6% vs. 5.0%), despite the fact that all patients were encouraged to take calcium and vitamin D supplements. Although, most episodes of hypocalcemia were generally mild, easily managed and rarely required hospital admission, fatal cases of hypocalcemia have been reported. Therefore it is advised to correct pre-existing hypocalcemia prior to the start of denosumab treatment and to monitor calcium levels while on treatment and administer calcium, magnesium, and vitamin D if necessary (Amgen Inc. Denosumab: full prescribing information. http://pi.amgen.com/united_states/xgeva/xgeva_pi.pdf (accessed 22 June 2012)). Moreover, the calcium levels should be monitored more frequently when denosumab is administered in combination with calcium lowering drugs or in patient with pre-existent impaired renal function due to an increased risk of hypocalcemia.

The most important adverse event associated with the use of bisphosphonates in oncology is osteonecrosis of the jaw (ONJ). This seems also the case for denosumab. In the three large prospective phase 3 studies among patients with metastatic bone disease, comparing denosumab with zoledronic acid, regular oral assessment was incorporated [12-14]. In all three studies, a similar proportion of denosumab-treated and zoledronic acid-treated patients developed ONJ (1.8 vs. 1.3%, respectively) [15]. In total, 89 confirmed cases of ONJ occurred among 5,677 patients who received one or more doses of either agent. However, the median time period of patient participation in the study was only approximately two years. Since studies have shown that ONJ can still occur after a longer duration of bisphosphonate treatment and that the incidence seems to increase with time to exposure [16,17], the low percentages of ONJ described by Saad et al. [15] could be an underestimation. As a consequence, longer follow-up is necessary to draw final conclusions.

ONJ is a serious complication which is a real challenge for the physician who has seldom experience with this rare event. Detailed recording of the severity and the course of the ONJ by Saad et al. [15] gave relevant information on the true incidence and outcome of this condition. ONJ was diagnosed as early as four months after the start of the bone-targeting agents or at 30 months at the latest. Over half of the patients (54%) were treated conservatively using analgesics or antibiotics. Approximately 41% of the patients underwent limited surgical intervention (sequestrectomy, extraction or superficial debridement) with only 4 of 89 patients (4%) requiring resection of the necrotic bone. The symptoms lasted a median duration of 14 months and resolved (defined as mucosal coverage of the exposed bone) in 36% of patients (29.7% for zoledronic acid and 40.4% for denosumab) [15].

In the search for potential risk factors, tooth extraction (while on treatment) was a major risk factor for developing ONJ among patients treated with denosumab. As a consequence, it is currently advised to perform an oral examination and appropriate preventive dentistry prior to the initiation of denosumab treatment as well as periodically during denosumab treatment. Patients must be informed about oral hygiene practices and invasive dental procedures should be avoided whenever possible (Amgen Inc. Denosumab: full prescribing information. http://pi.amgen.com/united_states/xgeva/xgeva_pi.pdf (accessed 22 June 2012)).

A key difference between the safety profiles of denosumab and zoledronic acid is the lack of renal toxicity of denosumab. Since denosumab is a monoclonal antibody, clearance is likely to occur via the reticulo-endothelial system, resulting in normal dosing of denosumab in the presence of renal (or hepatic) failure. In the randomized phase 3 trial comparing denosumab with zoledronic acid in advanced breast cancer patients the incidence of renal toxicity was less frequent with denosumab than with zoledronic acid (adverse events potentially associated with renal toxicity 4.9 vs. 8.5%, respectively and serious renal adverse events 0.9 vs. 1.5%, respectively). Moreover, in patients with baseline renal clearance between 30-60 ml/min, the renal adverse events were only 5.9% with denosumab compared to 20% with zoledronic acid [14]. Denosumab can thus be administered in case of renal impairment and can be administered concomitantly with nephrotoxic chemotherapy. Moreover, it does not require dose-adjustment for reduced creatinine clearance and obviates the need for assessment of the patient's renal function before each denosumab dose (Amgen Inc. Denosumab: full prescribing information. http://pi.amgen.com/united_states/xgeva/xgeva_pi.pdf (accessed 22 June 2012)).

Remaining issues to be addressed

From the currently published data, denosumab as a bone modifying agent seems to be a valuable drug in the aim to prevent SREs in breast cancer patients suffering bone metastases. Nevertheless, many questions still remain.

The first issue concerns the optimal duration of denosumab treatment. Until now, the phase 3 trials in patients with bone metastases examined a monthly dose of 120 mg denosumab with the longest median duration on treatment 17 months [14]. A possible concern could be the risk of long term toxicity. Potential benefits should be weighed against potential side effects from continuing denosumab beyond two years of treatment until more mature data are available.

Second, the question what the optimal treatment is after developing a SRE while on denosumab treatment. In the current studies, denosumab has just been continued, however, it could be argued that in these patients the activated RANK/RANKL system is not repressed well enough. Patient-tailored treatment (see below) might be an option to further explore this possibility. Alternatively, other mechanisms than the activated RANK/RANKL pathway could be responsible for developing SREs, Src activation being one of these. Src is a membrane-associated non-receptor tyrosine kinase with a pivotal role in all kinds of cellular functions and also plays an essential role in the activation of osteoclasts [18]. Dasatinib, an oral tyrosine kinase inhibitor, which targets among others Src tyrosine kinase has shown potent anti-resorptive bone activity as well as inhibition of osteoclast proliferation in rat models. Moreover, two trials are currently ongoing to evaluate the role of dasatinib either alone or in combination with zoledronic acid among breast cancer patients with bone metastases (http://www.clinicaltrials.gov. Identifiers NCT00566618 and NCT00410813). Thus, further study is needed to find the optimal treatment once patients have developed SREs while on monthly denosumab treatment.

Third, the issue whether denosumab can be used directly after pre-treatment with bisphosphonates.

Breast cancer patients with bone metastases are nowadays almost always treated with intravenous bisphosphonates. In case renal function deteriorates, a switch to denosumab could theoretically be considered. Since both drugs have a long elimination half-life, the question is whether a switch from a bisphosphonate to denosumab would lead to increased toxicity which for example has been described for patients treated with intravenous pamidronate who were switched to intravenous zoledronic acid (e.g. increased percentages of ONJ) [17].

Recently the results of the two year open label extension treatment phase of the earlier mentioned phase 3 trial in bone metastatic breast cancer were reported [19]. All patients who remained on treatment after a median time on study in the phase 3 trial of 17 months were offered open-label denosumab in a prespecified two year extension treatment phase ($n = 752$). Eighty-nine per cent of the patients chose to receive open label denosumab. Patients who did not participate in the open-label treatment were followed for survival every twelve weeks for up to two years after their last dose of investigational product in the double-blinded treatment phase. The total median cumulative denosumab exposure was 19.3 months (range 0.9-59.8 months). Adverse events were comparable between the two groups. An additional 20 patients in the denosumab group and 18 patients in the group, who received zoledronic acid in the double-blinded phase of the study, reported osteonecrosis of the jaw, resulting in a cumulative incidence for the entire study period of 4.7% for denosumab treated patients and 3.5% for patients treated with denosumab after treatment with zoledronic acid. Overall survival was similar between groups over the entire study.

Based on these data, switching from bisphosphonates to denosumab treatment seems to be safe and well tolerated, although the number of patients treated are small and the treatment duration was only short. Therefore, abstention in switching patients from bisphosphonates to denosumab is advised, until more mature safety and efficacy data are available in this particular setting.

Finally, the theoretical concern that denosumab could be associated with an increased risk of opportunistic infections. Besides the expression in bone, RANKL and RANK are also expressed by activated T- and B-lymphocytes, and dendritic cells and is thereby involved in the regulation of the immune system [20,21]. Since denosumab binds to RANKL in general, it could be hypothesised that inhibition of RANKL expressed by cells of the immune system could alter the immune function. Preclinical data have indeed suggested that the RANKL-RANK interaction modifies immune responses in specific tissues such as the skin. A recent meta-analysis of three randomized clinical trials among postmenopausal women with low bone mass treated with denosumab or placebo (total number of patients: $n = 996$) reported a significantly increased risk of infections, that required hospitalisation (OR 4.5; $p = 0.03$) for those women treated with denosumab [22-24]. This meta-analysis did not incorporate safety data on the large pivotal FREEDOM trial, which randomized postmenopausal women with low bone mass to denosumab or placebo [25]. In a post hoc analysis, the incidence, type, and details in individual subjects ($n = 7,808$ women) of adverse events of infections were investigated [26]. The overall incidence as well as percentage of serious infections was similar between the placebo and denosumab groups (54.4 vs. 52.9%; $p = 0.17$ and 3.4 vs. 4.1%; $p = 0.14$ respectively). However,

serious adverse events in terms of cellulitis and erysipelas resulting in hospitalisation occurred more frequently with denosumab compared to placebo, although the number of events was low (n = 15 (0.4%) vs. n = 3 (<0.1%); p <0.05) [26].

In the three large prospective phase 3 studies among patients with metastatic bone disease, comparing zoledronic acid with denosumab, in which the concentration of denosumab is much higher (120 mg every month continuously) than compared to doses given for osteoporosis, special attention was paid to the occurrence of infectious adverse events. In the trial with advanced breast cancer patients there were no differences between the zoledronic acid and denosumab group in percentage of infectious adverse events (48.8 vs. 46.4, respectively) as well as percentage of infectious serious adverse events (8.2 vs. 7.0, respectively), however skin infections were not separately mentioned [14]. The same holds through for the other two phase 3 trials [12,13]. Taken together, there is no increased risk of infections in general in patient with bone-metastatic solid tumors treated with denosumab 120 mg monthly compared to zoledronic acid. However, based on the results of the larger series with lower doses of denosumab in the treatment of osteoporosis the risk of serious infectious of the skin at higher doses is still a matter of uncertainty.

Patient-tailored treatment

Currently, the main aim of using denosumab in breast cancer patients with bone metastases is to prevent SREs by suppressing osteoclast activity, thereby impairing bone resorption. Although complete inhibition of osteoclast activity might be considered most effective in preventing SREs, a patient-tailored denosumab treatment with a more controlled inhibition of osteoclast activity seems far more desirable. By such an approach, less denosumab might be necessary for many patients thereby ameliorating adverse events and decreasing costs.

Non-invasive measuring of bone resorption markers, such as N-telopeptide and C-telopeptide, might be useful tools both for identifying patients at risk of developing SREs and for monitoring response to therapy. The levels of bone markers in patients with breast cancer seem to correlate with response to bone modifying agents, the risk of developing SREs, bone metastases progression and prognosis [27-29]. Moreover, among breast cancer patients normalization of bone markers with zoledronic acid within 111 days of follow-up reduces the risk of SREs (RR = 0.51; 95% CI 0.33-0.78) and overall survival (RR = 0.52; 95% CI 0.34-0.78) compared to those patients in which bone markers did not normalise [30].

Since the bone resorption marker urinary N-telopeptide (uNTx) normalized for urinary creatinine (uNTx/Cr) correlates with osteoclast activity, this marker has been used to provide the optimal dose of denosumab for clinical use. Model-based pharmacodynamic analyses from six clinical trials investigating denosumab for the prevention of SREs in patients with cancer and bone metastases, came up with denosumab 120 mg administered every 4 weeks as the standard dose because this resulted in a normalizations of uNTx/Cr (i.e. <50 nM/mM) in over 95% of the subjects [31]. Interestingly, approximately 56% of the subjects with denosumab 30 mg administered every four weeks had an undetectable low uNTx/Cr (i.e. <1 nM/mM), compared to 64% for those administered denosumab

120 mg. This suggests that in more than half of the patients a 30 mg dose is enough to inhibit osteoclast activity sufficiently.

Moreover, in a study in which 111 patients with breast, prostate or other solid cancers were randomized to subcutaneous denosumab 180 mg every four weeks or every twelve weeks, or continued intravenous bisphosphonate therapy (every four weeks) for 25 weeks, all patients had elevated uNTx levels at screening despite ongoing treatment with intravenous bisphosphonates [32]. After twelve weeks, the proportion of patients achieving normalized uNTx levels (defined as an uNTx/Cr <50 nM/mM) was significantly greater among individuals who received denosumab every four weeks (78%; p <0.001) and every twelve weeks (64%; p ≤0.005) than in those treated with bisphosphonates (29%) [32]. These data suggest that at least for a substantial proportion of the patients (64%) a longer (twelve weeks) interval in between denosumab administrations is feasible. Finally, the duration of suppression was dose dependent. In a phase 1 study in cancer patients with bone lesions, a single subcutaneous dose of denosumab (0.1, 0.3, 1.0 and 3.0 mg/kg) led to a decrease in uNTx/Cr levels of up to 58% in patients with breast cancer one day after dosing. Administration of doses of >0.3 mg/kg provided sustained reductions throughout the study period of twelve weeks [11]. Taken together these data plead for patient-tailored approach with the use of biochemical markers to monitor the degree of bone resorption to guide denosumab treatment rather than treating all patients with the currently licensed and recommended fixed once monthly dose of 120 mg subcutaneous denosumab. This should however first be tested in a randomized clinical trial to ensure similar efficacy in reducing SREs of both dose schedules.

DENOSUMAB IN EARLY BREAST CANCER

Denosumab effective against cancer-therapy induced bone loss (CTIBL)?

Patients with breast cancer often develop bone loss secondary to cancer treatment itself. Several mechanisms of bone loss due to cancer treatment have been identified. Firstly, there is bone loss as a result of estrogen deprivation. In premenopausal women bone density loss averages 8% in the first year of treatment with premature ovarian suppression due to chemotherapy induced amenorrhea [33]. Secondly, there is bone loss due to endocrine anticancer therapies. The effects of tamoxifen, a selective estrogen receptor modulator (SERM), on bone are dependent on the actual physiologic estrogen concentration. Tamoxifen causes bone loss in premenopausal women, but is bone protective in postmenopausal women [34]. Aromatase inhibitors in postmenopausal women lower the estrogen level by suppressing the conversion of androstenedione to estradiol. As a consequence of the estrogen deprivation, on average a 2.6% loss of bone density in the first year of breast cancer treatment has been found [35]. In contrast, bone loss during natural menopause is typically 1% per year. Finally, chemotherapies and supportive drugs, such as steroids, affect bone density directly or do so indirectly. Chemotherapy treatment causes bone loss by directly damaging bone architecture or inducing early menopause in premenopausal women.

The role of denosumab in preventing aromatase inhibitor induced bone loss has been studied in the Hormone Ablation Bone Loss Trial in Breast Cancer (HALT-BC) study [24]. This trial examined the efficacy of denosumab (60 mg sc every six months for two years) vs. placebo for preventing bone loss among 252 postmenopausal women with early-stage breast cancer who were receiving an aromatase inhibitor. After 24 months of follow-up, a significant difference of 7.6% in lumbar spine bone density of patients treated with denosumab compared to placebo was found. Similarly, a significant difference of 4.7% was detected in total hip bone density in advantage of the denosumab treated group. Although the results of this trial were promising, the results of the large Austrian Breast and Colorectal Cancer Study Group Trial 18 (ABCSG18-trial; http://www.clinicaltrials.gov. Identifier: NCT00556374) have to be awaited. In this ongoing trial approximately 3,460 postmenopausal breast cancer patients receiving adjuvant aromatase inhibitor therapy will be randomized between denosumab (60 mg sc every six months) and placebo, with time to first clinical fracture as the primary endpoint. This study will provide the essential information on fracture rates, disease recurrence, and long term data safety to decide whether or not denosumab treatment could be given in this setting.

Denosumab effective in preventing (bone)metastases?

The "seed and soil" hypothesis of cancer metastases was proposed over a century ago, and stated that cancer cells (the seed) are only able to become metastases in organs where the micro-environment (the soil) permissive the growth of these cancer cells (the seed) [36]. Osteotropic cancer cells express several proteins that are thought to specifically adhere to surface markers expressed by bone marrow endothelial cells. One of these proteins is supposed to be RANK by which it interacts with the RANKL expressed on bone stromal cells. Interestingly, RANKL may also be involved in the epithelial-mesenchymal transitions, invasion and homing of tumor cells to bone [37].

It could therefore be speculated that by altering the micro-environment of the bone by interfering the RANKL/RANK system, the development of bone metastases could be postponed or even be prevented and maybe also dissemination to other organs. Denosumab is a specific inhibitor of the RANKL pathway for osteoclast activation and function but is primate-specific and thus cannot be tested in rat models of bone metastases. In animal models, inhibition of RANKL activity is achieved by binding to recombinant antibody constructs of either osteoprotegerin-Fc or RANK-Fc [38]. In animal models with advanced breast cancer, RANKL inhibition reduces the burden of tumor in bone [39]. Moreover, in another breast cancer mouse model, inhibition of the RANKL pathway prevented tumor progression and increased survival [40]. Finally, treatment of transgenic mice with RANK-Fc significantly decreased development of spontaneous lung metastases [41]. As such denosumab administration in humans could reduce rates of distant metastases by manipulating the bone micro-environment or potentially by a direct antitumor effect.

A large phase 3 randomized controlled trial among men (n = 1,432) with castration resistant prostate cancer indeed showed that denosumab (120 mg sc every four weeks) significantly delayed the onset of detectable bone lesions compared to placebo (33.2 vs. 29.5 months; HR 0.85; 95% CI 0.73-0.98; p =

0.028) [42]. Progression free and overall survival did not differ between the groups, suggesting that inhibiting bone resorption alone might be insufficient to prevent the subsequent development of metastases outside the bone. Denosumab is also being evaluated in the adjuvant setting among breast cancer patients in an ongoing trial (D-CARE trial; http://www.clinicaltrials.gov. Identifier: NCT01077154). In this trial 4,500 women with high-risk early breast cancer (stages II and III) will be randomized to denosumab or placebo for five years. The primary endpoint compares bone metastases free survival between denosumab and placebo. Secondary endpoints include disease free and overall survival and distance recurrence-free survival. The D-CARE trial has accrued more than 4,000 patients thus far, with no significant safety concerns to date, and will provide more data on the possible role of denosumab as adjuvant therapy to prevent bone metastases as well as potential adverse events with longer term treatment.

Of particular interest would be to test the efficacy of denosumab in a group of patients at high risk of developing bone metastases. Accordingly, in the near future a non-randomized phase 2 study will be started in women with early breast cancer with a significant number of disseminated tumor cells in the bone marrow after (neo)adjuvant chemotherapy (http://www.clinicaltrials.gov. Identifier: NCT01545648). These patients will receive denosumab for one year. Primary endpoint is reduction of bone marrow disseminated tumor cells at six and twelve months of treatment, compared to baseline. Another possibility of preselecting a group of patients with a high risk of developing bone metastases could be by using a genetic signature of the primary tumor [43].

DENOSUMAB EFFECTIVE AGAINST BREAST CARCINOGENESIS?

Progesterone is an important risk factor for breast carcinogenesis. From large epidemiological studies it became clear that postmenopausal women on hormone replacement therapy have an increased risk of developing breast cancer when taking combined estrogens and progestins, however not when taking estrogens alone [44]. The precise mechanism through which progesterone could case carcinogenesis is however unclear. Recently, preclinical research has shed new light on this issue, by the observation that progesterone was found to play an indirect role in carcinogenesis via the RANKL/RANK system.

Normal breast development in the mouse involves hormonal stimulation of a differentiated progesterone-receptor positive luminal epithelial cell population, as well as a progesterone-receptor negative (basal) stem cell population [45,46]. Progesterone-receptor positive luminal cells express RANKL in response to progesterone. RANKL on its turn binds to RANK on progesterone negative (basal) stem cells causing proliferation of these stem cells, i.e. increasing their number as well as their regenerating activity. By using a combination of progesterone derivate (progestin medroxyprogesterone acetate) and a well-known mutagenic agent (7,12-dimethylbenz(a) anthracene (DMBA)), mammary carcinomas could be induced in mice, a process fully regulated by the RANKL-RANK system. Overexpression of RANK by means of a mouse mammary tumor virus (MMTV)-driven

transgenic accelerated hyperplasia but also tumor formation. Pharmacological inhibition of RANKL in the mammary epithelium decreased incidence and delayed onset of carcinogenesis in this system [38]. An important question is whether denosumab has an anti-carcinogenesis effect in the clinical setting, as is reported in preclinical models for recombinant antibody constructs of RANK-Fc in animals. Immunostainings of human breast cancers reveal that RANKL is expressed in 11% of human tumors, whereas RANK was detected in 6% of tumors. There is no evidence for co-localization within the epithelium for receptor and ligand but expression of RANKL is found in some stromal cells [41]. This raises the possibility that the scenario is more complex in human breast cancer than in the present mouse tumor models with infiltrating immune cells providing the ligand.

In the earlier mentioned meta-analysis of three randomized clinical trials among post-menopausal women ($n = 996$) with low bone mass no significant decrease in cancer risk was seen in the patients treated with denosumab compared to placebo [22]. Also in the FREEDOM trial ($n = 7,868$), comparing denosumab with placebo in postmenopausal women with low bone mineral density, no differences were seen between the group in the incidence of malignancies [25]. Similarly, in the HALT-BC trial ($n = 252$), comparing denosumab vs. placebo in women with non-metastatic breast cancer and low bone mass who were receiving adjuvant aromatase inhibitor therapy, no new primary breast cancers were seen in both groups [24]. Finally, in the earlier mentioned trial among patients with bone metastases of breast cancer and the trial of bone metastases from other solid tumors or multiple myeloma, comparing denosumab with zoledronic acid, again no significant differences in the development of new primary cancer were seen (respectively 0.5 vs. 0.5% and 0.3 vs. 0.6%; $p = 0.73$) [13,14].

Taken together, the potential effects of denosumab on the carcinogenesis of the breast are currently unknown. However, based on the current clinical (indirect) evidence the preventive effect of denosumab on carcinogenesis, if any, seems relatively small. Further maturation of the safety data of the above mentioned trials and the results of the earlier mentioned currently ongoing ABCSG-18 and D-CARE study have to be awaited before definite conclusions can be drawn.

CONCLUSION

Denosumab is an interesting new member in the group of the bone-modifying agents with very promising results in breast cancer patients with bone metastases. Nevertheless, several questions still remain which have been discussed in this review. The most urgent question for tomorrows' daily practice is where to place denosumab relating to bisphosphonates. The ASCO clinical practice guidelines has recently been updated and recommend either denosumab or bisphosphonate therapy and state that there is currently insufficient evidence to demonstrate that one bone-modifying agent is to be preferred over the other [47]. Since no difference in survival was reported in breast cancer patients treated with denosumab compared to zoledronic acid, the benefit will need to be considered in reducing morbidity by reducing the risk of SREs and increasing patients' quality of life measured by diminished side effects and a more convenient way of administration compared to bisphosphonates.

Clearly, costs will also be an important factor to consider. Denosumab is significantly more expensive than e.g. zoledronic acid and might therefore be not cost-effective compared to treatment with zoledronic acid in preventing SREs [48]. Further research will be essential and investigating whether patient-tailored denosumab treatment for breast cancer patients with bone metastases is as effective as fixed dose denosumab is of great value.

Whether the indications for denosumab treatment could be expanded to secondary prevention (preventing the development of bone metastases) and maybe to anticancer treatment or primary prevention (prevent development of breast cancer) also, is currently under investigation. The results of the ABCSG-18 and D-CARE study are eagerly awaited.

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9

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

Introduction

In the Western world, breast cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in women [1]. The high incidence and prevalence of this disease makes this disease extremely suitable for scientific research and major advances have been made in the past decades. Both for early and metastatic breast cancer many new treatment options became available in the recent years. Unfortunately, all treatment modalities are associated with the risk of acute and/or late toxicity. Acute toxicity of chemotherapy could for example be manifest as bone marrow suppression, nausea and vomiting; long term toxicity could manifest as the development of other (breast) cancers, cardiotoxicity, premature postmenopausal state and osteoporosis. One of the major challenges in breast cancer research is to increase efficacy of anticancer treatments without increasing clinical relevant toxicity or to decrease toxicity of anticancer treatment without decreasing its efficacy.

Impaired DNA repair mechanism related to increased treatment-associated toxicity

BRCA1 and *BRCA2* (*BRCA1/2*) mutation carriers have decreased levels of functional BRCA1 and BRCA2 proteins, leading to homologous recombination deficiency, resulting in inadequate repair of double strand DNA breaks. Both ionizing radiation and various chemotherapeutic agents induce DNA damage by several mechanisms including induction of double strand DNA breaks. Patient with *BRCA1/2*-associated breast cancer might therefore be at increased risk for treatment-associated toxicity.

Ionizing radiation

BRCA1/2 mutation carriers are at increased risk for the development of breast cancer and once they developed breast cancer they are at increased risk for the development of a second ipsilateral or contralateral new primary breast cancer [2-4]. This makes that a possibly increased risk of developing (primary or second primary) breast cancer as a result of ionizing radiation might be proved easier in this population.

In **Chapter 2** we provide an overview and interpretation of the literature about the risk for the development of a first and second primary breast cancer as late toxicity of diagnostic and/or therapeutic ionizing radiation, with special attention for *BRCA1/2* mutation carriers. There is a clear positive radiation dose-risk relation, which is modified by age, whereby young age at exposure is associated with an increased breast cancer risk. Furthermore, studies suggest a minimal latency period of 10-12 years before an increased breast cancer risk becomes apparent. For sporadic breast cancer patients, diagnosed above the age of 45 years, there is no reason to withhold radiotherapy in adjuvant setting. For younger sporadic patients, however, no definite conclusion can be drawn based on the current data. Data on the deleterious effects of radiation for the subgroup of *BRCA1/2* mutation carriers are sparse. Nevertheless, for screening purposes there seems to be enough evidence to incorporate

mammography in breast cancer screening programs for *BRCA1/2* mutation carriers, only after the age of 30 years. For those *BRCA1/2* mutation carriers who developed breast cancer above the age of 30 years and opting or breast conserving therapy, there are no hard data regarding a possibly increased carcinogenic effect of adjuvant radiotherapy with respect to a second primary breast cancer. Caution with regard to breast conserving surgery and radiotherapy seems warranted in *BRCA1/2* mutation carriers, aged <30 years at breast cancer diagnosis.

We added new data to the important topic of the possible deleterious effects of adjuvant radiotherapy by increasing the risk of ipsilateral and contralateral breast cancer in *BRCA1/2* mutation carriers. In **Chapter 3** we present the results of a single center retrospective cohort study from the Rotterdam Family Cancer Clinic database. In this study we include all proven or obligate *BRCA1/2* mutation carriers treated at the Erasmus MC Cancer Institute for early stage breast cancer diagnosed between January 1, 1980 and January 1, 2013 ($n = 790$). Objective of this study was to estimate the influence of adjuvant radiotherapy for primary breast cancer on the risk of contralateral breast cancer in *BRCA1/2* mutation carriers. Additionally, tendencies in locoregional treatments and rates of contralateral risk-reducing mastectomy over time were explored. No association between radiotherapy for primary breast cancer and risk of contralateral breast cancer was observed in the total group, nor in the patients irradiated before the age of 40 years. Importantly, the number of patients at risk for developing contralateral breast cancer decreased substantially over time since a large proportion of patients were censored after contralateral risk-reducing mastectomy or breast cancer recurrence. As a consequence, the number of patients at risk after 10 and 15 years of follow-up was too small to definitively exclude harmful effect of radiotherapy on the development of contralateral breast cancer among young *BRCA1/2* mutation carriers. Over the years, increasing preference for mastectomy without radiotherapy was found ranging from less than 30% in 1995 to almost 50% after 2010. The rate of contralateral risk-reducing mastectomy increased over the past decades from less than 40% in 1995 to more than 60% after 2010. Further increase in patients choosing for risk-reducing mastectomy is expected, due to the increased awareness of breast cancer risks and risk-reducing possibilities in this population, also called the Angelina Jolie effect [5,6]. These trends in locoregional treatments eventually decreased the proportion of patients at risk for radiation-induced contralateral breast cancer.

Nevertheless, the question whether adjuvant radiotherapy has deleterious effect on contralateral breast cancer risk is still important for those patients who want to conserve their (ipsilateral and) contralateral breast. Moreover, in the nearby future a larger proportion of patients potentially might opt for breast conserving treatment and abstain from contralateral risk-reducing mastectomy, due to an increased use of endocrine therapy as chemoprevention, improved diagnostic imaging techniques for screening and improved effectiveness of adjuvant systemic therapy. Future research in larger study populations with minimal follow-up of 10 years is needed to achieve a better understanding of the true toxic effect of radiotherapy on the contralateral breast cancer risk in *BRCA1/2*-associated breast cancer patients.

Chemotherapy

Besides the possibly increased toxicity of ionizing radiation in *BRCA1/2* mutation carriers, one could also argue that the toxicity of chemotherapy might be increased in these patients, since chemotherapy causes cell damage by induction of double strand DNA breaks. In **Chapter 4** we describe the results of a single center retrospective cohort study on differences in (neo)adjuvant chemotherapy related toxicity between *BRCA1/2* mutation carriers and sporadic breast cancer patients. For this study we selected all female patients who were treated at the Erasmus MC with adjuvant or neoadjuvant chemotherapy for primary breast cancer or local/locoregional recurrence (PBC/LR). Further eligibility criteria concerned: chemotherapy regimen consisting of anthracyclines and/or taxanes, chemotherapy treatment started between January 1, 2004 and December 31, 2014. In total, 701 patients were eligible for data-analyses, of whom one *BRCA1* mutation carrier and two sporadic patients were treated with two separate episodes of (neo)adjuvant chemotherapy for a PBC/LR during the study period. 85 patients (12%) were *BRCA1/2* mutation carriers (n = 67 *BRCA1* and n = 18 *BRCA2*). Primary outcome was the relative total dose intensity (RTDI), a measure of delivered (actual) dose-intensity (i.e. administered dose over the total time course of treatment), relative to the planned dose intensity. The RTDI therefore expresses the effect of reductions, delays as well as premature discontinuations of a treatment. We found no differences in RTDI (both for anthracyclines and taxanes) between breast cancer patients with a *BRCA1/2* mutation and sporadic breast cancer patients. Furthermore, we found no differences in the occurrence of febrile neutropenia, in delay of chemotherapy administration or alteration of the chemotherapy regimen due to toxicity between these groups. This absence of increased acute toxicity due to (neo)adjuvant chemotherapy in *BRCA1/2* mutation carriers, compared to sporadic breast cancer patients, shows that the DNA damage repair mechanism of non-cancer cells with only one normal copy of either the *BRCA1* gene or the *BRCA2* gene is sufficiently functional to handle acute chemotherapy-associated toxicity of currently used chemotherapy regimens. Both platinum derivatives and PARP inhibitors are increasingly used in *BRCA1/2*-associated breast cancer [7-11]. These drugs have a much higher capacity in inducing double strand DNA breaks, compared to anthracyclines and might therefore induce more toxicity in *BRCA1/2* mutation carriers. Further data on the toxicity of these regimens in *BRCA1/2* mutation carriers compared to sporadic patients should follow from currently ongoing and new studies.

Could toxicity be used to optimize treatment?

Interestingly, toxicity might be associated with efficacy and might therefore be used to increase efficacy by increasing systemic therapy dose in patients without or with only limited toxicity. Especially the amount of hematological toxicity in breast cancer patients treated with chemotherapy seems to be related to efficacy [12-14]. The amount of toxicity could thus be used as a marker in dose escalation studies. In **Chapter 5** we describe the results of a single center prospective study on the feasibility of neutrophil-guided dosing of anthracycline-cyclophosphamide containing chemotherapy in sporadic breast cancer patients. Thirty-two patients planned for three-weekly anthracycline-cyclophosphamide

containing chemotherapy, either in adjuvant or metastatic setting, were enrolled in this study. The first treatment cycle was administered in a standard BSA (body surface area)-adjusted dose. For patients with none or mild (CTC grade 0-2) neutropenia and no other dose limiting toxicity, we performed a 10-25% dose escalation of the second cycle with the opportunity to a further 10-25% dose escalation of the third cycle. Two out of 23 patients (9%) treated with FEC (fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2) did not develop grade 3-4 neutropenia after the first treatment cycle. Dose escalation was performed in these two patients (30% in one and 15% in the other patient). During dose escalation, there were no complications like febrile neutropenia. No patients treated with FAC (fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2) or AC (doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2) could be escalated, since all of them developed grade 3-4 neutropenia. We conclude that asymptomatic grade 3-4 neutropenia is likely to be achieved in the majority of patients with breast cancer treated with anthracycline-cyclophosphamide-containing chemotherapy according to presently advocated BSA-based dose levels. Escalation of currently advocated doses of anthracycline-cyclophosphamide-containing chemotherapy without G-CSF, with a target of grade 3-4 neutropenia, is feasible, but only possible in a small proportion of patients. Since no other dosing algorithms are available for anthracycline-cyclophosphamide-containing chemotherapy, BSA-guided dosing remains standard practice at this moment. For the vast majority of classical cytotoxic anticancer agents, BSA-guided dosing remains still standard practice. Further research on neutrophil-guided dosing might be useful for other chemotherapeutic agents. For a number of (oral) targeted agents, flat-fixed dosing and a posteriori dose reduction in case of severe toxicity is a standard practice. For a small number of targeted anticancer agents an association between toxicity and efficacy has been found. Increased skin toxicity of anti-EGFR antibodies is associated with increased response rates in metastatic colorectal cancer [15]. For adjuvant endocrine therapy it has been suggested that development of toxicity might be used as a biomarker of better response to therapy [16].

Treatment related toxicity, compliance and therapeutic drug monitoring

Bothersome toxicity of anticancer agents, influencing quality of life, might be a reason for early discontinuation of treatment. For adjuvant endocrine therapy, which often has to be used for years, it has been found that, despite their proven benefit, adherence to and persistence with the medications is poor [17]. Therapy adherence, change in environmental factors and possibly resistance mechanisms might influence the systemic exposure over time. The best way forward for individualization of tamoxifen therapy seems to be therapeutic drug monitoring (TDM). The often used method of TDM by serial measurements of plasma concentrations of drugs and/or metabolites over time might be difficult to incorporate in clinical practice. A method, which gives (in retrospect) information about the course of concentrations of anticancer drugs (and its metabolites) over time, could be the measurement of these concentrations in scalp hair, which gives the opportunity to study the historic pattern of drug concentrations (depending on the length of hair collected), which

makes serial venous blood collections unnecessary [18]. In **Chapter 6** we describe the validation of an earlier developed high-performance highly sensitive ultra performance liquid chromatography/ tandem mass spectrometry (UPLC-MS/MS) method for quantification of tamoxifen and its three main metabolites (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen, and 4-hydroxy-N-desmethyl-tamoxifen) in scalp hair. In that study we conclude that measurement of the concentration of tamoxifen and its main metabolites in hair is possible, with the selective, sensitive, accurate and precise UPLC-MS/MS method. However for tamoxifen, it seems not possible to determine exposure over time with segmental analysis of hair, probably largely due to the effect of UV irradiation. Further research should therefore focus on quantification of other anticancer drugs, in segmental scalp hair, that are less sensitive to UV irradiation. For studies on therapeutic drug monitoring in patients treated with tamoxifen, measurement of endoxifen concentrations in plasma remains gold standard.

Prevention and treatment of toxicity

One of the important toxicities of breast cancer treatment is premature bone loss. This therapy-induced bone loss can develop by a variety of mechanisms: premature ovarian failure due to adjuvant chemotherapy and/or adjuvant endocrine therapy, lower levels of estrogens by aromatase inhibitors and the direct influence of chemotherapy and supportive drugs, such as steroids. Furthermore, the bone is the most common site to which breast cancer metastasizes [19]. The occurrence of skeletal related events, defined as pathological fractures, necessity for radiation or surgery due to (threaten) spinal cord compression or hypercalcemia due to bone metastases, has a negative impact on patient's quality of life and generally portends a worse prognosis.

For years, bisphosphonates have been the cornerstone for the prevention and management of both skeletal related events in metastatic breast cancer and cancer-treatment induced bone loss in early breast cancer [19,20]. Denosumab, a fully human monoclonal antibody that binds to the receptor activator of nuclear factor kappa-B ligand (RANKL) has been developed as a new targeted bone therapy, which can be used both in early and metastatic breast cancer [21-23]. In **Chapter 7** we describe the current indications for denosumab in the various settings of breast cancer treatment and concludes that there are still a number of questions to be answered such as where to place denosumab relating to bisphosphonates, whether patient-tailored dosing, for example based on markers of bone turnover, is possible and whether there is a place for denosumab in the prevention of bone metastases or even in the prevention of the development of breast cancer. Important information on the value of denosumab in the (neo)adjuvant setting will follow from the D-CARE study (https://clinicaltrials.gov/ct2/show/NCT01077154).

Postponing (cyto)toxic therapy an option in HER2-positive metastatic breast cancer?

Once patients develop metastatic breast cancer or local recurrence without surgical or radiotherapeutic options, treatment is aimed at maintaining or improving quality of life and improving progression free and overall survival. When treating patients with metastatic breast cancer minimizing toxicity

is therefore an important goal. For the subgroup of HER2-positive breast cancer, the combination of trastuzumab and a taxane (either docetaxel or paclitaxel) was standard first line treatment until recently [24,25]. Progress in this subgroup of patients might be made in different ways. One way could be to increase efficacy of treatment by adding a new agent, with limited toxicity. Another option could be to try to decrease toxicity by delaying the start of chemotherapy by adding a second targeted agent with limited toxicity to trastuzumab and only start chemotherapy at progression under this regimen. Bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), could be one of the possible agents to be used in these strategies [26,27].

In **Chapter 8** we describe the results of an open label, randomized, non-comparative, phase 2 study, in which 84 patients were randomized (1:1) between HAT (the combination of bevacizumab, trastuzumab and weekly paclitaxel) and HA-HAT (the regimen of upfront trastuzumab and bevacizumab and adding weekly paclitaxel after progression). The progression free rate at 1 year was 74.4% (95% confidence interval, CI 61.8-89.4) and 62.2% (95% CI 49.6-89.4) in the HAT and HA-HAT arm, respectively. The median progression free survival in the HAT arm was 19.8 months (95% CI 14.9-25.6), and the median overall progression free survival in the HA-HAT arm was 19.6 months (95% CI 12.0-32.0). In the HA-HAT arm the median progression free survival for treatment with only bevacizumab and trastuzumab was 10.4 months (95% CI 6.2-15.0) and the median progression free survival for treatment with trastuzumab, bevacizumab and paclitaxel after first progression was 8.2 months (95% CI 7.0-12.6). The median overall survival was 36.8 months (95% CI 29.1 to not applicable) in the HAT group and 54.0 months (95% CI 37.4 to not applicable) in the HA-HAT group. Number and severity of adverse events were comparable between the arms and were in favor of starting with only bevacizumab and trastuzumab.

Based on comparison with historical controls, the data suggest that bevacizumab enhances the antitumor activity of the trastuzumab-based regimens as applied in this study. With the publication of the CLEOPATRA trial showing median overall survival benefit of 15.7 months for the combination of trastuzumab, pertuzumab and docetaxel compared to the combination of trastuzumab and docetaxel, this regimen has become the standard first line therapy in patients with HER2-positive metastatic breast cancer [28,29]. When taking this solid data and the favorable toxicity profile of pertuzumab, compared to bevacizumab into account, it is unlikely that further phase 3 trials on the combination of bevacizumab, trastuzumab and chemotherapy will be performed. The results from our study however shows that the concept of starting with a less toxic chemotherapy-free approach with only biologicals is an approach deserving further study. From a quality of life viewpoint, it would be of great interest to develop treatment schedules starting with less toxic therapies. New trials about chemotherapy-free treatments should therefore also incorporate newer HER2-targeted agents such as pertuzumab, trastuzumab-emtansine and novel agents, but also endocrine therapy in hormone receptor positive patients.

Conclusion

Altogether we may conclude that, despite all the improvements in the treatment of both early and metastatic breast cancer, the disease remains a serious health problem with high morbidity and mortality. Unfortunately, all treatments are associated with toxicity and one of the important goals in breast cancer research should be decreasing treatment-associated acute and long term toxicity. It is therefore essential to continue preclinical and clinical trials as well as prospective and retrospective observational studies. For future studies it is more and more important to design studies in subgroups of patients with specific patient and tumor characteristics. For example, for patients with a *BRCA1/2* gene mutation it is extremely important to design studies specific for these subgroups since they might differ in both efficacy and toxicity of systemic therapy but also the baseline risk for the development of primary and second primary ipsilateral and contralateral breast cancer are increased compared to the sporadic population. This will only be possible by combining study populations through collaborative efforts on a national, or even international level. Furthermore future research should not only incorporate possible biomarkers that better predict which patients will benefit from a certain treatment, but also which patients will or will not develop toxicity from that treatment.

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Appendix

Samenvatting

Dankwoord

Curriculum vitae

List of publications

PhD portfolio

SAMENVATTING

Inleiding

Borstkanker is de meest voorkomende kankersoort bij vrouwen in de westerse wereld. Daarnaast is het de op één na belangrijkste oorzaak van overlijden aan kanker onder vrouwen. Het aantal nieuwe patiënten met borstkanker is de afgelopen jaren gestegen. Dit kan voor een deel verklaard worden door de toegenomen levensverwachting en de toenemende vergrijzing. In Nederland is het risico voor vrouwen om borstkanker te krijgen in de loop van het leven opgelopen tot bijna 14%. Door wetenschappelijk onderzoek is de laatste jaren duidelijke vooruitgang geboekt in de behandeling van borstkanker. Zowel voor vroegstadium (niet uitgezaaide) borstkanker als voor uitgezaaide borstkanker zijn nieuwe behandelmogelijkheden beschikbaar gekomen.

Vroegstadium borstkanker

Borstkanker wordt bij vrouwen meestal in een vroeg stadium ontdekt, waarbij de ziekte niet uitgezaaid is voorbij de regionale lymfklierstations. De behandeling is dan gericht op genezing en bestaat uit lokale behandeling (operatie en in veel gevallen nabehandeling met bestraling) vaak in combinatie met perioperatieve medicamenteuze behandeling (chemotherapie, endocriene therapie en/of doelgerichte therapie). Deze medicamenteuze behandelingen kunnen gegeven worden vóór (neoadjuvant) of na (adjuvant) resectie van de tumor. Deze medicamenteuze behandelingen hebben ten doel om zowel het risico op een lokaal recidief als het risico op uitzaaiingen op afstand te verminderen. De beslissing om (neo)adjuvante behandeling te geven en vervolgens in welke vorm, wordt genomen op basis van kenmerken van de tumor en van de patiënten. Een groot deel van de patiënten die behandeld worden voor vroegstadium borstkanker ontvangt postoperatieve bestraling van de borst/thoraxwand en/of locoregionale lymfklierstations ter vermindering van het risico van lokaal recidief en verbetering van de overleving.

Uitgezaaide borstkanker

Helaas ontwikkelt een deel van de patiënten die behandeld zijn voor vroegstadium borstkanker op enig moment een lokaal recidief, uitgezaaide borstkanker of een tweede primaire borstkanker. Daarnaast presenteert ongeveer 5% van de borstkankerpatiënten zich bij diagnose reeds met uitzaaiingen op afstand.

Wanneer patiënten uitzaaiingen op afstand hebben ontwikkeld is genezing in het algemeen niet meer mogelijk. Behandeling is dan gericht op levensverlenging en behoud of verbetering van kwaliteit van leven. De levensverwachting wisselt sterk tussen patiënten met uitgezaaide borstkanker en hangt sterk af van tumorkenmerken, de uitgebreidheid en de locaties van de uitzaaiingen en de mate waarin de uitzaaiingen reageren op behandelingen. De beslissing om medicamenteuze anti-kanker behandeling te geven en vervolgens in welke vorm hangt ook bij uitgezaaide borstkanker sterk af van de kenmerken van de tumor, maar ook van kenmerken en wensen van de patiënt. Bij

patiënten met botuitzaaiingen kunnen osteoclastremmers (bisfosfonaten of denosumab) het risico op botcomplicaties verminderen. Helaas geldt ook bij de behandeling van uitgezaaide borstkanker dat alle middelen geassocieerd zijn met bijwerkingen, die een negatief effect kunnen hebben op de kwaliteit van leven.

Eén van de grote uitdagingen in het wetenschappelijk onderzoek naar de behandeling van borstkanker is om effectiviteit van behandelingen te verbeteren, zonder toename van ernstige bijwerkingen (toxiciteit) of het verminderen van toxiciteit zonder verlies van effectiviteit. Dit proefschrift beschrijft wetenschappelijk onderzoek verricht met als doel het optimaliseren van de behandeling van borstkanker. Toxiciteit kan hier op verschillende manier in gebruikt worden. De behandeling kan verbeterd worden door: (1) het verminderen van toxiciteit, met behoud van effectiviteit, (2) verbeteren van effectiviteit, zonder klinisch relevante toename van toxiciteit, (3) verbeteren van effectiviteit door dosisescalatie op basis van toxiciteit en (4) voorkomen van (complicaties van) toxiciteit van anti-kanker behandelingen.

Inadequaat DNA reparatie-mechanisme en toename van bijwerkingen

Vrouwen (en mannen) met een mutatie in het *BRCA1 of BRCA2 (BRCA1/2)* gen hebben een verhoogd risico op het krijgen van borstkanker. In 5-10% van de vrouwen met borstkanker speelt een mutatie in één van deze genen een oorzakelijke rol. De BRCA1 en BRCA2 eiwitten hebben een belangrijke rol in het repareren van dubbelstrengs DNA breuken via het mechanisme van homologe recombinatie. Zowel ioniserende straling als chemotherapeutica leiden tot DNA schade door verschillende mechanismes zoals het induceren van dubbel strengs DNA breuken. Patiënten met *BRCA1/2*-geassocieerde borstkanker zouden daardoor een verhoogd risico kunnen hebben op toxiciteit ten gevolge van deze behandelingen die noodzakelijk zijn voor de behandeling van de kanker.

Ioniserende straling

BRCA1/2 genmutatie draagsters hebben een verhoogd risico op het krijgen van borstkanker. Daarnaast hebben patiënten met *BRCA1/2*-geassocieerde borstkanker een verhoogd risico op het ontwikkelen van een tweede primaire ipsilaterale of contralaterale borstkanker. Ioniserende straling leidt tot het ontstaan van DNA schade en is op de langere termijn een risicofactor voor het ontstaan van borstkanker. Patiënten met een *BRCA1/2* mutatie zouten gevoeliger kunnen zijn voor de negatieve effecten van ioniserende straling. In **Hoofdstuk 2** geven we een overzicht en interpretatie van de literatuur over het risico op het ontwikkelen van een eerste en tweede primaire borstkanker als late toxiciteit van diagnostische en/of therapeutische ioniserende straling (=radiotherapie) met speciale aandacht voor *BRCA1/2* genmutatie draagsters. Er blijkt een duidelijke relatie te zijn tussen radiatie-dosis en risico op borstkanker. Jonge leeftijd ten tijde van blootstelling aan ioniserende straling is geassocieerd met een verhoogd risico op borstkanker. Gegevens over de negatieve effecten van ioniserende straling in de subgroep van *BRCA1/2* genmutatie draagsters zijn zeldzaam. Terughoudendheid met ioniserende

straling lijkt gerechtvaardigd voor jonge patiënten met een *BRCA1/2* genmutatie.

Met het in **Hoofdstuk 3** beschreven onderzoek hebben we nieuwe inzichten toegevoegd met betrekking tot het belangrijke vraagstuk over het mogelijke negatieve effect van adjuvante radiotherapie op het verhogen van het risico op ipsilaterale of contralaterale borstkanker bij *BRCA1/2* genmutatie draagsters. In dit hoofdstuk beschrijven we de resultaten van een onderzoek vanuit de database van de polikliniek erfelijke tumoren van het Erasmus MC in Rotterdam. In dit onderzoek includeerden we alle bewezen of obligate (niet getest, maar zeker op basis van geteste familieleden) *BRCA1/2* genmutatie draagsters die behandeld werden voor vroegstadium borstkanker, gediagnosticeerd tussen 1 januari 1980 en 1 januari 2013 (n = 790). Doel van dit onderzoek was om de invloed van adjuvante radiotherapie voor primaire borstkanker op het risico op contralaterale borstkanker bij *BRCA1/2* genmutatie draagsters te bepalen. Er werd geen associatie gevonden tussen bestraling voor primaire borstkanker en het risico op contralaterale borstkanker in de totale groep, maar ook niet in de patiënten die bestraald werden voor de leeftijd van 40 jaar. De aantallen zijn echter te klein om definitieve conclusies te trekken. Aanvullend werd in kaart gebracht of er tendensen waren in het aandeel van contralaterale risico-reducerende mastectomie en keuzes voor de verschillende locoregionale behandelingen over de tijd. Het aantal patiënten dat een contralateraal borstkanker zou kunnen ontwikkelen, nam substantieel af over de tijd, omdat een groot deel van de patiënten gecensureerd (censurering = uitval uit een studie als gevolg van redenen die ook samenhangen met de uitkomst) werden na contralaterale risico-reducerende mastectomie of terugkeer van de borstkanker. Over de jaren werd daarnaast een toenemende voorkeur gezien voor lokale behandeling middels mastectomie zonder bestraling, oplopend van minder dan 30% in 1995 tot bijna 50% na 2010. Het percentage patiënten dat kiest voor contralaterale risico-reducerende mastectomie nam in de afgelopen decades toe van minder dan 40% in 1995 tot meer dan 60% na 2010. Verdere toename in het aantal patiënten dat kiest voor risico-reducerende mastectomie is te verwachten door de toenemende bewustwording van het risico op borstkanker en de risico-reducerende mogelijkheden in de *BRCA1/2* populatie.

Desondanks is de vraag of adjuvante radiotherapie een negatieve invloed heeft op het risico op contralaterale borstkanker nog steeds relevant voor patiënten die hun (ipsilaterale en) contralaterale borst willen behouden. Op basis van de in hoofdstuk 2 beschreven literatuur en dit onderzoek kan geconcludeerd worden er geen hard bewijs is betreffende een mogelijk verhoogd carcinogeen effect van adjuvante radiotherapie op het ontwikkelen van een tweede primaire borstkanker voor *BRCA1/2* genmutatie draagsters die borstkanker ontwikkelen boven de leeftijd van 30 jaar en kiezen voor borstsparende behandeling. Gezien het mogelijk verhoogd risico op een tweede primaire (contralaterale) tumor, lijkt terughoudendheid met betrekking tot borstsparende chirurgie en bestraling gerechtvaardigd voor *BRCA1/2* genmutatie draagsters, die jonger dan 30 jaar zijn bij diagnose van borstkanker. Verder onderzoek in grotere studie populaties met een minimale follow-up duur van 10 jaar is nodig om een beter inzicht in de werkelijke negatieve effecten van bestraling op het risico van contralaterale borstkanker bij *BRCA1/2*-geassocieerde borstkanker patiënten te krijgen.

Chemotherapie

Naast de mogelijk verhoogde toxiciteit van ioniserende straling bij *BRCA1/2* genmutatie draagsters, kan men ook veronderstellen dat de toxiciteit van chemotherapie toegenomen zou zijn bij deze patiënten, omdat meerdere soorten chemotherapie werken via het induceren van dubbelstrengs DNA breuken. In **Hoofdstuk 4** bespreken we de resultaten van een retrospectieve cohort studie naar verschillen in toxiciteit van (neo)adjuvante chemotherapie tussen *BRCA1/2* genmutatie draagsters en sporadische borstkankerpatiënten. Voor deze studie werden alle vrouwelijke patiënten geselecteerd die in het Erasmus MC werden behandeld met adjuvante of neoadjuvante chemotherapie (bestaande uit anthracyclines en/of taxanen) voor primaire borstkanker of lokaal/ locoregionaal recidief (PB/LR) zonder afstandsmetastasen tussen 01-01-2004 en 31-12-2014. Totaal konden de gegevens van 701 patiënten gebruikt worden voor de analyses. Er waren 85 patiënten (12%) met een *BRCA1/2* genmutatie (*n* = 67 *BRCA1* en *n* = 18 *BRCA2*). Als primaire uitkomst werd relatieve totale dosis-intensiteit (RTDI) genomen, een maat voor toegediende (actuele) dosis-intensiteit (toegediende dosis over de hele tijdsperiode van de behandeling), gerelateerd aan de geplande dosis-intensiteit. Door het gebruik van RTDI worden de effecten van dosisreducties, uitstel en vroegtijdig staken van behandeling meegenomen. We vonden geen verschillen in RTDI (zowel voor anthracyclines als voor taxanes) tussen borstkanker patiënten met een *BRCA1/2* genmutatie en sporadische borstkankerpatiënten. Ook vonden we geen verschillen tussen beide groepen in het optreden van aantal patiënten waar neutropene koorts optrad, uitstel van chemotherapie toediening of verandering van chemotherapie schema ten gevolge van toxiciteit nodig was. De afwezigheid van toegenomen acute toxiciteit ten gevolge van (neo)adjuvante chemotherapie onder *BRCA1/2* genmutatie draagsters, vergeleken met sporadische borstkankerpatiënten, laat zien dat de reparatie mechanismes voor dubbelstrengs DNA schade van gezonde lichaamscellen met slechts één normale kopie van ofwel het *BRCA1* gen ofwel het *BRCA2* gen voldoende functioneel zijn zodat niet meer ernstige acute chemotherapie geassocieerde toxiciteit van de onderzochte chemotherapie schema's gezien wordt in *BRCA1/2* genmutatie draagsters vergeleken met sporadische borstkankerpatiënten. Echter platinum derivaten en in de toekomst mogelijk ook PARP remmers worden steeds vaker gebruikt bij *BRCA1/2*-geassocieerde borstkanker. Deze geneesmiddelen hebben een veel hogere capaciteit om dubbelstrengs DNA breuken te induceren vergeleken met antracyclines. Gegevens over de toxiciteit van deze middelen bij *BRCA1/2* genmutatie draagsters zullen dan ook moeten volgen uit lopende en nieuwe studies.

Kan toxiciteit gebruikt worden om behandeling te optimaliseren?

Het is een interessant gegeven dat het optreden van toxiciteit door anti-kankertherapie geassocieerd kan zijn met hogere effectiviteit. Meerdere studies hebben laten zien dat de mate van hematologische toxiciteit door behandeling met chemotherapie bij borstkankerpatiënten, gerelateerd lijkt aan de effectiviteit. Hierdoor kan toxiciteit gebruikt worden om effectiviteit van bijvoorbeeld medicamenteuze anti-kanker therapie te verhogen door de dosering te verhogen bij patiënten met geen of slechts beperkte toxiciteit. In **Hoofdstuk 5** beschrijven we de resultaten van een single-center prospectief

onderzoek naar de haalbaarheid van dosisverhoging van anthracycline-cyclofosfamide bevattende chemotherapie bij sporadische borstkankerpatiënten op geleide van het aantal neutrofielen. Tweeëndertig patiënten die startten met drie-wekelijkse anthracycline-cyclofosfamide bevattende chemotherapie zonder G-CSF, in adjuvante dan wel gemetastaseerde setting, werden geïncludeerd in deze studie. De eerste chemotherapie kuur werd gegeven in een standaard dosis, gebaseerd op lichaamsoppervlak. Bij patiënten met geen of milde verlaging van de neutrofielen (CTC graad 0–2) en geen andere dosis-limiterende toxiciteit werd de dosis van de tweede kuur met 10-25% geëscaleerd, met de mogelijkheid voor een eventuele verdere dosisescalatie van 10-25% bij de derde kuur.
Twee van de 23 patiënten (9%) die behandeld werden met FEC (fluorouracil 500 mg/m^2, epirubicine 100 mg/m^2 en cyclofosfamide 500 mg/m^2) ontwikkelden, na de eerste FEC-kuur toediening slechts een milde verlaging van het aantal neutrofielen. Bij deze twee patiënten werd dosisescalatie toegepast (30% bij de ene en 15% bij de andere patiënte). Na dosisescalatie waren er geen complicaties, zoals neutropene koorts. Bij geen van de patiënten die behandeld werden met FAC (fluorouracil 500 mg/m^2, doxorubicine 50 mg/m^2 en cyclofosfamide 500 mg/m^2) of AC (doxorubicine 60 mg/m^2 en cyclofosfamide 600 mg/m^2) kon dosisescalatie worden toegepast omdat al deze patiënten reeds na de eerste kuur ernstige verlaging van het neutrofielen aantal (CTC graad 3-4) ontwikkelden. Escalatie van de huidige standaard dosis van anthracycline-cyclofosfamide bevattende chemotherapie zonder G-CSF is slechts mogelijk in een klein deel van de patiënten, maar lijkt dan wel haalbaar. Onderzoek naar dosisverhoging op geleide van het aantal neutrofielen ten einde de effectiviteit te verhogen zou wellicht zinvol kunnen zijn voor andere chemotherapeutica.

Bijwerkingen van behandeling, therapietrouw en spiegelbepalingen

Adjuvante endocriene therapie, die vaak jarenlang gebruikt moet worden, is een belangrijk onderdeel van de behandeling van hormoonreceptor positieve borstkanker met bewezen gunstig effect. Desondanks is aangetoond dat er vaak sprake is van verminderde therapietrouw, vaak ingegeven door hinderlijke bijwerkingen, die een belangrijke invloed kunnen hebben op de kwaliteit van leven. Therapietrouw, verandering in leefstijlfactoren en mogelijk resistentiemechanismen kunnen de blootstelling van een geneesmiddel over de tijd veranderen. De beste manier om voortgang te boeken in individualiseren van behandeling lijkt 'therapeutic drug monitoring' (TDM) te zijn. De vaak gebruikte methode van TDM door het herhaaldelijk meten van plasmaconcentraties van geneesmiddelen of metabolieten over de tijd is moeilijk in te bouwen in de klinische praktijk gezien de belasting voor de patiënten. Een methode, die (achteraf) informatie geeft over de gemiddelde concentratie van antikanker geneesmiddelen (en de metabolieten) over de tijd, zou gevonden kunnen worden in het meten van deze concentraties in hoofdhaar. Dit geeft de mogelijkheid om, afhankelijk van de lengte van het verzamelde haar, het gemiddelde van geneesmiddel concentraties over de tijd te bestuderen. Dit maakt herhaaldelijke bloedafnames onnodig. In **Hoofdstuk 6** beschrijven we de validatie van een eerder ontwikkelde UPLC-MS/MS (ultra performance liquid chromatography/ tandem mass spectrometry) methode voor het kwantificeren van tamoxifen en de drie belangrijkste

metabolieten (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen, en 4-hydroxy-N-desmethyl-tamoxifen) in hoofdhaar. Met de gegevens uit dit onderzoek kunnen we concluderen dat het meten van de concentratie van tamoxifen en de belangrijkste metabolieten in haar mogelijk is met de UPLC-MS/MS methode. Het lijkt voor tamoxifen echter niet mogelijk om de blootstelling over de tijd vast te stellen met segmentele analyse van haar, meest waarschijnlijk grotendeels ten gevolge van het effect van UV straling, waardoor tamoxifen en de metabolieten afgebroken worden. Verder onderzoek naar de waarde van geneesmiddelconcentraties in hoofdhaar zou zich daarom dan ook moeten toespitsen op het kwantificeren van anti-kanker geneesmiddelen, die minder gevoelig zijn voor UV straling. Voor onderzoek naar TDM bij patiënten die behandeld worden met tamoxifen, blijft het meten van endoxifen concentraties in plasma de gouden standaard.

Preventie en behandeling van toxiciteit

Eén van de belangrijke bijwerkingen van de behandeling van borstkanker is het optreden van vroegtijdig verlies van botdichtheid. Dit kan ontstaan door verschillende mechanismes: vroegtijdige menopauze door chemotherapie en/of endocriene therapie, lagere spiegels van oestrogeen ten gevolge van aromatase-remmers en de directe invloeden van chemotherapie en ondersteunende medicatie, zoals steroïden. Daarnaast zijn botuitzaaiingen vaak de eerste uiting van uitgezaaide borstkanker. Het ontstaan van botcomplicaties, gedefinieerd als pathologische breuken, noodzaak voor bestraling of operatie ten gevolge van (dreigende) compressie van het ruggenmerg of hypercalciëmie ten gevolge van botmetastasen, heeft een negatieve invloed op de kwaliteit van leven. Lange tijd zijn bisfosfonaten de hoeksteen geweest van de preventie en behandeling van botcomplicaties zowel bij uitgezaaide borstkanker als bij botverlies ten gevolge van behandeling bij vroegstadium borstkanker. Denosumab, een volledig gehumaniseerd monoklonaal antilichaam, dat bindt aan de receptoractivator van het nucleaire factor kappa-B ligand (RANKL) is ontwikkeld als een nieuwe botgerichte therapie, die gebruikt kan worden in zowel vroegstadium als uitgezaaide borstkanker. In **Hoofdstuk 7** beschrijven we de huidige indicaties voor denosumab zowel bij vroegstadium als uitgezaaide borstkanker. We concluderen dat er nog steeds een aantal vragen om beantwoording vragen, zoals waar denosumab te plaatsen ten opzichte van bisfosfonaten, of individualisering van de dosering (bijvoorbeeld op basis van bot turnover marker) mogelijk is en of er een plaats is voor denosumab in de preventie van het ontstaan van botmetastasen of mogelijks zelfs in de preventie van het ontstaan van borstkanker. Desalniettemin is denosumab een zeer effectief middel om botcomplicaties door osteoporose of botuitzaaiingen van borstkanker te voorkomen, met beperkte bijwerkingen.

Uitstellen van toxische chemotherapie bij HER2-positieve uitgezaaide borstkanker?

De standaard eerstelijns behandeling voor de subgroep van HER2-positieve uitgezaaide borstkanker bestond tot recent uit een combinatie van trastuzumab met een taxaan (docetaxel of paclitaxel). Vooruitgang in de behandeling van deze subgroep van patiënten kan op meerdere manieren

gemaakt worden. Een manier zou kunnen zijn het verhogen van de effectiviteit van de behandeling door het toevoegen van een nieuw medicament met beperkte bijwerkingen. Een andere optie zou kunnen zijn om te proberen de toxiciteit te verlagen door het starten van chemotherapie uit te stellen door het toevoegen van een nieuw medicament met beperkte toxiciteit aan trastuzumab en alleen te starten met chemotherapie bij progressie onder dit schema. Bevacizumab, een monoklonaal antilichaam tegen vasculaire endotheliale groei factor (VEGF), zou een van de medicamenten kunnen zijn om in de genoemde strategieën te gebruiken.

In **Hoofdstuk 8** beschrijven we de resultaten van een open-label, gerandomiseerde, niet-vergelijkende fase 2 studie, waarbij 84 patiënten werden gerandomiseerd tussen HAT (de combinatie van bevacizumab, trastuzumab en wekelijks paclitaxel) en HA-HAT (starten met trastuzumab en bevacizumab met toevoegen van wekelijks paclitaxel bij progressie). Het percentage patiënten dat progressie-vrij was na een jaar was 74.4% (95% betrouwbaarheidsinterval, BI 61.8-89.4) en 62.2% (95% BI 49.6-89.4) in respectievelijk de HAT en HA-HAT arm. De mediane progressie-vrije overleving in de HAT arm was 19.8 maanden (95% BI 14.9-25.6), en de mediane totale progressie-vrije overleving in de HA-HAT arm was 19.6 maanden (95% BI 12.0-32.0). In de HA-HAT arm was de mediane progressie-vrije overleving voor behandeling met alleen bevacizumab en trastuzumab 10.4 maanden (95% BI 6.2-15.0) en de mediane progressie-vrije overleving voor behandeling met trastuzumab, bevacizumab en paclitaxel na eerste progressie was 8.2 maanden (95% BI 7.0-12.6). Het aantal en de ernst van de bijwerkingen was vergelijkbaar tussen de twee armen en was in het voordeel van starten met alleen bevacizumab en trastuzumab.

Wanneer vergeleken wordt met historische controles, suggereren de uitkomsten dat bevacizumab de antitumor activiteit verhoogd van de trastuzumab-gebaseerde schema's, zoals gebruikt in dit onderzoek. Met de publicatie van de resultaten van de CLEOPATRA trial, waarin een mediane totale overlevingsvoordeel van 15.7 maanden gezien werd voor de combinatie van trastuzumab, pertuzumab en docetaxel, vergeleken met de combinatie van trastuzumab en docetaxel, is dit schema de standaard eerstelijnsbehandeling geworden voor patiënten met HER2-geassocieerde uitgezaaide borstkanker. Deze gegevens en het gunstige bijwerkingenprofiel van pertuzumab vergeleken met bevacizumab in ogenschouw nemende, is het onwaarschijnlijk dat er nog fase 3 studies gedaan zullen worden naar de combinatie van trastuzumab, bevacizumab en chemotherapie. De resultaten van ons onderzoek laten wel zien dat het concept van starten met een minder toxische, chemotherapie-vrije behandeling met alleen monoklonale antilichamen een strategie is die verder onderzoek verdient. Vanuit het oogpunt van kwaliteit van leven gezien, is het van groot belang om behandelingsschema's te ontwikkelen waarbij gestart wordt met minder toxische behandelingen.

Conclusie

In de afgelopen jaren is duidelijke voortuitgang geboekt in de behandeling van zowel vroegstadium als uitgezaaide borstkanker. Deze vooruitgang gaat helaas (deels) ten koste van toxiciteit. Eén van de belangrijke doelen in het onderzoek naar de behandeling van borstkanker moet dan ook zijn het

verminderen van de met de behandeling samenhangende acute en lange-termijns toxiciteit. Daarom is het van groot belang om zowel preklinisch onderzoek, klinisch onderzoek en observationeel onderzoek (prospectief en retrospectief) te blijven verrichten.

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DANKWOORD

Dit hoofdstuk zal waarschijnlijk één van de meest gelezen hoofdstukken van dit boekje zijn. Dit terwijl de wetenschappelijke waarde van dit hoofdstuk zeer beperkt is. Desondanks is er veel reden om iedereen te bedanken die een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik hier in het bijzonder bedanken.

Als eerste wil ik mijn promotor noemen, prof.dr. S. Sleijfer. Beste Stefan. Heel veel dank voor het vertrouwen dat je me de afgelopen jaren hebt gegeven. Toen ik net gestart was met de deelspecialisatie interne oncologie, vroeg ik je een keer of er mogelijkheden waren voor wetenschappelijk onderzoek binnen de afdeling. Kort daarna kwam je al met opties voor onderzoek wat zou kunnen resulteren in een promotie-onderzoek. Zowel tijdens de periode dat ik in het Erasmus MC werkte, als na mijn vertrek voor een baan in een niet-academisch (perifeer klinkt zo afgelegen) ziekenhuis, ben je altijd zeer betrokken en laagdrempelig toegankelijk geweest. Genoemd moet ook worden de snelheid waarmee jij manuscripten beoordeelde, met altijd een paar zinvolle globale suggesties voor verbetering, maar ook oog voor details.

Als tweede (of gedeelde eerste, zoals dat met auteursplaatsen bij wetenschappelijke artikelen ook gaat) mijn promotor, dr. A. Jager. Beste Agnes, veel dank voor de begeleiding in de afgelopen jaren. Jouw reacties op manuscripten lieten soms wat langer op zich wachten, maar waren dan in het algemeen ook uitgebreid en op een manier, waarop het verbeteren van een manuscript voor mij ook een leertraject was. Dank ook voor het feit dat je bij herhaling meedacht over nieuw onderzoek, op de momenten dat een voorgenomen onderzoek niet door kon gaan vanwege financiering of vanwege onvoldoende resultaten in de pilot fase. Dank voor alle tijd die je in mijn begeleiding gestoken hebt.

Leden van de kleine commissie, prof.dr. J.P. Pignol, prof.dr. C. Verhoef en prof.dr. E. van der Wall, jullie wil ik hartelijk bedanken voor jullie bereidheid en tijd om mijn proefschrift te beoordelen. Fijn dat jullie alle drie ook bij de verdediging aanwezig hopen te zijn. Daarnaast wil ik ook de leden van de grote commissie, prof.dr. C.C.D. van der Rijt, dr. L.B. Koppert, dr. J.R. Kroep, hartelijk bedanken voor de tijd en moeite om het proefschrift te beoordelen en de bereidwilligheid om aanwezig te zijn bij de verdediging. De behandeling van borstkanker is nagenoeg altijd multidisciplinair. Een multidisciplinaire borstkankerpatiëntenbespreking kan, zonder tekort te doen aan de andere participerende disciplines, in de praktijk niet van start gaan zonder aanwezigheid van een oncologisch chirurg, een medisch oncoloog, en een radiotherapeut. Dit geldt mijns inziens ook voor de beoordeling, maar ook voor de verdediging van dit proefschrift. Dank dat jullie hier dan ook zorg voor dragen.

Bij de multidisciplinaire behandeling van borstkanker zijn nog vele anderen medisch specialisten en zorgprofessionals betrokken, zoals: fysiotherapeut, klinisch geneticus, maatschappelijk werkende,

mammacare verpleegkundige, medisch psycholoog, oncologieverpleegkundige, palliatief verpleegkundige, patholoog, plastisch chirurg, radioloog, verpleegkundig specialist enzovoorts.

Dit geldt evenzeer voor dit proefschrift. Zonder de hulp, ondersteuning en bijdrage van velen was dit proefschrift er nooit gekomen. Een aantal van hen wil ik met name noemen.

Beste Delal, dank voor het meedenken en meeschrijven aan het artikel over radiotherapie en risico op contralaterale borstkanker (Hoofdstuk 3). Dit artikel is voor jou ook het eerste manuscript voor jouw proefschrift. Heel veel succes met al het werk dat nog voor je ligt. Daniëlle, dank voor het opmaken van de figuren in dit hoofdstuk.

Beste Nyree en Nasrin. Dank voor jullie bijdrage aan het statusonderzoek wat ten grondslag ligt aan Hoofdstuk 4. Annette, veel dank voor het verrichten van de statistische analyses en het meedenken over deze studie.

Beste Tilly, dank voor je meedenken en inclusie van patiënten in de 'Tamoxifen in haar' studie (Hoofdstuk 6). Dankzij jou hadden we snel de benodigde patiënten bij elkaar. Peter, dank voor het verrichten van en de uitleg over de analyses, zoals die in jouw laboratorium verricht zijn. Ron, dank voor de begeleiding bij dit onderdeel van het proefschrift.

Beste Harm, Harriet, Steffen en Elise, dank voor jullie ondersteuning bij dataverzameling, interpretatie en logistiek van de HAT studie (Hoofdstuk 7).

Beste Caroline, Maartje, Margreet en Inge-Marie, dank voor jullie kritisch meedenken en meeschrijven met één of meerdere studies in dit proefschrift.

Medewerkers van de afdeling hyperthermie en genetica, dank voor jullie bijdrage in het hyperthermie biopten project. Helaas heeft dit project tot nog toe geen beschrijvenswaardig wetenschappelijk resultaat opgeleverd.

Dit laatste geldt ook voor de AMH studie, met dit verschil dat de resultaten wel op niet al te lange termijn tegemoet gezien kunnen worden. Collega's uit Erasmus MC, Maasstad, SFG en Ikazia, dank voor de gestage inclusie in deze studie. Mandy, dank voor het overnemen van de logistiek van deze studie.

Alle co-auteurs wil ik bedanken voor hun kritisch meedenken, meeschrijven en doen van suggesties voor verbetering van de manuscripten.

De stafleden van de afdeling interne oncologie van het Erasmus MC wil ik bedanken voor hun betrokkenheid en de inclusie van patiënten in een deel van de gedane studies.
Veel dank aan alle secretaresses (van beide locaties), datamanagers, (research)verpleegkundigen en verpleegkundig specialisten van de afdeling interne oncologie van het Erasmus MC, die op welke manier dan ook, een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Silvia, mijn secretaresse in de periode als chef de clinique in het Erasmus MC, veel dank voor het puzzelen met alle afspraken die gemaakt moesten worden. Dank ook dat je in de periode dat ik formeel geen recht meer had op secretariële ondersteuning toch de dingen voor me deed die een secretaresse veel sneller en efficiënter kan doen dan een dokter.

Van mijn mede-promovendi wil ik een aantal met name noemen. Evelien Kuip, Astrid Oosten en Annemieke van der Padt. Wij zaten min of meer in het zelfde schuitje: beginnen aan een promotieonderzoek aan het eind van of zelfs na de opleiding tot internist-oncoloog. Favoriet gespreksonderwerp was dan ook vaak de moeilijkheden, maar ook de voordelen en uitdagingen die het combineren van klinische taken, staftaken en promotieonderzoek met zicht meebrengt. Ik heb er alle vertrouwen in dat ik ook van jullie (binnenkort) een boekje te zien krijg.

Mijn huidige collega's, intussen maten, in het Ikazia Ziekenhuis: Ad, Adrienne, Claudia, Esther, Felix, Fransien, Geert, Ginette, Hestia, Marieke, Michel, Pieter en Rob. Dank voor de fijne samenwerking die we al weer twee jaar mogen hebben. Veel dank voor de mogelijkheid, die jullie gegeven hebben om de eerste periode dat ik in het Ikazia werkte, voldoende tijd vrij te maken om aan dit proefschrift te werken. Zonder die mogelijkheid, had dit proefschrift langer op zich laten wachten. Nu ik toegetreden ben tot de maatschap en dit boekje af is, hoop ik meer tijd vrij te kunnen maken voor andere niet-patiënt gebonden taken.

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Daarnaast bijzondere dank voor Felix de Jongh, die tijdens mijn periode als arts-assistent in het Ikazia voor een eerste echte kennismaking met de interne oncologie zorgde. Mede dankzij jou, heb ik uiteindelijk besloten om me te specialiseren in de interne oncologie. Dank ook voor de mogelijkheid die je bood om de analyses en het manuscript van de ANCHOR studie uit te werken, zodat deze studie in dit proefschrift opgenomen kon worden (Hoofdstuk 5). Dit kwam erg gelegen op het moment dat twee andere veel belovende projecten strandden. Heel fijn dat je me op de dag van de verdediging ter zijde wil staan als paranimf.

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Beste schoonvader, heel veel dank voor uw interesse in mij en mijn werkzaamheden. U heeft van heel dichtbij met borstkanker te maken gehad. Ook na het overlijden van uw vrouw aan de indirecte gevolgen van de ziekte waarover dit proefschrift gaat, heeft u altijd oprechte interesse getoond. Daarnaast stond u regelmatig klaar voor Arine en de jongens, als ik even geen tijd voor hen had. Dank ook voor de afleiding, onder andere door de familievakanties in Limburg. Hartelijk dank daarvoor.

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Lieve Arine, dank je wel voor alles wat je voor mij betekent. Je zal het me niet kwalijk nemen, dat ik dat 'alles' niet helemaal uitleg in dit dankwoord. Dank voor je onvoorwaardelijke liefde. Dank voor alle emotionele, psychische, maar ook praktische hulp.

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Boven alles past dankbaarheid aan God. Hij gaf mij de talenten, maar ook de kracht en lust om dit werk te doen.

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CURRICULUM VITAE

Jan Cornelis Drooger werd geboren op 3 april 1982 te Rotterdam. In 2000 voltooide hij het gymnasium aan het Wartburg college, locatie Guido de Brès te Rotterdam. In hetzelfde jaar begon hij de studie Geneeskunde aan het Erasmus MC. In 2004 behaalde hij zijn doctoraal examen. In 2003 startte hij met een Master of Sciene programma bij het Netherlands Institute for Health Science, wat hij in 2004 afrondde met behalen van de titel Master of Science in Public Health. In 2006 deed hij artsexamen in het Erasmus MC. Aansluitend werkt hij als arts niet in opleiding tot specialist in het Ikazia Ziekenhuis in Rotterdam, alwaar hij 1 januari 2008 aan de opleiding tot internist begon (opleiders prof.dr. J.L.C.M. van Saase en dr. A. Dees). In 2010 vervolgde hij zijn opleiding in het Erasmus MC. Vanaf 2011 specialiseerde hij zich binnen de interne geneeskunde in de medische oncologie (opleider dr. A. van der Gaast). Tegelijkertijd werd gestart met wetenschappelijk onderzoek binnen de afdeling Interne Oncologie van het Erasmus MC Kanker Instituut, onder supervisie van dr. A. Jager en prof.dr. S. Sleijfer. Vanaf september 2013 werkte hij als internist-oncoloog in het Erasmus MC Kanker Instituut, locatie Daniel den Hoed. Vanaf 1 februari 2014 werkt hij als internist-oncoloog in het Ikazia Ziekenhuis. Tot 1 februari 2015 had hij nog een gedeeltelijke aanstelling in het Erasmus MC voor het afronden van zijn wetenschappelijk onderzoek, wat uiteindelijk heeft geresulteerd in dit proefschrift.

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PHD PORTFOLIO (2011-2016)

	Year	Workload ECTS
1. PhD training		
General courses		
- Good clinical practice	2011-2014	1
- Integrity in research, Erasmus MC	2014	1
- BROK (basiscursus Regelgeving Klinisch Onderzoek) course	2015	1
Specific courses (e.g. Research school, Medical Training)		
Seminars and workshops		
- opleidingsdagen/avonden jNVMO	2011-2013	1
- Course 'communicatie in de oncologie'	2012	1
- Course 'Desiderius cursus ziekenhuismanagement'	2013	1
- Roche4young, 'nascholing voor jonge oncologen'	2014-2015	1
Presentations		
- Klinische research bespreking, oral presentations	2011-2014	1
- IKNL netwerkdagen, Middelburg, oral presentation	2012	1
- OVUM overleg, oral presentation,	2012	0.5
- Mamma beleidsgroep, oral presentation	2013	0.5
- Radiotherapie researchbespreking, oral presentation	2013	0.5
- San Antonio Breast Cancer Symposium, poster presentation	2014	1
- Oncology TV, oral presentation	2014	0.5
- Scientific meeting Medical Oncology, oral presentation	2015	1
- NABON/BOOG meeting, oral presentation	2015	1
- European Breast Cancer Conference, poster presentation	2016	1
(Inter) national conferences		
- Oncologiedagen	2011-2016	1
- Internistendagen	2012, 2016	1
- ESMO Annual meeting, Vienna, Austria	2012	1
- Jaarsymposium Continuüm oncologie	2013	1
- San Antonio Breast Cancer Symposium, San Antonio, USA	2014	1
- Symposium borstkanker behandeling beter	2011-2016	1
- European Breast Cancer Conference, Amsterdam	2016	1
Other		
- Monodisciplinair onderwijs Medical Oncology, Erasmus MC	2011-2013	1
- OMBO training, Erasmus MC	2011-2014	1
- Refereereerbijeenkomst Medical Oncology, Erasmus MC	2011-2014	1
- Polikliniekbespreking Medical Oncology, Erasmus MC	2011-2014	1.5
- Complicatiebespreking Medical Oncology, Erasmus MC	2011-2014	0.5
- Scientific meeting Medical Oncology, Erasmus MC	2012-2015	1
- Borstkanker research bespreking, Erasmus MC	2014	0.5
- Regiobijeenkomst fertiliteitsbehoud	2015	0.5

	Year	Workload ECTS
2. Teaching		
Lecturing		
- biennially education radiation therapy operators	2012-2015	1
- education oncology nurses, Ikazia	2015	0.5
- education AIOS/ANIOS, Ikazia	2015	0.5
- NPV Barendrecht: lecture 'Borstkanker, en dan…'	2016	0.5
Supervision		
- supervision review article minor students	2014	0.5
- supervision medical students clinical research	2014-2015	1