RECEPTOR CONVERSION IN DISTANT BREAST CANCER METASTASES

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Receptor conversion in distant breast cancer metastases
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RECEPTOR CONVERSION IN DISTANT BREAST CANCER METASTASES

RECEPTOR CONVERSIE IN AFSTANDSMETASTASEN VAN BORSTKANKER

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Promotoren: Prof. dr. P.J. van Diest
Prof. dr. E. van der Wall

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CHAPTER 1

General introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide with over 1.3 million new cases per year[1]. In The Netherlands, 12-13% of women will develop breast cancer during their life. Despite substantial advances in early diagnostics, optimal surgery and adjuvant systemic therapy, one third of breast cancer patients will eventually develop distant metastases, the most common sites being the bone, liver and lungs[2]. There is an urgent need to understand the process of distant metastases formation, as they are the main cause of death in breast cancer patients. Women diagnosed with metastatic breast cancer face the double burden of an illness associated with substantial symptoms and the knowledge that metastatic breast cancer is ultimately incurable, although treatable[3]. The median overall survival for metastatic breast cancer is 18 to 24 months, though this varies between patients from a few months to many years[4].

The estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are therapeutically and prognostically important and predictive biomarkers in the management of breast cancer. About 70% of primary breast carcinomas express ERα and/or (ERα-regulated) PR, referred to as "ERα or PR positive". These receptors play a critical role in breast tumorigenesis by stimulating breast epithelial cell proliferation on estrogen and/or progesterone stimulation. Hormone receptors are considered the main predictive marker of clinical benefit of hormonal therapy, like tamoxifen (a selective ERα modulator (SERM) that competes with estrogens) or aromatase inhibitors (which block the production of estrogens by inhibiting aromatase)[5, 6]. Immunohistochemistry (IHC) is the predominant method for assessing ERα and PR status in clinical practice. In Europe, tumors with 10% or more immunopositive malignant cells are classified as ERα or PR positive, while the American Society of Clinical Oncology (ASCO) uses a 1% threshold for receptor positivity[7]. Hormonal therapy is the preferred option for hormone receptor positive disease, both in the adjuvant and metastatic settings[8, 9]. The use of adjuvant hormonal therapy reduces the annual breast cancer death rate by no less than 31%[10]. Among women with newly diagnosed metastatic disease, approximately 30 to 40% will have an objective response to hormonal therapy and a clinically significant period of disease stability. Although usually better tolerated than chemotherapy, hormonal therapy is associated with side effects like thromboembolic events, osteoporosis and increased risk of endometrial cancer[6].
Amplification of the HER2 oncogene (also referred to as HER-2/*neu*) and overexpresssion of the corresponding HER2 encoded protein are found in 10-20% of invasive breast cancers, and is associated with enhanced aggressiveness and worse prognosis. Amplification or overexpression of HER2 (the latter as determined by IHC, scored using the DAKO scoring system as 0, 1+, 2+ and 3+ according to standardized criteria[11], considering 3+ cases as positive) predicts response to trastuzumab, a monoclonal antibody targeting the HER2 receptor. Trastuzumab was originally approved for the treatment of metastatic HER2 positive breast cancer, because of a significant survival benefit when given in combination with chemotherapy. More recently adjuvant treatment of primary HER2 positive breast cancer with trastuzumab has been shown to improve patient outcome[12]. However, given the therapy side effects (cardiotoxicity) and high costs (€30.000-40.000 per year), accurate patient selection is essential.

When breast cancer patients develop distant metastases, the choice of systemic treatment with chemotherapy, hormonal therapy, or HER2 targeted therapy is currently based on receptor assessment of the primary tumor by routine histopathology, IHC, and/or molecular analysis, since metastatic tumors are not often biopsied because of limited accessibility of these metastases, or because it is deemed unnecessary for further therapeutic decision making. However, previous studies reveal lack of stability of the hormonal and/or HER2 markers during tumor progression whereby the receptor status of breast cancer metastases may differ from the primary tumor, generally denoted "receptor conversion".
These observations, if confirmed, have important clinical consequences, since this would mean that a number of patients are withheld adequate systemic treatment for their metastases. In addition, if receptor conversion would occur in high frequency, this would make it clinically very relevant to biopsy distant metastases to re-evaluate receptor status to guide treatment in the metastatic setting. Unfortunately, previous receptor conversion studies suffer from several limitations. Therefore, the available data may not be sufficiently reliable to change current clinical practice. The aim of this thesis was therefore to evaluate differences in tumor characteristics by IHC and/or molecular analysis between primary breast cancer and corresponding distant metastases and possible implications for clinical practice. We were able to establish a large series of 233 patients with rare biopsy material of both metastases and primary breast cancers by making use of the pathology archives of many hospitals in The Netherlands.

Outline of the thesis

In **chapter 2** we study receptor conversion for ERα, PR, and HER2 in a large group of distant (non-bone) breast cancer metastases by re-staining all primary tumors and metastases with current optimal immunohistochemistry on full sections. Next, in **chapter 3**, we evaluated the prognostic value of receptor conversion for ERα and PR. If receptor conversion would occur in high frequency, this would make it clinically very relevant to biopsy distant metastases to re-evaluate receptor status. However this may not always be feasible because of limited lesion accessibility or potential complications. Therefore, accurate prediction of receptor conversion in metastatic breast cancer patients obviating a biopsy would be valuable. **Chapter 4** describes the development of prediction models for hormone receptor conversion in metastatic breast cancer patients, using routinely available clinicopathological information. Previous studies have been done on single distant metastases, while patients often develop multiple metastases. We therefore aimed to study discordance of receptor status between different distant metastases from the same patient in **chapter 5**.
Also other predictive biomarkers may show conversion in distant metastases. In **chapter 6**, we therefore compared expression of phosporylated (p-mTOR) in breast cancer metastases with their primary tumors in patients who received adjuvant endocrine therapy.
We initially made the deliberate choice not to include bone metastases in our receptor conversion studies to avoid false negative immunohistochemical results due to potential

decalcification artifacts. Evidence for this was however limited. We therefore studied in **chapter 7** the influence of different decalcification techniques in receptor expression in breast cancer. In **chapter 8**, we touch on the molecular background of distant breast cancer metastases formation by comparing copy number of common breast cancer genes between primary breast carcinoma and distant metastases. We conclude with a summarizing discussion of the contents of this thesis (**chapter 9**).

REFERENCES

1. Society AC: Global Cancer Facts & Figures 2nd Edition. 2011.
2. Rabbani SA, Mazar AP: Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer Metastasis Rev 2007, 26:663-674.
3. Cardoso F: Metastatic breast cancer patients: the forgotten heroes! Breast 2009, 18:271-272.
4. Greenberg PA, Hortobagyi GN, Smith TL, Ziegler LD, Frye DK, Buzdar AU: Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer. J Clin Oncol 1996, 14:2197-2205.
5. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF, Bast RC, Jr.: American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007, 25:5287-5312.
6. Riggs BL, Hartmann LC: Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. N Engl J Med 2003, 348:618-629.
7. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, et al: American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol, 28:2784-2795.
8. Cardoso F, Costa A, Norton L, Cameron D, Cufer T, Fallowfield L, Francis P, Gligorov J, Kyriakides S, Lin N, et al: 1st International consensus guidelines for advanced breast cancer (ABC 1). Breast, 21:242-252.
9. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E: Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol, 23 Suppl 7:vii11-19.
10. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005, 365:1687-1717.
11. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al.: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707-712.
12. Tuma RS: Trastuzumab trials steal show at ASCO meeting. J Natl Cancer Inst 2005, 97:870-871.


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metastases metastases

CHAPTER 2

Receptor conversion in distant breast cancer metastases

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ABSTRACT

Background

When breast cancer patients develop distant metastases, the choice of systemic treatment is usually based on tissue characteristics of the primary tumor as determined by immunohistochemistry (IHC) and/or molecular analysis. Several previous studies have shown that the immunophenotype of distant breast cancer metastases may be different from that of the primary tumor ("receptor conversion"), leading to inappropriate choice of systemic treatment. The studies published so far are however small and/or methodologically suboptimal. Therefore, definite conclusions that may change clinical practice could not yet be drawn. We therefore aimed to study receptor conversion for estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in a large group of distant (non-bone) breast cancer metastases by re-staining all primary tumors and metastases with current optimal immunohistochemical and in situ hybridization methods on full sections.

Methods

233 distant breast cancer metastases from different sites (76 skin, 63 liver, 43 lung, 44 brain and 7 gastro-intestinal) were IHC stained for ERα, PR and HER2, and expression was compared to that of the primary tumor. HER2 in situ hybridization (ISH) was done in cases of IHC conversion or when primary tumors or metastases showed an IHC 2+ result.

Results

Using a 10% threshold, receptor conversion by IHC for ERα, PR occurred in 10.3%, 30.0% of patients, respectively. In 10.7% of patients, conversion from "ER+ or PR+" to ER-/PR- and in 3.4% from ER-/PR- to "ER+ or PR+" was found. Using a 1% threshold, ERα and PR conversion rates were 15.1% and 32.6%. In 12.4% of patients conversion from "ER+ or PR+" to ER-/PR-, and 8.2% from ER-/PR- to "ER+ or PR+" occurred. HER2 conversion occurred in 5.2%. Of the 12 cases that showed HER2 conversion by IHC, 5 showed also conversion by ISH. One further case showed conversion by ISH, but not by IHC. Conversion was mainly from positive in the primary tumor to negative in the metastases for ERα and PR, while HER2 conversion occurred equally both ways. PR conversion occurred significantly more often in liver, brain and gastro-intestinal metastases.

Conclusion

Receptor conversion by immunohistochemistry in (non-bone) distant breast cancer metastases does occur, is relatively uncommon for ERα and HER2, and more frequent for PR, especially in brain, liver and gastro-intestinal metastases.

Introduction

With 1 million new cases causing 375,000 deaths worldwide per year, breast cancer is the leading cause of female cancer death worldwide [1]. Early detection, optimal surgery and adjuvant therapy are the key strategies to improving prognosis. Nevertheless, about one third of patients will develop distant metastases and eventually die of the disease. Patients who develop distant metastases usually undergo systemic therapy with chemotherapy, hormonal therapy and/or human epidermal growth factor receptor 2 (HER2) targeted therapy. Choice of therapy is currently personalized on the basis of the immunophenotype of the primary tumor, since distant metastases are often not biopsied, partly because of limited accessibility of these metastases, but also because it is not considered necessary for further therapeutic decision making.

However, previous studies[2-23] have indicated that receptor status of breast cancer metastases may differ from the primary tumor, generally denoted "receptor conversion". The published studies suggest that, compared to the primary tumors, estrogen receptor (ERα) and progesterone receptor (PR) are more frequently negative in distant metastases, whereas HER2 is more often positive. These observations, if confirmed, have important clinical consequences, since this would mean that a number of patients are withheld adequate systemic treatment for their metastases. In addition, if immunophenotype conversion would occur in high frequency, this would make it clinically very relevant to biopsy (even difficult to access) distant metastases to assess hormone receptor and HER2 status. An alternative for taking biopsies could be molecular imaging methods like PET and SPECT that are currently being developed to functionally assess immunophenotype of breast cancer metastases [24-26].

Unfortunately, the previous conversion studies suffer from several limitations: small size (six studies contain < 30 cases and eight studies ≤ 62 cases) [2, 3, 6, 7, 9, 10, 12-15, 18-20, 22], only one metastatic site studied[9, 18], using a ligand-binding assay [2, 3, 7, 10], which, especially in the case of metastases, may be biased by low cellularity and contamination by nonmalignant cells, inclusion of decalcified bone metastases [2, 4, 6, 8-10, 12, 16, 19, 20, 22] that may give rise to false negative immunohistochemistry , extraction of original immunohistochemistry results from the pathology report instead of repeating the staining [3, 5, 8, 10, 11, 23], and/or use of tissue arrays [21] which may introduce sampling bias. Therefore, the available data may not sufficiently reliable to change current clinical practice, although several guidelines already advise to rebiopsy distant metastases when possible [27, 28]. Consequently, for most patients with metastatic breast cancer hormone receptor and HER2 status in the primary tumor are still used to guide therapy.

We have now performed a large study analyzing metastases from different sites, while restaining metastases and primary tumors side-by-side with optimal current immunohistochemical methods for ERα, PR and HER2 on full sections to asses the conversion rate of ERα, PR and HER2 status in distant metastases compared to the primary breast carcinomas.

Materials and Methods

Patients

Two hundred and thirty three primary breast carcinomas and corresponding metachronous non-bone distant metastases from female patients were obtained from the departments of pathology of the University Medical Center Utrecht, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Centre, the Canisius Wilhelmina Hospital Nijmegen, the Netherlands Cancer Institute Amsterdam, the Medical Center Alkmaar, the Medical Center Zaandam, the University Medical Center Groningen, the St. Antonius Hospital Nieuwegein, the Diakonessenhuis Utrecht, the Free University Medical Center Amsterdam, and the Laboratory for Pathology Dordrecht, all in The Netherlands. Original diagnoses were made between January 1985 and March 2009, and these cases comprised all the paired cases that could be retrieved from the participating labs during this period, minimizing selection bias. All histological specimens had been fixed for 12-24 hours in neutral buffered formaldehyde. The vast majority of primary specimens were paraffin blocks of breast or lumpectomies, except for 17 cases were core biopsies from the primary tumors were used (no cytology). For 11 cases this information was not available. The sites of the distant metastases are shown in Table 1. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in hospitals in The Netherlands [29]. Ethical approval was not required.
For each case, hematoxylin-eosin stained slides of the paraffin blocks were reviewed by a single pathologist (PJvD) to confirm the presence of malignancy in tumor samples.
Histologic type was assessed according to the World Health Organization. Histologic grade was assessed according to the Nottingham modification of the Bloom-Richardson system, applying standardized mitotic counts [30]. Clinicopathologic characteristics are shown in Table 1.

Immunohistochemistry

Immunohistochemical analysis was carried out on 4-µm sections. We did not use the tissue microarray approach, to avoid sampling bias due to tumor heterogeneity. All primary tumors and metastases were restained by the same person (LCDH) according to the same protocol to allow optimal pair-wise comparisons. For all stainings, slides were deparaffinized in xylene and rehydrated in decreasing ethanol dilutions. Endogenous peroxidase activity was blocked with H_2O_2 in phosphate buffered saline (PBS) followed by antigen retrieval. For ERα and HER2, antigen retrieval was performed in an autoclave with the slides placed in an EDTA buffer, pH=9. For PR antigen retrieval was performed in citrate buffer, pH=6 (20 min, 100°C). A cooling off period of 30 min preceded the incubation (60 min, room temperature) with the primary antibodies.
Mouse monoclonal antibodies used were: ERα (M7047, 1:80, DAKO, Glostrup, Denmark), PR (M3569, 1:50, DAKO) and HER2 (RM-9103-S, 1:100, Neomarkers, Lab Vision Corporation, Fremont, California, USA). For detection of the primary antibodies a poly HRP anti Mouse/Rabbit/Rat IgG (ready to use; Powervision, Immunovision Technologies, Brisbane, California, USA) was used. Between steps, slides were washed with PBS. Finally, peroxidase activity was developed with diaminobenzidin, slides were lightly counter-stained with hematoxylin, dehydrated in increasing alcohol dilutions and cover slipped. Appropriate negative and positive

Table 1. Clinicopathologic characteristics of 233 invasive breast cancer patients studied for receptor conversion in distant metastases.

Feature	Grouping	N or value	%
Age (years)	Mean	53.9	
	Range	25-93	
Tumor size (cm)	≤2	73	31.3
	>2 and ≤5	80	34.3
	>5	12	5.2
	Not available	68	29.2
Histologic type	Invasive ductal cancer	192	82.4
	Invasive lobular cancer	20	8.6
	Others	20	8.6
	Not available	1	0.4
Histologic grade	1	8	3.4
	2	61	26.2
	3	161	69.1
	Not available	3	1.3
MAI (per 2 mm2)	Mean	25	
	Range	0-172	
	≤12	71	30.5
	≥13	156	67.0
	Not available	6	2.5
Lymph node status	Positive	119	51.1
	Negative	81	34.8
	Not available	33	14.2
Site of distant metastasis	Brain	44	18.9
	Lung	43	18.5
	Liver	63	27.0
	Skin	76	32.6
	Gastro-intestinal	7	3.0

MAI, mitotic activity index.

controls were used throughout. We regularly participate in EQA schemes to monitor our performance with these routine antibodies.

If HER2 status differed between primary tumor and metastases, or when either primary tumor or metastasis were IHC 2+ (see below), silver in situ hybridization (SISH) analysis [31] was performed with a fully automated technique (INFORM, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's guidelines.

Scoring of IHC slides was performed by one observer (PJvD) in random order, blinded to other data in the paired samples. For ERα and PR, the percentage of positively stained nuclei was estimated. In primary tumor samples, the adequacy of staining was checked by also evaluating

the normal breast parenchyma when present. Samples with 10% or more immunopositive malignant cells were classified as ERα- or PR positive as usual [8, 11, 12]. In order to also comply with the most recent ASCO guidelines [32], we also used the 1% threshold that is now widely used in the USA. HER2 expression was scored using the DAKO scoring system as 0, 1+, 2+ and 3+ according to standardized criteria [33], considering 3+ cases as positive. SISH results were evaluated by one observer (MJvdV) according to the manufacturer's instructions blinded to other data in the paired samples and immunohistochemistry results. According to the ASCO/CAP guidelines [34], tumors with <6 HER2 copies/tumor cell nucleus were scored as HER2 non-amplified; and tumors with 6 or more HER2 copies/tumor cell nucleus were scored as HER2 amplified.

Statistical analysis

Percentages of nuclei expressing ERα and PR in primary tumors and their metastases were compared by paired T-test (SPSS). The frequency of receptor expression (positive vs negative) in the primary tumors and distant metastases was calculated. Percentages of conversion were calculated for the whole group, and for subgroups of metastatic sites (10% threshold for ERα and PR only). As steroid receptor conversion is especially important if a patient converts from "ER+ or PR+" to ER-/PR-, or from ER-/PR- to "ER+ or PR+", we calculated the percentages for these conversions as well. Conversion rates for the different distant sites (10% threshold for ERα and PR) were compared by chi-square test.

Results

The percentage of nuclei expressing ERα or PR was generally lower in the distant metastases than in the primary tumor (Figure 1), but significance was only reached for PR ($p<0.001$). Receptor conversion exceeding the threshold of 10% occurred for ERα in 10.3% and for PR in 30.0% of the patients (Table 2). Such conversion was mainly from positive to negative: 10.7% of the patients converted from "ER+ or PR+" to ER-/PR-, and 3.4% from ER-/PR- to "ER+ or PR+". Receptor conversion exceeding the threshold of 1% occurred for ERα in 15.1% and for PR in 32.6% of the patients (Table 2), while 12.4% of the patients converted from "ER+ or PR+" to ER-/PR-, and 8.2% from ER-/PR- to "ER+ or PR+".

Receptor conversion for ERα and PR (10% threshold) seemed to occur especially in liver (ERα 12.7%, PR 41.2%) and brain metastases (ERα 13.7%, PR 36.3%) (Table 3). For PR, conversion was significantly more often seen for brain, liver and gastro-intestinal metastases ($p=0.04$).

For HER2, receptor conversion by IHC occurred in 5.2% of patients, about half of them from negative to positive and the other half from positive to negative (Table 2). Receptor conversion for HER2 seemed to occur especially although not significantly in liver metastases (9.5%) (Table 3). Of the 12 cases that showed HER2 conversion by IHC, 5 showed also conversion by SISH. One further case showed conversion by ISH, but not by IHC (Table 4).

Examples of conversion of ERα, PR and HER2 from primary breast cancers to distant metastases are shown in Figure 2.

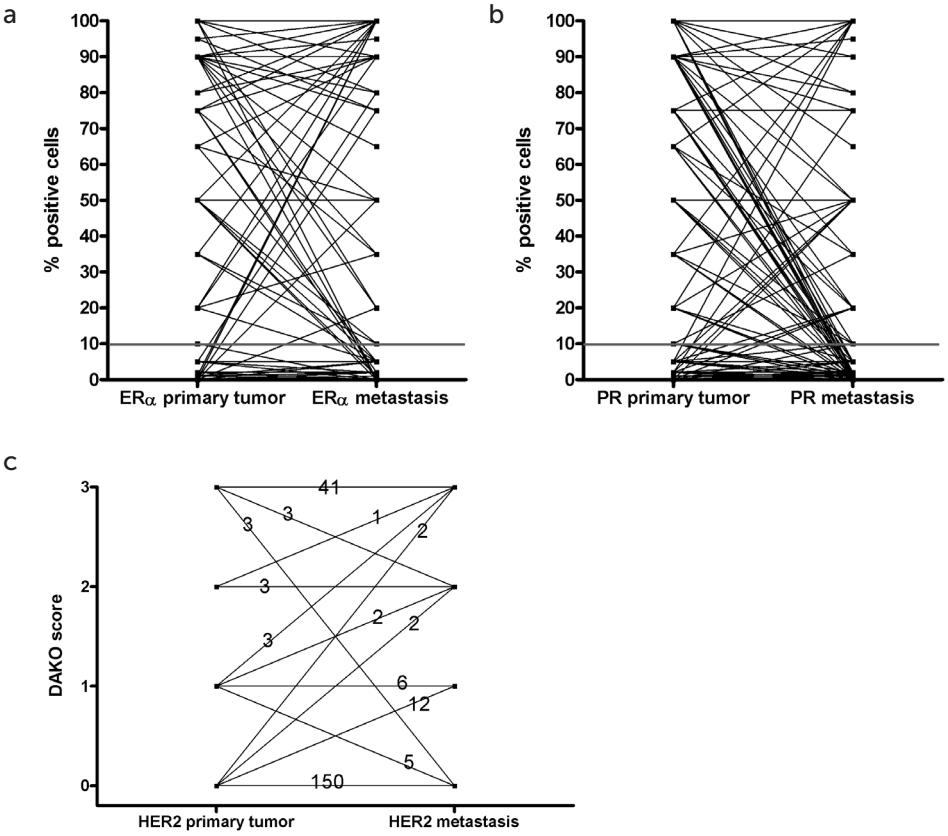

Figure 1. Immunophenotype for ERα (a), PR (b) and HER2 (c) in 233 primary breast tumors and their corresponding metastases.

Table 2. HER2 and ERα, PR expression by immunohistochemistry in paired primary breast cancers and their distant metastases.

		Distant metastases					
Primary tumor	status	-		+		total	
HER2	-	180 (77.2%)		6 (2.6%)		186	
	+	6 (2.6%)		41 (17.6%)		47	
		10% threshold			1% threshold		
		-	+	total	-	+	total
ERα	-	79 (33.9%)	7 (3.0%)	86	47 (20.2%)	12 (5.2%)	59
	+	17 (7.3%)	130 (55.8%)	147	23 (9.9%)	151 (64.8%)	174
PR	-	92 (39.5%)	12 (5.1%)	104	33 (14.2%)	27 (11.6%)	60
	+	58 (24.9%)	71 (30.5%)	129	49 (21.0%)	124 (53.2%)	173

For ERα and PR, data are shown using both the traditional 10% and new ASCO 1% thresholds.
HER2, human epidermal growth factor receptor 2; ERα, estrogen receptor alpha; PR, progesterone receptor.

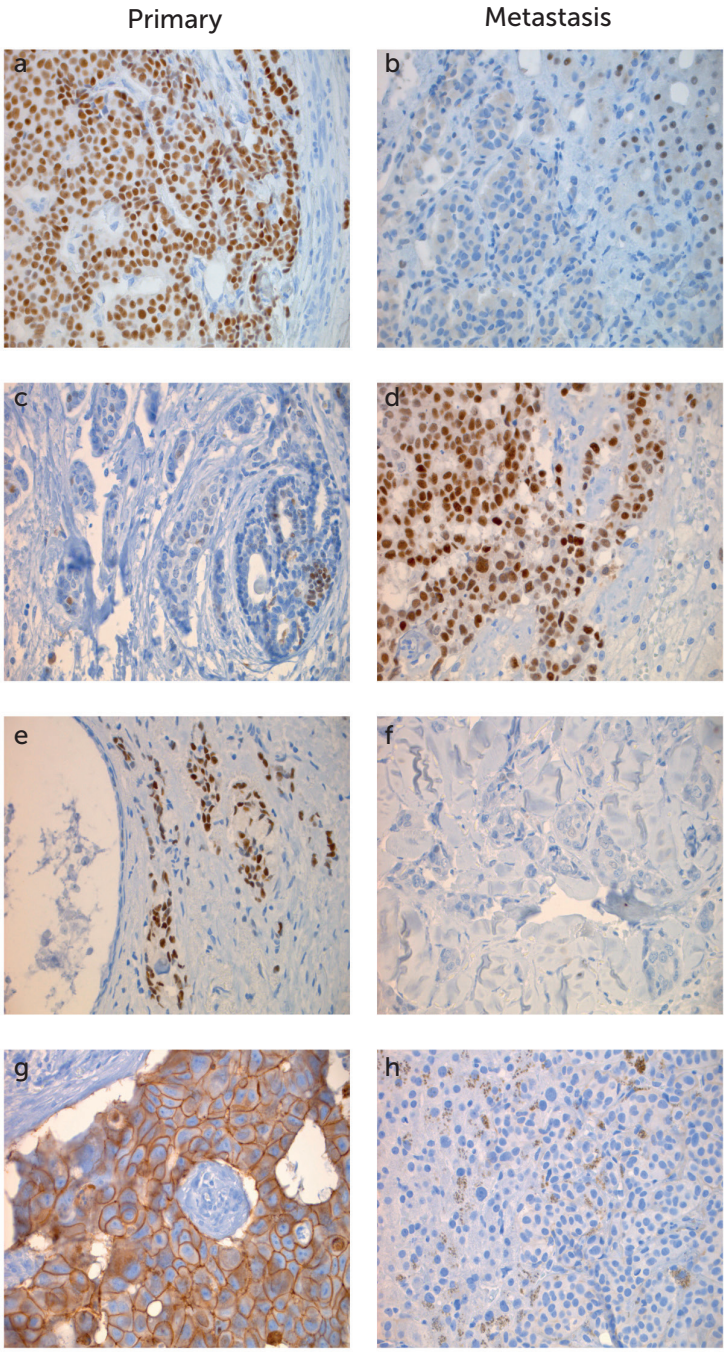

Figure 2. Examples of receptor conversion in distant metastases of primary breast cancers in the same patient. ERα positive primary tumor (**a**) with ERα negative liver metastasis that bears ERα expression in hepatocytes (**b**). ERα negative primary tumor (**c**) with ERα positive brain metastases (**d**). PR positive primary tumor (**e**) with PR negative skin metastasis (**f**). HER2 positive (3+) primary tumor (**g**) with HER2 negative liver metastasis (**h**).

Table 3. Receptor conversion for ERα, PR (10% threshold) and HER2 in distant breast cancer metastases according to site.

		% conversion		
		ERα	PR	HER2
	N	N (%)	N (%)	N(%)
Brain	44	6 (13.7)	16 (36.3)*	1 (2.3)
Lung	43	4 (9.4)	8 (18.6)	2 (4.7)
Liver	63	8 (12.7)	26 (41.2)*	6 (9.5)
Skin	76	5 (6.6)	17 (22.3)	2 (2.6)
Gastro- intestinal	7	1 (14.3)	3 (42.9)*	1 (14.3)

* statistically significantly more often than for lung and skin metastases (p=0.04, chi-square test).
ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Discussion

Previous studies have shown that distant breast cancer metastases may show receptor conversion, potentially leading to inappropriate choices of systemic treatment in these patients. These previous studies however suffered from several limitations not allowing to draw definite conclusions that may change clinical practice. We therefore set out to reevaluate receptor conversion in a large group of non-bone distant breast cancer metastases using optimal methodology. Receptor conversion by IHC for ERα, PR and HER2 occurred in 10.3%, 30.0% and 5.2% of patients, respectively, using the traditional 10% threshold. When using the new 1% ERα and PR threshold according to the ASCO guidelines, conversion rates were even higher at 15.1% and 32.6%.

Previous studies (all using the 10% threshold when indicated) reported ERα receptor conversion rates from 12% to 54%, clearly higher than in the present study. The explanation for this finding may be that previous ERα studies did not restain both the primary and metastatic lesions [3, 5, 8, 10, 11], used ligand-binding assays [2, 3, 7, 10] (where the result is influenced by differences in the percentage of non-tumor cells in the samples), or included bone metastases that may suffer from false negative IHC results due to decalcification [2, 4, 6, 8-10, 12]. In addition, with 233 cases our series is much larger than most previous ERα studies. Only two previous studies report on comparable numbers of cases (200 and 211, respectively), but in these studies the original immunohistochemistry results from the pathology report were used instead of renewed stainings. Previous studies (all using the 10% threshold when indicated) reported PR conversion rates from 28% to 61%, again higher than in the present study. The explanation for this may be similar to what has been mentioned above: previous studies did not restain both the primary and metastatic lesions [5, 8, 10, 11], used ligand-binding assays [2, 7, 10], or included bone metastases [2, 6, 8-10, 12]. Further, our series is much larger with 233 compared to most previous PR studies with 9-59 cases. There were two previous studies with respectively 173 and 211 cases, but these used the original immunohistochemistry results from the pathology report instead of renewed stainings.

Conversion for ERα and PR was mainly from positive in the primary tumor to negative in the metastases as has been described before [2, 4, 6, 8, 10-12]. This finding may well be explained by clonal selection of less differentiated receptor negative cells during the metastatic process, e.g. elicited by adjuvant hormonal treatment [12, 35, 36]. However, in a few cases conversion from negative in the primary tumor to positive in the metastases occurred. This phenomenon has also been described before [2, 3, 8, 10, 11], but is more difficult to explain. Although false negative primary tumor results cannot be fully excluded, we also assessed the adequacy of staining by analysis of staining of epithelial cells in the normal ducts and lobules as an internal control. Perhaps in these cases small receptor positive clones within the primary tumor preferentially metastasized [37]. Alternatively, this phenomenon could be a result of genetic drift during tumor progression [38].

Previous studies reported HER2 immunophenotype conversion rates from 0% to 58.3%, which is generally higher than the 5.2% conversion rate we have found in the present study.

Table 4. Silver in situ hybridization results for breast cancer cases showing HER2 receptor conversion in distant metastases by immunohistochemistry or 2+ scores by immunohistochemistry in either the primary tumor or the metastasis.

Case	HER2 (IHC) primary tumor	HER2 (SISH) primary tumor	HER2 (IHC) metastasis	HER2 (SISH) metastasis	Metastatic site
1	0	No amplification	2+	No signal	lung
2	0	No amplification	2+	No signal	liver
3	0	High amplification	3+	High amplification	liver
4	0	Low amplification	3+	Low amplification	skin
5	1+	No amplification	2+	High amplification	lung
6	1+	No amplification	2+	No amplification	skin
7	1+	No amplification	3+	High amplification	skin
8	1+	No amplification	3+	High amplification	liver
9	2+	High amplification	2+	High amplification	liver
10	2+	High amplification	2+	High amplification	liver
11	2+	Low amplification	2+	Low amplification	liver
12	2+	Low amplification	3+	High amplification	liver
13	2+	Low amplification	3+	High amplification	liver
14	3+	High amplification	0	No amplification	liver
15	3+	High amplification	0	No amplification	liver
16	3+	High amplification	0	No amplification	gastro-intestinal
17	3+	High amplification	2+	High amplification	brain
18	3+	High amplification	2+	No signal	lung
19	3+	High amplification	2+	High amplification	lung

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; SISH, silver in situ hybridization.

As mentioned above, previous HER2 studies did not restain both the primary and metastatic lesions [10, 11, 23], used TMAs [21] or included bone metastases [10, 12, 16, 19, 20, 22]. Further, our series is much larger with 233 cases compared to most previous studies with 12-211 cases, and in the only larger study (N=382) restaining was not performed. By SISH, only half of these IHC conversions were accompanied by a difference in HER2 gene amplification status. Therefore, for only six out of the 233 patients (3%) a "true" conversion on the gene level of HER2 status between primary tumor and distant metastasis could be demonstrated. The fact that scoring of HER2 SISH signals is more straightforward than interpretation of HER2 IHC may play a role here. Contrary to ERα and PR that preferentially converted from positive in the primary tumors to negative in the metastases, HER2 receptor conversion occurred both ways. Although from a tumor progression model one would expect HER2 conversion to preferentially occur from negative to positive, conversion both ways has been described before [17, 18, 20-23]. One mechanism of conversion from positive to negative may well be explained by clonal selection of HER2 negative cells during the metastatic process, e.g. elicited by trastuzumab therapy [39].

When considering the different metastatic sites, receptor conversion seemed to occur mostly in liver and brain metastases, but only for PR conversion this was significant. The reason for this observation is unclear, and these results need to be interpreted with caution.

One limitation to receptor conversion studies is the lack of internal control cells in samples from most of the metastatic sites. An exception to this is the liver that bears ERα and to a lesser extent PR expression in hepatocytes. Since most receptor conversion was seen in the liver, and the fact that most biopsies from breast cancer metastases are small and therefore probably quickly and well fixed, it is unlikely that these issues play an important role. A further limitation was the deliberate choice not to include the preferential metastatic site of breast cancer: the bone (marrow). This was to avoid false negative results due to decalcification artefacts. Such false negative results are not easy to trace since internal positive control cells in the bone marrow are lacking. Future studies selectively studying small bone biopsies that were not decalcified may shed further light on percentages of conversion on this metastatic site.

Nearly 11% of the patients converted from ER+ or PR+ to ER-/PR- and 3.4% from ER-/PR- to ER+ or PR+ (using the 10% threshold) ; in these cases steroid receptor conversion could be especially clinically relevant.

Together with the HER2 conversion rate of 5.2% by IHC, in about 19% of metastatic patients the choice of systemic therapy is suboptimal when solely based on IHC of the primary tumor. However, before concluding that metastases should be biopsied when possible, there are a few issues to consider. Clinicians would probably be inclined to treat patients with positively converted distant metastases (3.4+2.6=6%) with the matching (hormonal or trastuzumab) systemic treatment, but for patients with negatively converted distant metastases this is probably more complicated. First, technical problems in cases with receptor negative metastases cannot be fully excluded. Second, there may be heterogeneity between distant metastases from which only one may get biopsied [40]. Third, there are few clinical data on response to systemic treatment in negatively converted patients. Therefore, clinicians might be inclined to consider (hormonal or trastuzumab) systemic treatment even in negatively converted patients. Nevertheless, when a biopsy of a distant metastasis is available, hormone receptor and HER2 status should be

reassessed in these biopsies and the tests results should be critically evaluated in conjunction with ER, PR and HER2 status of the primary tumor. In the future, non-invasive assessment of the receptor status by molecular imaging may form an alternate and more functional way of assessing receptor status of distant metastases [24-26], especially for metastases at inaccessible sites, also providing information on heterogeneity of receptor status between distant metastases.

Conclusions

In conclusion, receptor conversion in distant non-bone breast cancer metastases indeed occurs, is relatively uncommon for ERα and HER2, more frequent for PR, and seems to be more frequent in liver and brain metastases. In a considerable number of patients such conversion could theoretically have consequences for the systemic therapeutic regimen. For this reason, receptor status should therefore be reassessed on available biopsies from distant metastases. Whether distant breast cancer metastases should be more routinely biopsied when possible, will likely be the subject of further discussion. In the future, non-invasive assessment of the receptor status by molecular imaging may be an attractive alternative, especially for metastases that are difficult to biopsy.

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REFERENCES

1. Bray F, McCarron P, Parkin DM: The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res 2004, 6:229-239.
2. Holdaway IM, Bowditch JV: Variation in receptor status between primary and metastatic breast cancer. Cancer 1983, 52:479-485.
3. Hull DF, 3rd, Clark GM, Osborne CK, Chamness GC, Knight WA, 3rd, McGuire WL: Multiple estrogen receptor assays in human breast cancer. Cancer Res 1983, 43:413-416.
4. Brunn Rasmussen B, Kamby C: Immunohistochemical detection of estrogen receptors in paraffin sections from primary and metastatic breast cancer. Pathol Res Pract 1989, 185:856-859.
5. Li BD, Byskosh A, Molteni A, Duda RB: Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. J Surg Oncol 1994, 57:71-77.
6. Kuukasjärvi T, Kononen J, Helin H, Holli K, Isola J: Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J Clin Oncol 1996, 14:2584-2589.
7. Branković-Magić M, Janković R, Nesković-Konstantinović Z, Nikolić-Vukosavljević D: Progesterone receptor status of breast cancer metastases. Journal of Cancer Research and Clinical Oncology 2002, 128:55-60.
8. Lower EE, Glass EL, Bradley DA, Blau R, Heffelfinger S: Impact of metastatic estrogen receptor and progesterone receptor status on survival. Breast Cancer Res Treat 2005, 90:65-70.
9. Amir E, Ooi WS, Simmons C, Kahn H, Christakis M, Popovic S, Kalina M, Chesney A, Singh G, Clemons M: Discordance between receptor status in primary and metastatic breast cancer: an exploratory study of bone and bone marrow biopsies. Clin Oncol (R Coll Radiol) 2008, 20:763-768.
10. Broom RJ, Tang PA, Simmons C, Bordeleau L, Mulligan AM, O'Malley FP, Miller N, Andrulis IL, Brenner DM, Clemons MJ: Changes in estrogen receptor, progesterone receptor and Her-2/neu status with time: discordance rates between primary and metastatic breast cancer. Anticancer Res 2009, 29:1557-1562.
11. Liedtke C, Broglio K, Moulder S, Hsu L, Kau SW, Symmans WF, Albarracin C, Meric-Bernstam F, Woodward W, Theriault RL, Kiesel L, Hortobagyi GN, Pusztai L, Gonzalez-Angulo AM: Prognostic impact of discordance between triple-receptor measurements in primary and recurrent breast cancer. Ann Oncol 2009.
12. Simmons C, Miller N, Geddie W, Gianfelice D, Oldfield M, Dranitsaris G, Clemons MJ: Does confirmatory tumor biopsy alter the management of breast cancer patients with distant metastases? Ann Oncol 2009, 20:1499-1504.
13. Masood S, Bui MM: Assessment of Her-2/neu overexpression in primary breast cancers and their metastatic lesions: an immunohistochemical study. Ann Clin Lab Sci 2000, 30:259-265.
14. Shimizu C, Fukutomi T, Tsuda H, Akashi-Tanaka S, Watanabe T, Nanasawa T, Sugihara K: c-erbB-2 protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. J Surg Oncol 2000, 73:17-20.
15. Tanner M, Jarvinen P, Isola J: Amplification of HER-2/neu and topoisomerase IIalpha in primary and metastatic breast cancer. Cancer Res 2001, 61:5345-5348.
16. Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M, Verhest A, Bernard-Marty C, Piccart MJ, Larsimont D: Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 2002, 13:1036-1043.
17. Edgerton SM, Moore D, 2nd, Merkel D, Thor AD: erbB-2 (HER-2) and breast cancer progression. Appl Immunohistochem Mol Morphol 2003, 11:214-221.
18. Lear-Kaul KC, Yoon HR, Kleinschmidt-DeMasters BK, McGavran L, Singh M: Her-2/neu status in breast cancer metastases to the central nervous system. Arch Pathol Lab Med 2003, 127:1451-1457.
19. Gong Y, Booser DJ, Sneige N: Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 2005, 103:1763-1769.
20. Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C, Hadary A: Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 2005, 93:552-556.
21. Tapia C, Savic S, Wagner U, Schönegg R, Novotny H, Grilli B, Herzog M, Barascud AD, Zlobec I, Cathomas G, Terracciano L, Feichter G, Bubendorf L: HER2 gene status in primary breast cancers and matched distant metastases. Breast Cancer Res 2007, 9:R31.
22. Santinelli A, Pisa E, Stramazzotti D, Fabris G: HER-2 status discrepancy between primary breast cancer and metastatic sites. Impact on target therapy. Int J Cancer 2008, 122:999-1004.

23. Lower EE, Glass E, Blau R, Harman S: HER-2/neu expression in primary and metastatic breast cancer. Breast Cancer Res Treat 2009, 113:301-306.
24. Munnink THO, Nagengast WB, Brouwers AH, Schröder CP, Hospers GA, Hooge MNL-d, Wall Evd, Diest PJv, Vries EGEd: Molecular imaging of breast cancer. The Breast 2009, 18:S66-S73.
25. Perik PJ, Lub-De Hooge MN, Gietema JA, van der Graaf WT, de Korte MA, Jonkman S, Kosterink JG, van Veldhuisen DJ, Sleijfer DT, Jager PL, de Vries EG: Indium-111-labeled trastuzumab scintigraphy in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer. J Clin Oncol 2006, 24:2276-2282.
26. Hospers GA, Helmond FA, de Vries EG, Dierckx RA, de Vries EF: PET imaging of steroid receptor expression in breast and prostate cancer. Curr Pharm Des 2008, 14:3020-3032.
27. National Comprehensive Cancer Network: Practice Guidelines in Oncology: Breast Cancer - V1.2010.
28. National Cancer Institute PDQ. Breast Cancer Treatment. In; 2010.
29. van Diest PJ: No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002, 325:648-651.
30. van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH, Kwee WS: Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 1992, 23:603-607.
31. Powell RD, Pettay JD, Powell WC, Roche PC, Grogan TM, Hainfeld JF, Tubbs RR: Metallographic in situ hybridization. Hum Pathol 2007, 38:1145-1159.
32. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, Hicks DG, Lester S, Love R, Mangu PB, McShane L, Miller K, Osborne CK, Paik S, Perlmutter J, Rhodes A, Sasano H, Schwartz JN, Sweep FC, Taube S, Torlakovic EE, Valenstein P, Viale G, Visscher D, Wheeler T, Williams RB, et al.: American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol, 28:2784-2795.
33. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al.: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707-712.
34. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007, 25:118-145.
35. Cui X, Schiff R, Arpino G, Osborne CK, Lee AV: Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy. J Clin Oncol 2005, 23:7721-7735.
36. Kurbel S: Selective reduction of estrogen receptor (ER) positive breast cancer occurrence by estrogen receptor modulators supports etiological distinction between ER positive and ER negative breast cancers. Med Hypotheses 2005, 64:1182-1187.
37. Kerbel RS: Growth dominance of the metastatic cancer cell: cellular and molecular aspects. Adv Cancer Res 1990, 55:87-132.
38. Kuukasjarvi T, Karhu R, Tanner M, Kahkonen M, Schaffer A, Nupponen N, Pennanen S, Kallioniemi A, Kallioniemi OP, Isola J: Genetic heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. Cancer Res 1997, 57:1597-1604.
39. Wu JM, Halushka MK, Argani P: Intratumoral heterogeneity of HER-2 gene amplification and protein overexpression in breast cancer. Hum Pathol, 41:914-917.
40. Wu JM, Fackler MJ, Halushka MK, Molavi DW, Taylor ME, Teo WW, Griffin C, Fetting J, Davidson NE, De Marzo AM, Hicks JL, Chitale D, Ladanyi M, Sukumar S, Argani P: Heterogeneity of breast cancer metastases: comparison of therapeutic target expression and promoter methylation between primary tumors and their multifocal metastases. Clin Cancer Res 2008, 14:1938-1946.

APPENDIX

Testing for discordance at metastatic relapse of breast cancer matters

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To the editor

In an editorial[1] accompanying two papers on receptor conversion of distant breast cancer metastases[2, 3], Chia states that "The contribution to this discordance from preanalytic and analytic variables or from true biology based on heterogeneity of clonal populations in breast cancer and treatment is unclear". We agree that this is an important issue, and indeed, most studies on receptor conversion may have suffered from these variables. However, recently we have published a study on receptor conversion where we have at least ruled out the analytical features that Chia mentions[4]. Our series was large with 233 paired cases. Bone metastases were excluded since they may provide problems with antigenicity due to decalcification[2], and only biopsies were used, no fine needle aspirations. We used whole sections, no tissue arrays, to rule out tumor heterogeneity problems. Fresh sections were cut and stained with current, optimal, standardized staining methods to rule out differences in antigen retrieval, antibodies and antibody concentrations, and detection methods. Human epidermal growth factor receptor 2 (HER2) discrepancies between primary tumors and distant metastases were further evaluated by in situ hybridization. All sections were scored by one experienced pathologist to prevent interobserver variation. In addition, normal tissue next to tumor was carefully evaluated as internal control to also (partly) deal with preanalytical variables.

Even with all these variables excluded, we still found receptor conversion in 10.3%, and 30% of patients for estrogen receptor (ERα) and progesterone receptor (PR), respectively, when using a decision threshold of 10%. In 10.7% of patients, conversion from ER+ or PR+ to ER-/PR- and in 3.4% from ER-/PR- to ER+ or PR+ occurred, which is obviously relevant for the decision on hormonal therapy. For the 1% decision threshold, ERα and PR conversion rates were 15.1% and 32.6%. In 12.4% of patients, conversion from ER+ or PR+ to ER-/PR-, and in 8.2% from ER-/PR- to ER+ or PR+ occurred. Together with a 5.2% conversion rate for HER2, this means that a potentially clinically relevant receptor conversion occurred in more than 19% of patients using the 10% threshold (10.7% +3.4% +5.2%) and almost 26% (12.4% +8.2% +5.2%) for the 1% threshold. Conversion for ERα and PR was mainly from positive to negative, while HER2 conversion was equally both ways[4].

In a follow up study, we additionally showed that receptor conversion for ERα and PR, being positive in the primary tumor and converted to negative in the distant metastases, has unfavorable prognostic impact[5].

All in all, these data show that despite potential preanalytical and analytical variables in different published studies, receptor conversion in distant breast cancer metastases can still be demonstrated in a significant percentage of patients when ruling out most of these error sources. Such receptor conversion could result in not only withholding patients effective endocrine- or HER2 blocking agents, but could also unjust administration of these same agents. At the same time, receptor conversion for ERα and PR has prognostic value. Like Amir et al[2], and Niikura et al[3], we therefore believe that distant metastases should be biopsied whenever possible to re-evaluate receptor status.

REFERENCES

1. Chia S: Testing for discordance at metastatic relapse: does it matter? J Clin Oncol, 30:575-576.
2. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, Oldfield M, Dranitsaris G, Tomlinson G, Laupacis A, et al: Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol, 30:587-592.
3. Niikura N, Liu J, Hayashi N, Mittendorf EA, Gong Y, Palla SL, Tokuda Y, Gonzalez-Angulo AM, Hortobagyi GN, Ueno NT: Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. J Clin Oncol, 30:593-599.
4. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, et al: Receptor conversion in distant breast cancer metastases. Breast Cancer Res, 12:R75.
5. Hoefnagel LD, Moelans CB, Meijer SL, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, et al: Prognostic value of estrogen receptor alpha and progesterone receptor conversion in distant breast cancer metastases. Cancer, 118:4929-4935.


Breast cancer
Metastases

CHAPTER 3

Prognostic value of ERα and PR receptor conversion in distant breast cancer metastases

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ABSTRACT

Background

Changes in the receptor profile of primary breast cancers to their metastases ("receptor conversion") have been described for the estrogen (ERα) and progesterone receptors (PR). The purpose of this study was to evaluate the impact of receptor conversion for ERα and PR on survival in a large group of distant (non-bone) breast cancer metastases.

Methods

Receptor conversion was studied by immunohistochemistry in a group of 233 metastatic breast cancer patients. Kaplan-Meier overall survival curves were plotted, and differences between the curves were analyzed by logrank analysis. Additional prognostic value of conversion to established prognosticators was studied by Cox regression.

Results

Overall survival of patients showing conversion from positive to negative ERα or PR, from negative to positive ERα or PR or remaining receptor negative was comparable, and significantly worse than patients remaining receptor positive. ERα or PR receptor conversion from positive in the primary breast tumor to negative in distant metastases has independent negative prognostic value.

Conclusion

ERα or PR receptor conversion from positive in the primary breast cancer to negative in distant metastases has negative prognostic value.

Introduction

Traditionally, metastatic tumors were rarely biopsied due to limited accessibility or it because it was deemed unnecessary for further therapeutic decision making. When breast cancer patients develop distant metastases, the choice of systemic treatment with chemotherapy, hormonal therapy, human epidermal growth factor receptor 2 (HER2) targeted therapy and/or or anti-angiogenesis therapy is in those cases based on assessment of the primary tumor characteristics by routine histopathology, immunohistochemistry (IHC), and/or molecular analysis. However, we and several previous studies have indicated that distant breast cancer metastases may show "receptor conversion" from primary tumors to their distant metastases[1-31]. This indicates that it may be clinically important to biopsy distant metastases to assess hormone receptor and HER2 status whenever possible. Several recent guidelines now include this recommendation[32-34], and distant metastases are now much more often biopsied.
Although ERα and PR expression levels of the primary breast tumors correlate with prognosis in patients[35, 36], little information is yet available whether survival is influenced by receptor conversion of ERα and PR in breast cancer metastases.
Therefore, we evaluated the prognostic value of receptor conversion for ERα and PR in a large group of distant (non-bone) breast cancer metastases by re-staining all primary tumors and metastases with current optimal immunohistochemical methods on full sections.

Materials and Methods

Patients

This retrospective study included 233 female breast cancer patients previously studied for receptor conversion of ERα and PR in their metachronous non-bone distant metastases, as described before[27]. Paraffin blocks of these patients were obtained from the departments of Pathology of the University Medical Center Utrecht, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Centre, the Canisius Wilhelmina Hospital Nijmegen, the Netherlands Cancer Institute Amsterdam, the Medical Center Alkmaar, the Medical Center Zaandam, the University Medical Center Groningen, the St. Antonius Hospital Nieuwegein, the Diakonessenhuis Utrecht, the VU University Medical Center Amsterdam, and the Laboratory for Pathology Dordrecht, all in The Netherlands. Original diagnoses were made between January 1985 and March 2009, and these cases comprised all the cases for which paired samples could be retrieved from the participating labs during this period, minimizing selection bias. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in hospitals in The Netherlands[37].

Immunohistochemistry

Immunohistochemical analysis was carried out on full 4-μm sections like described before[27]. In short, mouse monoclonal antibodies used were: ERα (M7047, 1:80, DAKO, Glostrup, Denmark) and PR (M3569, 1:50, DAKO) and HER2 (RM-9103-S, 1:100, Neomarkers, Lab Vision Corporation,

Fremont, California, USA). For detection of the primary antibodies a poly HRP anti Mouse/Rabbit/Rat IgG (ready to use; Powervision, Immunovision Technologies, Brisbane, California, USA) was used. Appropriate negative and positive controls were used throughout.
Scoring of IHC slides was performed by one observer (PJvD) in random order, blinded to other data in the paired samples. For ERa and PR, the percentage of positively stained nuclei was estimated. In primary tumor samples, the adequacy of staining was checked by also evaluating the normal breast parenchyma when present.

Statistical analysis

Table 1 summarizes the characteristics of patients and their primary tumors. Treatment and follow up data were obtained from the IKNL, The Netherlands (http://www.ikcnet.nl/). Treatment data were available for 221 and follow-up data for 225 patients.
For ERα- and PR, both the European 10% and ASCO 1% thresholds were tested[32]. Overall survival Kaplan-Meier curves were plotted, and differences between the curves were analyzed by logrank analysis. Additional prognostic value of conversion to established prognosticators[38-40] was studied by Cox proportional hazard model, all variables with $p < 0.05$ in univariate analysis were entered into the model. Data are presented as hazard ratios (HR) with 95% confidence intervals (95% CI). All statistical analyses were conducted with SPSS, regarding two-sided p-values below 0.05 as significant.

Results

Using the 10% threshold, receptor conversion occurred for ERα in 10.3% and for PR in 30.0% of the patients. Such conversion was mainly from positive to negative: 10.7% of the patients converted from ER+ or PR+ to ER-/PR-, and 3.4% from ER-/PR- to ER+ or PR+, like described before[27]. For the 1% threshold, receptor conversion % occurred for ERα in 15.1% and for PR in 32.6% of the patients, while 12.4% of the patients converted from ER+ or PR+ to ER-/PR-, and 8.2% from ER-/PR- to ER+ or PR+, like described before[27]. Figures 1-4 show Kaplan-Meier overall survival curves for the four groups based on ERα and/or PR status of the primary tumors and their metachronous metastatic lesions.
Using the 10% threshold, patients who remained ERα positive in their metastasis had a significantly better overall survival (median: 3595 days) than the other patients, while patients that remained ERα negative (median: 1066 days) or showed ERα conversion from positive to negative (median: 2336 days) or from negative to positive (median: 1161 days) had similar survival ($p \leq 0.001$, Figure 1, Table 2). The same significant differences in survival were seen for PR (PR: median: 4258 days versus median: 1168, 2079, 3106 days, respectively ($p \leq 0.001$, Figure 2, Table 2).
Similarly, for the 1% threshold, patients who remained ERα/PR positive in their metastasis did significantly better than the other patients (ERα: median: 2829 days versus median: 1063, 1530, 669 days, respectively) (PR: median: 2829 days versus median: 1063, 1833, 1358 days, respectively (ERα, PR $p \leq 0.001$, Figure 3, 4, Table 2).

Table 1a. Clinicopathologic characteristics of 233 invasive breast cancer patients for ERα and PR (threshold 10%) studied for prognostic value of receptor conversion in distant metastases.

Feature	Grouping	ERα		PR	
		Conversion – (N=209)	Conversion + (N=24)	Conversion – (N=163)	Conversion + (N=70)
Age (years)	Mean	53.76	53.21	53.20	54.87
	Range	25-88	40-79	25-84	28-88
	≤2	34.5%	16.7%	37.4%	21.4%
Tumor size at diagnosis (cm)	>2 and ≤5	36.8%	45.8%	33.8%	47.1%
	>5	9.6%	8.3%	10.4%	7.1%
	Not available	19.1%	29.2%	18.4%	24.4%
	Invasive ductal cancer	82.3%	83.3%	84.7%	77.1%
Histologic type	Invasive lobular cancer	8.6%	8.3%	7.4%	11.4%
	Others	8.6%	8.4%	7.3%	10.1%
	Not available	0.5%	-	0.6%	1.4%
	≤12	29.2%	41.7%	28.2%	35.7%
MAI (per 2 mm^2)	≥13	67.9%	58.3%	69.3%	61.4%
	Not available	2.9%	-	2.5%	2.9%
	1	3.8%	-	4.3%	1.4%
Histologic grade	2	25.4%	33.3%	22.1%	35.8%
	3	69.4%	67.7%	72.4%	61.4%
	Not available	1.4%	-	1.2%	1.4%
	Positive	51.7%	45.8%	50.9%	51.4%
Lymph node status	Negative	34.4%	37.5%	37.4%	28.6%
	Not available	13.9%	16.7%	11.7%	20.0%
	Yes	67.4%	29.2%	22.1	41.4%
Hormonal therapy	No	27.8%	62.5%	73.6%	51.5%
	Not available	4.8%	8.3%	4.3%	7.1%
	Yes	42.6%	50.0%	47.2%	34.3%
Chemotherapy	No	52.6%	41.7%	48.5%	58.6%
	Not available	4.8%	8.3%	4.3%	7.1%

ERα, estrogen receptor alpha; PR, progesterone receptor; MAI, mitotic activity index.

Irrespective of the threshold used, conversion from an ERα/PR positive tumor to a negative metastasis was associated with a significantly worse survival compared to patients remaining receptor positive (ERα $p=0.004$ and $p\leq0.001$, PR $p\leq0.001$ and $p=0.001$, Table 2). In contrast, there was no significant difference in survival between patients showing conversion from an ERα/PR negative tumor to an ERα/PR positive metastasis compared to patients remaining negative (Table 2).

Multivariate analysis was carried out including age at diagnosis (continuous), tumor size (continuous), and tumor grade. After adjustment for these covariates, patients who showed ERα

conversion from positive to negative (threshold 1%) still had a worse survival compared with patients who remained positive in their metastases (HR 2.20; 95% CI: 1.23-3.92; p=0.008) (Table 3). A similar trend was seen for PR (threshold 10%) (HR 2.207; 95% CI: 1.34-3.63; p=0.002) (Table 3).
Within the subgroup of patients receiving hormonal therapy for their primary tumor, using the 10% threshold for conversion, patients with an ERα positive tumor had significantly worse survival when their metastases were ERα negative compared to patients remaining ERα positive (p=0.046). The same phenomenon was observed for PR (p=0.030). For the 1% threshold, no such significance was observed (ERα p=0.596, PR p=0.696).

Table 1b. Clinicopathologic characteristics of 233 invasive breast cancer patients for ERα and PR (threshold 1%) studied for prognostic value of receptor conversion in distant metastases.

		ERα		PR	
Feature	Grouping	Conversion – (N=198)	Conversion + (N=35)	Conversion – (N=157)	Conversion + (N=76)
Age (years)	Mean	53.98	52.11	53.75	53.61
	Range	27-88	25-74	27-88	25-84
	≤2	32.3%	34.3%	36.9%	23.7%
Tumor size at diagnosis (cm)	>2 and ≤5	33.9%	25.7%	35.7%	42.1%
	>5	9.1%	1.4%	9.6%	9.2%
	Not available	18.7%	28.6%	17.8%	25.0%
	Invasive ductal cancer	82.3%	82.9%	82.2%	82.9%
Histologic type	Invasive lobular cancer	9.6%	2.9%	9.6%	6.6%
	Others	7.6%	14.2%	7.6%	9.2%
	Not available	0.5%	-	0.6%	1.3%
	≤12	29.8%	34.3%	33.1%	25.0%
MAI (per 2 mm2)	≥13	67.2%	65.7%	64.4%	72.4%
	Not available	3.0%	-	2.5%	2.6%
	1	4.0%	-	4.5%	1.3%
Histologic grade	2	26.8%	22.9%	29.3%	19.7%
	3	67.7%	77.1%	65.6%	76.3%
	Not available	1.5%	-	0.6%	2.7%
	Positive	51.0%	51.4%	54.2%	44.7%
Lymph node status	Negative	35.4%	31.4%	33.1%	38.2%
	Not available	13.6%	17.2%	12.7%	17.1%
	Yes	31.3%	8.6%	31.8%	19.7%
Hormonal therapy	No	64.1%	82.%	63.7%	73.7%
	Not available	4.6%	8.6%	4.5%	6.6%
	Yes	40.4%	60.0%	40.8%	48.7%
Chemotherapy	No	55.1%	31.4%	54.7%	44.7%
	Not available	4.5%	8.6%	4.6%	6.6%

ERα, estrogen receptor alpha; PR, progesterone receptor; MAI, mitotic activity index.

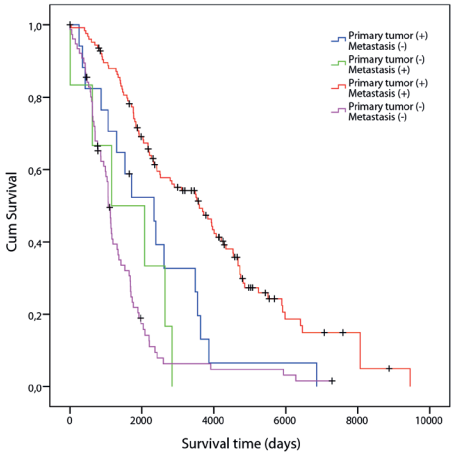

Figure 1. Overall survival curves for metastatic breast cancer patients with or without ERα conversion from the primary tumor to distant metastases according to the 10% threshold (p≤0.001).

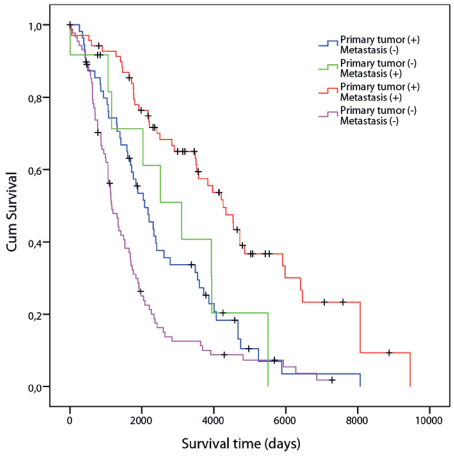

Figure 2. Overall survival curves for metastatic breast cancer patients with or without PR conversion from the primary tumor to distant metastases according to the 10% threshold (p≤0.001).

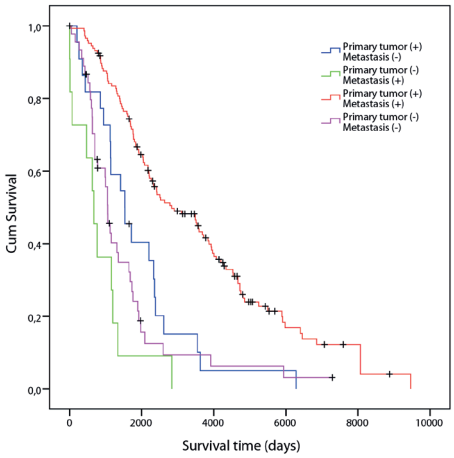

Figure 3. Overall survival curves for metastatic breast cancer patients with or without ERα conversion from the primary tumor to distant metastases according to the 1% threshold (p≤0.001).

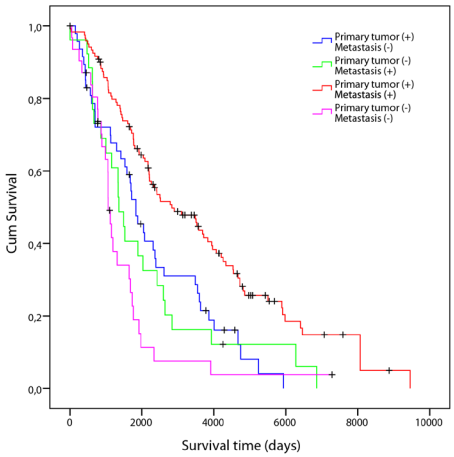

Figure 4. Overall survival curves for metastatic breast cancer patients with or without PR conversion from the primary tumor to distant metastases according to the 1% threshold (p≤0.001).

Table 2. Overall survival analysis results of conversion of metastatic breast cancer patients for ERα and PR according to different thresholds.

		Threshold			
		10%		1%	
Receptor	Grouping	numbers	p-value	numbers	p-value
	no conversion vs. conversion	202 vs. 23	0.078	192 vs. 33	<0.001
	no conversion + to + vs. no conversion – to –	126 vs. 76	<0.001	147 vs. 45	<0.001
	vs. conversion + to – vs. conversion – to +	vs. 17 vs. 6		vs. 22 vs. 11	
ERα	no conversion + to + vs. no conversion – to –	126 vs. 76	<0.001	147 vs. 45	<0.001
	no conversion + to + vs. conversion + to –	126 vs. 17	0.004	147 vs. 22	<0.001
	no conversion – to – vs. conversion – to +	76 vs. 6	0.415	45 vs. 11	0.124
	conversion + to – vs. conversion – to +	17 vs. 6	0.254	22 vs. 11	0.020
	no conversion vs. conversion	157 vs. 68	0.397	152 vs. 73	0.003
	no conversion + to + vs. no conversion – to –	69 vs. 88	<0.001	121 vs. 31	<0.001
	vs. conversion + to – vs. conversion – to +	vs. 56 vs. 12		vs. 47 vs. 26	
PR	no conversion + to + vs. no conversion – to –	69 vs. 88	<0.001	121 vs. 31	<0.001
	no conversion + to + vs. conversion + to –	69 vs. 56	<0.001	121 vs. 47	0.001
	no conversion – to – vs. conversion – to +	88 vs. 12	0.061	31 vs. 26	0.187
	conversion + to – vs. conversion – to +	56 vs. 12	0.591	47 vs. 26	0.850

ERα, estrogen receptor alpha; PR, progesterone receptor.

Table 3. Additional prognostic value of receptor conversion to negative in breast cancer metastases to established prognosticators (age, tumor size and tumor grade).

	10% threshold				1% threshold			
	ERα		PR		ERα		PR	
	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
Conversion	1.61 (0.82-3.14)	0.164	2.21 (1.34-3.63)	0.002	2.2 (1.23-3.92)	0.008	1.38 (0.90-2.14)	0.142
Age	1.01 (0.99-1.03)	0.543	1.01 (0.99-1.04)	0.351	1 (0.99-1.02)	0.809	1 (0.99-1.02)	0.722
Size	1.18 (1.07-1.30)	0.001	1.12 (0.97-1.30)	0.114	1.15 (1.06-1.26)	0.002	1.13 (1.03-1.24)	0.01
Grade	1.68 (1.06-2.67)	0.027	1.51 (0.92-2.48)	0.103	2.02 (1.33-3.09)	0.001	1.92 (1.25-2.96)	0.003

ERα, estrogen receptor alpha; PR, progesterone receptor; 95% CI, 95% confidence interval.

Discussion

Several studies have shown that useful therapy predictive factors are not stable during tumor progression. Distant breast cancer metastases may show receptor conversion, potentially leading to inappropriate choices for systemic treatment[1-31]. Little information is however available whether survival is influenced by receptor conversion. To this end we evaluated the impact of receptor conversion for ERα and PR on survival in a large group of distant (non-bone) breast cancer metastases by re-staining all primary tumors and metastases with current optimal immunohistochemical methods.

Patients who remained ERα or PR positive in their metastasis had a more favorable prognosis compared to patients remaining receptor negative or showing receptor conversion. This was independent from established prognosticators. We hypothesize that the decreased survival in patients converting to negative in their metastases may be due to the fact that endocrine treatment of stage IV disease was at the time initiated based on the primary tumor characteristics, whereas receptor negative cells are known to be fully resistant to endocrine treatment. It would therefore be important to know upfront which receptor positive patients are likely to form receptor negative metastases, in order to e.g. supplement their adjuvant hormonal therapy (to control their receptor positive metastatic cells) with adjuvant chemotherapy.

Our results are in line with two previous studies, but in both these studies original immunohistochemistry results from old pathology reports were used instead of repeated stainings, so the available data may not be optimally reliable[7, 24]. In the study of Lower *et al*, patients converting to ERα positive in the metastases had prolonged survival compared to those remaining ERα negative, where we only saw such a non-significant trend possibly related to the low number of patients converting to ER positive. Idirisinghe *et al*. observed shorter survival among women with ERα negative metastatic and locally recurrent tumors regardless of the primary tumor ERα status[28]. This finding did not reach statistical significance, also most likely due to the low number of patients.

In order to further explore the clinical implications of hormone receptor conversion in breast cancer patients, a randomized clinical trial in stage IV could be proposed randomizing patients based on receptor expression of the primary tumor only versus of the metastasis and primary tumor together. Further, studies evaluating predictors of receptor conversion would be interesting.

In conclusion, conversion from an ERα or PR positive primary breast carcinoma to a negative distant metachronous metastasis has a negative prognostic impact. Further elucidation of the exact clinical relevance of these findings is warranted, ideally in a prospective randomized clinical trial.

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REFERENCES

1. Holdaway IM, Bowditch JV: Variation in receptor status between primary and metastatic breast cancer. Cancer 1983, 52:479-485.
2. Hull DF, 3rd, Clark GM, Osborne CK, Chamness GC, Knight WA, 3rd, McGuire WL: Multiple estrogen receptor assays in human breast cancer. Cancer Res 1983, 43:413-416.
3. Brunn Rasmussen B, Kamby C: Immunohistochemical detection of estrogen receptors in paraffin sections from primary and metastatic breast cancer. Pathol Res Pract 1989, 185:856-859.
4. Li BD, Byskosh A, Molteni A, Duda RB: Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. J Surg Oncol 1994, 57:71-77.
5. Kuukasjärvi T, Kononen J, Helin H, Holli K, Isola J: Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J Clin Oncol 1996, 14:2584-2589.
6. Branković-Magić M, Janković R, Nesković-Konstantinović Z, Nikolić-Vukosavljević D: Progesterone receptor status of breast cancer metastases. Journal of Cancer Research and Clinical Oncology 2002, 128:55-60.
7. Lower EE, Glass EL, Bradley DA, Blau R, Heffelfinger S: Impact of metastatic estrogen receptor and progesterone receptor status on survival. Breast Cancer Res Treat 2005, 90:65-70.
8. Amir E, Ooi WS, Simmons C, Kahn H, Christakis M, Popovic S, Kalina M, Chesney A, Singh G, Clemons M: Discordance between receptor status in primary and metastatic breast cancer: an exploratory study of bone and bone marrow biopsies. Clin Oncol (R Coll Radiol) 2008, 20:763-768.
9. Gong Y, Han EY, Guo M, Pusztai L, Sneige N: Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. Cancer 2011, 117:705-713.
10. Masood S, Bui MM: Assessment of Her-2/neu overexpression in primary breast cancers and their metastatic lesions: an immunohistochemical study. Ann Clin Lab Sci 2000, 30:259-265.
11. Shimizu C, Fukutomi T, Tsuda H, Akashi-Tanaka S, Watanabe T, Nanasawa T, Sugihara K: c-erbB-2 protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. J Surg Oncol 2000, 73:17-20.
12. Tanner M, Jarvinen P, Isola J: Amplification of HER-2/neu and topoisomerase IIalpha in primary and metastatic breast cancer. Cancer Res 2001, 61:5345-5348.
13. Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M, Verhest A, Bernard-Marty C, Piccart MJ, Larsimont D: Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 2002, 13:1036-1043.
14. Edgerton SM, Moore D, 2nd, Merkel D, Thor AD: erbB-2 (HER-2) and breast cancer progression. Appl Immunohistochem Mol Morphol 2003, 11:214-221.
15. Lear-Kaul KC, Yoon HR, Kleinschmidt-DeMasters BK, McGavran L, Singh M: Her-2/neu status in breast cancer metastases to the central nervous system. Arch Pathol Lab Med 2003, 127:1451-1457.
16. Gong Y, Booser DJ, Sneige N: Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 2005, 103:1763-1769.
17. Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C, Hadary A: Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 2005, 93:552-556.
18. Tapia C, Savic S, Wagner U, Schönegg R, Novotny H, Grilli B, Herzog M, Barascud AD, Zlobec I, Cathomas G, et al: HER2 gene status in primary breast cancers and matched distant metastases. Breast Cancer Res 2007, 9:R31.
19. Santinelli A, Pisa E, Stramazzotti D, Fabris G: HER-2 status discrepancy between primary breast cancer and metastatic sites. Impact on target therapy. Int J Cancer 2008, 122:999-1004.
20. Fabi A, Di Benedetto A, Metro G, Perracchio L, Nistico C, Di Filippo F, Ercolani C, Ferretti G, Melucci E, Buglioni S, et al: HER2 protein and gene variation between primary and metastatic breast cancer: significance and impact on patient care. Clin Cancer Res 2011, 17:2055-2064.
21. Wilking U, Karlsson E, Skoog L, Hatschek T, Lidbrink E, Elmberger G, Johansson H, Lindstrom L, Bergh J: HER2 status in a population-derived breast cancer cohort: discordances during tumor progression. Breast Cancer Res Treat 2011, 125:553-561.

22. Guarneri V, Giovannelli S, Ficarra G, Bettelli S, Maiorana A, Piacentini F, Barbieri E, Dieci MV, D'Amico R, Jovic G, Conte P: Comparison of HER-2 and hormone receptor expression in primary breast cancers and asynchronous paired metastases: impact on patient management. Oncologist 2008, 13:838-844.
23. Broom RJ, Tang PA, Simmons C, Bordeleau L, Mulligan AM, O'Malley FP, Miller N, Andrulis IL, Brenner DM, Clemons MJ: Changes in estrogen receptor, progesterone receptor and Her-2/neu status with time: discordance rates between primary and metastatic breast cancer. Anticancer Res 2009, 29:1557-1562.
24. Liedtke C, Broglio K, Moulder S, Hsu L, Kau SW, Symmans WF, Albarracin C, Meric-Bernstam F, Woodward W, Theriault RL, et al: Prognostic impact of discordance between triple-receptor measurements in primary and recurrent breast cancer. Ann Oncol 2009, 20:1953-1958.
25. Lower EE, Glass E, Blau R, Harman S: HER-2/neu expression in primary and metastatic breast cancer. Breast Cancer Res Treat 2009, 113:301-306.
26. Simmons C, Miller N, Geddie W, Gianfelice D, Oldfield M, Dranitsaris G, Clemons MJ: Does confirmatory tumor biopsy alter the management of breast cancer patients with distant metastases? Ann Oncol 2009, 20:1499-1504.
27. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, et al: Receptor conversion in distant breast cancer metastases. Breast Cancer Res 2010, 12:R75.
28. Idirisinghe PK, Thike AA, Cheok PY, Tse GM, Lui PC, Fook-Chong S, Wong NS, Tan PH: Hormone receptor and c-ERBB2 status in distant metastatic and locally recurrent breast cancer. Pathologic correlations and clinical significance. Am J Clin Pathol 2010, 133:416-429.
29. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, Purdie CA: Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res 2010, 12:R92.
30. Bogina G, Bortesi L, Marconi M, Venturini M, Lunardi G, Coati F, Massocco A, Manfrin E, Pegoraro C, Zamboni G: Comparison of hormonal receptor and HER-2 status between breast primary tumours and relapsing tumours: clinical implications of progesterone receptor loss. Virchows Arch 2011:DOI 10.1007/s00428-00011-01097-00427.
31. Curigliano G, Bagnardi V, Viale G, Fumagalli L, Rotmensz N, Aurilio G, Locatelli M, Pruneri G, Giudici S, Bellomi M, et al: Should liver metastases of breast cancer be biopsied to improve treatment choice? Ann Oncol 2011:DOI 10.193/annonc/mdq751.
32. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, et al: American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol 2010, 28:2784-2795.
33. National Comprehensive Cancer Network: Practice Guidelines in Oncology: Breast Cancer - V2.2011.
34. National Cancer Institute PDQ. Breast Cancer Treatment. In; 2010.
35. Chang J, Clark GM, Allred DC, Mohsin S, Chamness G, Elledge RM: Survival of patients with metastatic breast carcinoma: importance of prognostic markers of the primary tumor. Cancer 2003, 97:545-553.
36. Bartlett JM, Brookes CL, Robson T, van de Velde CJ, Billingham LJ, Campbell FM, Grant M, Hasenburg A, Hille ET, Kay C, et al: Estrogen receptor and progesterone receptor as predictive biomarkers of response to endocrine therapy: a prospectively powered pathology study in the Tamoxifen and Exemestane Adjuvant Multinational trial. J Clin Oncol 2011, 29:1531-1538.
37. Coebergh JW, van Veen EB, Vandenbroucke JP, van Diest P, Oosterhuis W: One-time general consent for research on biological samples: opt out system for patients is optimal and endorsed in many countries. Bmj 2006, 332:665.
38. Solomayer EF, Diel IJ, Meyberg GC, Gollan C, Bastert G: Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. Breast Cancer Res Treat 2000, 59:271-278.
39. Rabbani SA, Mazar AP: Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer Metastasis Rev 2007, 26:663-674.
40. Largillier R, Ferrero JM, Doyen J, Barriere J, Namer M, Mari V, Courdi A, Hannoun-Levi JM, Ettore F, Birtwisle-Peyrottes I, et al: Prognostic factors in 1,038 women with metastatic breast cancer. Ann Oncol 2008, 19:2012-2019.


Breast cancer
Metastases

CHAPTER 4

Predicting hormone receptor conversion in distant breast cancer metastases

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Submitted

ABSTRACT

Background

Most contemporary guidelines advice to biopsy metastases for receptor status in disseminated breast cancer patients to guide therapy decisions, as receptor status may be discordant from the primary tumor (i.e. "receptor conversion"). Such biopsies may not always be easily carried out because of limited accessibility or potential complications. Therefore, accurate prediction of receptor conversion in metastatic breast cancer patients obviating a biopsy would be valuable. In the present study we evaluate routinely available clinicopathologic variables for their potential of predicting hormone receptor conversion in breast cancer metastases.

Methods

Data from a retrospective study in 233 metastatic breast cancer patients was used to establish prediction models for receptor conversion using logistic regression analysis. We aimed to develop decision rules for eight different patient groups: for predicting estrogen receptor alpha (ERα) as well as hormone receptor (HR; ERα and Progesterone Receptor (PR)) conversion from primary positive to negative metastasis and vice versa, and considering the traditional 10% or ASCO 1% threshold for receptor positivity. The models were internally validated by bootstrapping techniques to adjust for overfitting. Predictive performance was assessed by discrimination and calibration.

Results

All positive to negative conversion prediction models performed well across all ERα/HR and 1/10% threshold subgroups (models all based on percentage ERα and PR positive cells of the primary tumor supplemented with time between primary and metastasis for HR at 1%), and were robust under internal validation. Discrimination between patients with or without positive to negative receptor conversion was very good with overoptimism-corrected AUCs between 0.795–0.862. Depending on ERα/HR and 1/10% threshold subgroups, 22 to 45% of all metastatic breast cancer patients with primary receptor positive disease had a predicted conversion risk below 5%, potentially obviating the need to biopsy. The performance of the negative to positive ERα/HR conversion models was too low for clinical relevance.

Conclusion

We showed that the absence of positive to negative hormone receptor conversion can be accurately predicted based on percentage ERα and PR positive cells, potentially obviating the need for metastatic biopsy in this large group of patients.

Introduction

At the time of breast cancer metastatic relapse, the indication for endocrine treatment has for a long time been based on hormone receptor expression of the primary tumor, refraining from routine biopsies of the metastatic lesions as part of the standard workup. However, evidence is mounting that in a substantial proportion of metastatic breast cancer patients the expression of the estrogen receptor alpha (ERα), and progesterone receptor (PR) may differ between the primary breast tumor and distant metastases[1-6]. This receptor conversion could potentially lead to inappropriate use of targeted endocrine therapy in the metastatic setting if solely based on the receptor status of the primary tumor. Several recent guidelines thus recommend to biopsy distant metastases to reevaluate the receptor status[7-9].

Despite this clear rationale for a metastatic breast cancer biopsy, this may not always be feasible because of limited lesion accessibility. Furthermore, a biopsy is invasive and, besides being inconvenient, is associated with a small but relevant risk of complications. Non-invasive receptor assessment by molecular imaging is therefore being actively investigated as an attractive alternative, also facilitating the simultaneous evaluation of multiple lesions[10, 11]. Nevertheless, radionuclide molecular imaging tests are costly and need complicated infrastructure and logistics.

Therefore, prediction of receptor conversion in metastatic breast cancer patients based on routinely available information would be valuable. Such predictions may be helpful to healthcare professionals to identify patients with such a high chance of presence or absence of receptor conversion that receptor status reassessment of metastatic lesions may be obviated. Although little is known about what causes receptor conversion of ERα and PR breast cancer metastases, phenomena such as genomic evolution[12], selective clonal selection elicited by adjuvant systemic treatment or plain analytical variability associated with the assessment of these receptor have all been implicated. In the present study we evaluate routinely available clinicopathologic variables for their potential of predicting hormone receptor conversion in breast cancer metastases, using data of a retrospective study in 233 metastatic breast cancer patients[1].

Materials and Methods

Study population

Female patients with distant metastases were selected from pathology databases from the departments of pathology of 12 Dutch hospitals (the University Medical Center Utrecht, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Centre, the Canisius Wilhelmina Hospital Nijmegen, the Netherlands Cancer Institute Amsterdam, the Medical Center Alkmaar, the Medical Center Zaandam, the University Medical Center Groningen, the St. Antonius Hospital Nieuwegein, the Diakonessenhuis Utrecht, the VU University Medical Center Amsterdam, and the Laboratory for Pathology Dordrecht). We did not include bone metastases, because decalcification methods may give rise to false negative immuno-histochemistry (ref). Paraffin blocks of primary breast carcinomas and corresponding metastases

from 233 patients from the participating labs could be retrieved. Primary diagnoses were made between January 1985 and March 2009. Ethical approval was not required, as the use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in hospitals in The Netherlands[13].
For each case, hematoxylin-eosin stained slides of the paraffin blocks were reviewed by a single pathologist (PJvD) to confirm the presence of malignancy in tissue samples.

Definition of outcome

Receptor conversion was defined as discordant immunophenotype between primary and metastatic breast cancer as determined by immunohistochemistry, incorporating the direction of the conversion (i.e. from primary negative to metastasis positive and vice versa). To accommodate variations in endocrine treatment indication worldwide, we both evaluated receptor conversion solely based on the ERα status as well as based on hormone receptor (HR) status (i.e. the combination of ERα and PR status), and evaluated both the European 10% threshold and American Society of Clinical Oncology 1% threshold for receptor positivity[14]. HR was considered positive if either the ERα or PR was positive and negative otherwise.

Receptor assessment

Immunohistochemical analysis for ERα and PR status was carried out on full 4-µm sections using mouse monoclonal antibodies (ERα: ID5, 1:80, DAKO, Glostrup, Denmark; PR: PgR636, 1:50 DAKO). All primary tumors and metastases were restained for study purposes by the same researcher (LDCH) according to the same protocol to allow optimal pair-wise comparisons, as described before[1]. For detection of the primary antibodies a poly HRP anti Mouse/Rabbit/Rat IgG (ready to use; Powervision, Immunovision Technologies, Brisbane, California, USA) was used. Appropriate negative and positive controls were used throughout.
Scoring of IHC slides was performed by one breast cancer dedicated pathologist with 10+ years of experience (PJvD) in the presence of a second observer (LDCH) with patients and primary and metastatic lesions in random order and blinded to other data in the paired samples. For ERα and PR, the percentage of positively stained nuclei was estimated. In primary tumor and metastatic liver samples, the adequacy of staining was checked by also evaluating the normal breast and liver parenchyma when present.

Candidate predictors

We studied the following candidate predictors for the risk of receptor conversion: age, tumor size, histologic tumor type, tumor grade, percentage ERα and PR positive cells of the primary tumor, HER2 status of the primary tumor, use of hormonal therapy or chemotherapy and time between primary tumor and corresponding metastases. Age, tumor size and time to metastases (interval between day of surgery and day of metastasis biopsy) were checked in the pathological records. Histologic tumor type (assessed according to the World Health Organization) and tumor grade (assessed according to the Nottingham modification of the Bloom-Richardson system) were examined by one pathologist (PJvD). HER2 expression was scored using the DAKO scoring system as 0, 1+, 2+ and 3+ according to standardized criteria considering 3+

cases as positive HER2 (RM-9103-S, 1:100, Neomarkers, Lab Vision Corporation, Fremont, California,USA) [15]. When primary tumor was scored IHC 2+ silver in situ hybridization SISH analysis was performed [16]. According to the ASCO/CAP guidelines, tumors with <6 HER2 copies/tumor cell nucleus were scored as HER2 nonamplified; and tumors with 6 or more HER2 copies/ tumor cell nucleus were scored as HER2 amplified [17].
Adjuvant treatment data were obtained from the Dutch cancer registry (IKNL, The Netherlands (http://www.ikcnet.nl/)).

Statistical analysis

First, we used descriptive statistics to describe the baseline characteristics of the entire study population and subgroups according to ERα and HR receptor status of the primary tumor. We inspected data for missing values of candidate predictors, and baseline characteristics and conversion rates were compared between patients with and without complete data and tested using T-test, Mann Whitney and Chi-square statistics as appropriate. Six predictors (tumor size, histologic type, histologic grade, hormonal therapy, chemotherapy and time between primary tumor and metastases) contained missing data, with the amount of missing values varying between 0.4 and 16.7%. In total, 51 of 233 patients (21.9%) had incomplete information for one or more variables. As disregarding missing data in data-analysis may lead to biased results [18, 19] we multiply imputed missing data (iterative Markov Chain Monte Carlo (MCMC) method; 10 datasets) using all candidate predictors described above, including the outcome variables as well as information on year of diagnosis [18-21]. All below analyses were performed in each imputed dataset separately and then pooled using Rubin's rules (after Fisher's Z transformation for Nagelkerke'sR^2 results) [22, 23].
We aimed to develop decision rules for eight different patient groups: for predicting ERα as well as HR conversion from primary positive to negative metastasis and vice versa, and considering the 10% or 1% threshold for receptor positivity. For each of these eight patient groups, we first assessed the association between all candidate predictors and conversion univariably, and then proceeded to multivariable analyses using logistic regression. The number of conversions per patient group did not allow multivariable analysis of all candidate predictors simultaneously, and we therefore had to select predictors for multivariable modelling. As currently no empirical data are available to guide in selecting the most promising candidate predictors, we employed a forward selection approach using Nagelkerke's R^2 as a measure of explained variation, and selected the predictors with the highest univariable R^2 while fulfilling the one predictor per 10 events rule [24]. Furthermore, to limit the complexity of the models and in view of the amount of data available, we assumed linearity for each continuous predictor (age, tumor size, the percentage ERα and PR positive cells of the primary tumor, and time between primary tumor and metastases) and dichotomized candidate categorical predictors (histologic grade).
Besides the evaluation of performance based on Nagelkerke's R^2 values, we evaluated the predictive performance of each model for discrimination and calibration. Discrimination is the ability to distinguish patients with from those without receptor conversion. This was assessed with the area under the receiver operating characteristic curve (AUC) and the discrimination

slope. An AUC value of 0.5 indicates no discrimination, and a value of 1.0 indicates perfect discrimination. The discrimination slope denotes the absolute difference between mean predicted conversion probability in patients with and without receptor conversion, which shows how well the two outcome groups are separated by the model. To evaluate how close the model-derived predicted conversion probabilities reflect observed probabilities over the entire range of possible values, we made use of calibration plots.

As the modeling strategy to arrive at the decision rules was based on relatively scarce data and included candidate predictor selection, we internally validated the models using bootstrap resampling (n=500) to assess and correct for overoptimism of the prediction models, likely present if the apparent models would just be applied in new patients. Within each bootstrap resample the entire modeling process described above was repeated. This not only provided insight in predictor selection stability, but also yielded overoptimism-corrected estimates of the AUC and R^2. Furthermore, we used the bootstrap-derived shrinkage factor to adjust the estimated regression coefficients, which yielded, after updating the intercept, an internally validated decision rule. For promising decision rules we present ready-to-use score charts to easily calculate the probability of receptor conversion in individual patients.

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS, Inc., Chicago, Il, USA) and R version 2.15.3 (The R foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/) including package (Regression Modeling Strategies. http://CRAN.R-project.org/package=rms). We considered a two-sided p value < 0.05 as statistically significant, and report parameter estimates with 95% confidence intervals (CI; based on 2000 bootstrap resamples for discrimination slope and Nagelkerke's R^2 values). Negative R^2 values resulting from wide confidence intervals, or poorly performing models under bootstrap resampling validation were truncated to zero. Although this is not a diagnostic accuracy study, the report is in agreement with the STARD guidelines [25].

Results

The study included 233 metastatic breast cancer patients with a mean age at primary diagnosis of 53.7 years (SD 11.6; Table 1). Overall, 89% breast tumors were from ductal and 11% from lobular origin. Chemotherapy was the sole adjuvant treatment for 36% of patients, 20% received only adjuvant hormonal therapy, 10% received both and 35% did not receive any therapy at all. The median time between primary tumor and metastasis was 38 months (range 0-22 years). Biopsies were mainly taken from skin metastases (33%), followed by the liver (27%), brain (19%), lung (19%), and the gastro-intestinal tract (3%).

Following a 10% threshold, 147 primary cancers were ERα and 156 HR (i.e. ERα and/or PR) positive, which was 174 and 190 at the 1% threshold. Baseline characteristics for all ERα and HR subgroups are shown in Supplementary Table 1. Positive to negative receptor conversion occurred in 17 of 147 (12%) patients with ERα positive primary cancer at the 10% threshold, which was 23 of 174 (13%) at the 1% threshold, and 25 of 156 (16%) or 29 of 190 (15%) considering HR conversion (10% and 1% thresholds, respectively). Similarly, negative to positive receptor

Table 1. Clinicopathologic characteristics of 233 breast cancer patients studied for receptor conversion in distant metastases.

Feature		Study population (N=233)	Missing
Age at primary diagnosis(years)		53.7 (±11.6)[a]	0
Year of diagnosis		1998 (1985-2008)[b]	0
Tumor size (cm)		2.5 (0.6-16.0)[b]	39
Histologic type	Ductal	89% (206)	1
	Lobular	11% (26)	
Histologic grade	1	4% (8)	3
	2	27% (61)	
	3	70% (161)	
ERα primary*	Negative	37% (86)	0
	Positive	63% (147)	
PR primary*	Negative	45% (104)	0
	Positive	55% (129)	
HR primary*	Negative	33% (77)	0
	Positive	67% (156)	
HER2 primary	Negative	78% (181)	0
	Positive	22% (52)	
Adjuvant therapy	No therapy	35% (77)	12
	Chemotherapy	36% (79)	
	Hormonal therapy	20% (43)	
	Both chemo- and hormonal therapy	10% (22)	
Time to metastasis (months)		38(0-264)[b]	2
Site of distant metastasis	Brain	19% (44)	0
	Lung	19% (43)	
	Liver	27% (63)	
	Skin	33% (76)	
	Gastro-intestinal	3% (7)	

Percentages have been rounded and may not equal to 100%. * 10% threshold. [a] Mean (±sd). [b] Median (range). ERα, estrogen receptor alpha; PR, progesterone receptor; HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

conversion occurred in 7 of 86 (8%) and 12 of 59 (20%) patients with ERα negative primary cancer at the 10% and 1% thresholds, respectively, whereas 8 of 77 (10%) and 19 of 43 (44%) converted from primary HR negative to metastasis positive disease at the 10% and 1% thresholds, respectively.

Before developing decision rules for predicting receptor conversion we inspected patterns of missing predictor data (Supplementary Table 2). A total of 51 (22%) patients had missing data

for one or more predictors. Comparing this group with patients with complete data showed no large differences in predictor variables and conversion rates between the two groups, except that patients with missing data were on average diagnosed at an earlier date. Further analyses were therefore performed after multiple imputation of missing predictor data.

Univariable logistic regression showed that the strongest predictors of positive to negative receptor conversion were the percentage of ERα and PR positive cells of the primary tumor (Table 2). Each 10% increase in ERα positive cells decreased the risk of conversion between 25-35% across all 1% and 10% thresholds and ERα/HR subgroups (ORs between 0.645-0.746). Likewise, although less strong, each 10% increase in PR positive cells decreased the risk of conversion between 17-23% (ORs between 0.769-0.827; all statistical significant). Unlike for positive to negative ERα/HR receptor conversion, there were no prominent predictors for negative to positive conversion across patient subgroups (Table 3). Remarkably though, irrespective of the direction of conversion, primary HER2 positivity was associated with an increased risk of hormone receptor conversion (ORs between 1.28-7.90), albeit not statistically significant in all subgroups (Table 2 and 3).

Table 4 shows the performance of the full prediction models, after selecting the strongest univariable predictors in view of the number of conversions in each subgroup. The performance of all negative to positive ERα/HR conversion models was too low for clinical relevance, with poor discrimination between those with and without conversion (all overoptimism-corrected AUCs<0.65; Table 4b), and we refrained from further evaluation of those models.

By contrast, all prediction models for positive to negative receptor conversion performed well across all ERα/HR and 1/10% threshold subgroups, and were robust under internal validation (Table 4a). All these models included the percentage ERα and PR positive cells of the primary tumor, with the HR at 1% threshold model also including the time between primary and metastasis given the larger number of conversions in this group allowing more predictors (Table 2). Discrimination between patients with or without positive to negative receptor conversion was very good with overoptimism-corrected AUCs between 0.795–0.862 for all models, with the best model for HR conversion using the 10% threshold (Table 4a). Furthermore, the models were able to separate patients with and without conversion fairly well, with the predicted conversion probability in those with actual receptor conversion being on average 13-32% higher across the models than in patients without conversion (overoptimism-corrected discrimination slope), again with the HR at 10% threshold model performing best.

All models were better able to identify a group of patients with very low risk of conversion than a group with very high risk (Table 5). For example, a metastatic breast cancer patient, whose primary tumor was 100% ERα and 100% PR positive, would have a 4% risk of HR negative metastatic disease (given 10% threshold for positivity, see regression equation in Table 5). This in contrast to a woman whose primary tumor was 10% ERα and 10% PR positive, indicating a 76% risk of HR negative metastatic disease. Considering a 5% risk of positive to negative receptor conversion as the threshold below which targeted hormone therapy can be initiated without metastatic receptor status confirmation, a metastatic biopsy could be prevented in between 22 to 45% of all metastatic breast cancer patients with primary receptor positive disease (depending on ERα/HR and 1/10% threshold subgroups; Table 5). Within our data, the predicted

conversion probabilities closely agreed with the observed conversion probabilities, especially in the lower risk regions (Figure 1). To facilitate easy use in clinical practice, conversion risk score charts based on these models are available in Supplementary Figure 1. Allowing one more predictor to each model than strictly acceptable according to the one predictor per 10 events rule, did not materially improve the above models (data not shown).

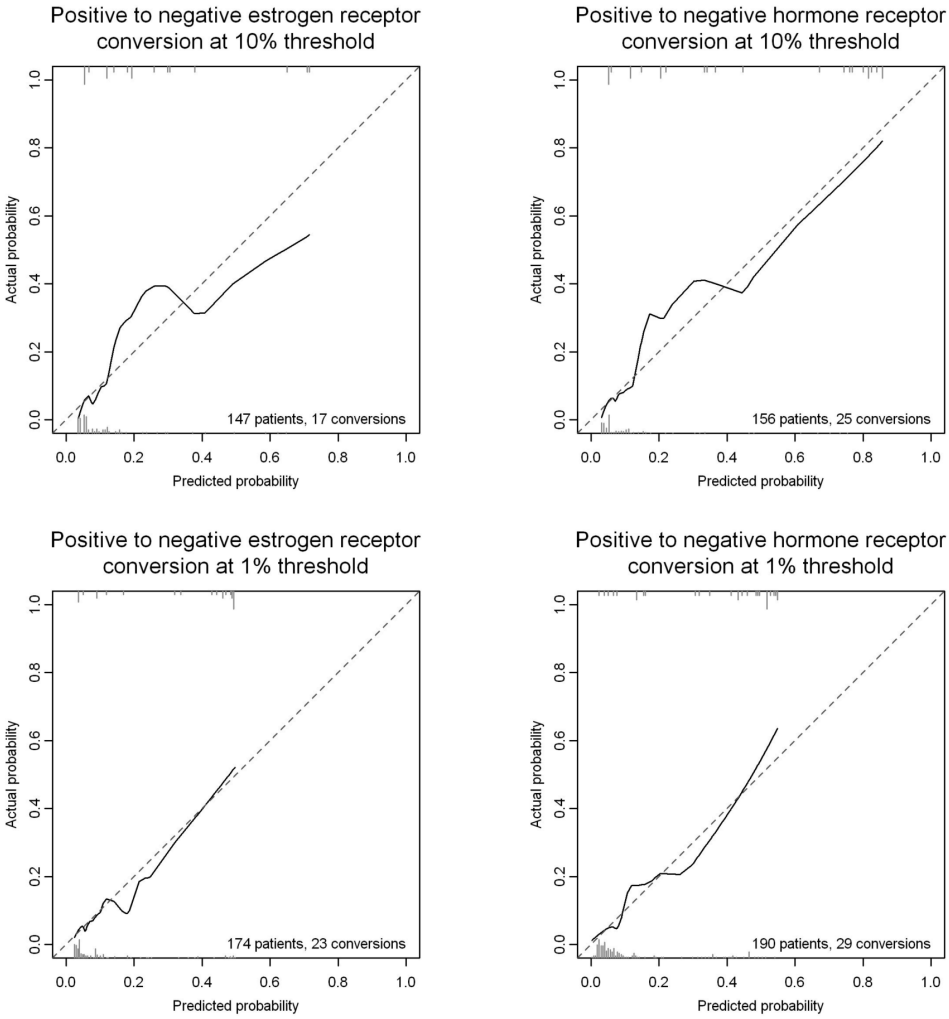

Figure 1. Calibration plots for positive to negative ERα and HR receptor conversion, and 1% and 10% thresholds for receptor positivity.

Table 2a. Association between clinicopathologic variables and positive to negative ERα receptor conversion from primary tumor to distant breast cancer metastases.

Risk of conversion from ERα positive in the primary to negative in the metastasis 10% threshold)

		No conversion (N=130)	Conversion (N=17)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2 (%)	OR (95%CI)	p-value
Age (years)		53.8 (11.8)[a]	55.1 (10.8)[a]	1.009 (0.967-1.054)	0.675	0.2		
	<=50 yrs	88 (56)	13 (8)					
	>50 yrs	89 (74)	11 (9)					
Tumor size (cm)		2.4 (0.6-16) [b]	2.9 (1.5-7.5) [b]	1.092 (0.858-1.390)	0.474	1.3		
Histologic type	Ductal	88 (107)	12 (15)	1.0 (ref)		0.6		
	Lobular	92 (23)	8 (2)	0.62 (0.13-2.89)	0.54			
Grade	Low	90 (56)	10 (6)	1.0 (ref)		0.5		
	High	87 (74)	13 (11)	1.39 (0.48-3.98)	0.542			
ERα primary (%)		95 (20-100)[b]	75 (10-100)[b]	0.680 (0.562-0.823)*	<0.001	20	0.721 (0.590-0.882)*	0.001
PR primary (%)		90 (0-100) [b]	20 (0-90) [b]	0.827 (0.725-0.944) *	0.005	10.9	0.878 (0.759-1.015)*	0.078
HER2 primary	Negative	91 (113)	9 (11)	1.0 (ref)		6		
	Positive	74 (17)	26 (6)	3.63 (1.19-11.09)	0.024			
Hormonal therapy	No	89 (79)	11 (10)	1.0 (ref)		0.1		
	Yes	88 (51)	12 (7)	1.11 (0.39-3.14)	0.848			
Chemotherapy	No	90 (84)	10 (10)	1.0 (ref)		0.5		
	Yes	86 (46)	14 (7)	1.37 (0.48-3.95)	0.561			
Time to metastasis (months)		51.5 (0-264)[b]	38.1 (0-96)[b]	0.988 (0.973-1.002)	0.1	4.3		

Table 2a. Continued

Risk of conversion from ERα positive in the primary to negative in the metastasis (1% threshold)

		No conversion (N=151)	Conversion (N=23)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2 (%)	OR (95%CI)	p-value
Age (years)		54.1 (11.9)[a]	51.9 (13.8)[a]	0.985 (0.949-1.022)	0.419	0.7		
	<=50 yrs	86 (65)	14 (11)					
	>50 yrs	88 (86)	12 (12)					
Tumor size (cm)		2.5 (0.6-16)[b]	2.3 (0.9-15)[b]	1.058 (0.874-1.280)	0.562	0.6		
Histologic type	Ductal	85 (127)	15 (22)	1.0 (ref)		2.9		
	Lobular	96 (24)	4 (1)	0.24 (0.03-1.86)	0.172			
Grade	Low	90 (60)	10 (7)	1.0 (ref)		0.8		
	High	85 (91)	15 (16)	1.51 (0.59-3.88)	0.395			
ERα primary (%)		90 (1-100)[b]	5 (1-100)[b]	0.738 (0.655-0.831)*	<0.001	28.1	0.791 (0.689-0.909)*	0.001
PR primary (%)		75 (0-100)[b]	5 (0-90)[b]	0.772 (0.676-0.883)*	<0.001	18.7	0.877 (0.748-1.029)*	0.108
HER2 primary	Negative	87 (124)	13 (18)	1.0 (ref)		0.2		
	Positive	84 (27)	16 (5)	1.28 (0.44-3.74)	0.657			
Hormonal therapy	No	82 (92)	18 (20)	1.0 (ref)		6.8		
	Yes	95 (59)	5 (3)	0.24 (0.07-0.86)	0.028			
Chemotherapy	No	91 (95)	9 (9)	1.0 (ref)		4.7		
	Yes	80 (56)	20 (14)	2.59 (1.02-6.57)	0.046			
Time to metastasis (months)		47.7 (0-264)[b]	33.4 (0-96)[b]	0.982 (0.967-0.997)	0.017	7.7		

* Data are reported as OR (95%CI) per 10% increase. [a] Mean (±sd). [b] Median (range).
ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 2b. Association between clinicopathologic variables and positive to negative HR receptor conversion from primary tumor to distant breast cancer metastases.

Risk of conversion from HR positive in the primary to negative in the metastasis (10% threshold)

		No conversion (N=131)	Conversion (N=25)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2 (%)		
Age (years)		53.7 (11.8)[a]	54.5 (11.3)[a]	1.006 (0.969-1.043)	0.767	0.1		
	<=50 yrs	83 (57)	17 (12)					
	>50 yrs	85 (74)	15 (13)					
Tumor size (cm)		2.5 (0.6-16)[b]	2.5 (1.1-8.4)[b]	1.066 (0.861-1.32)	0.555	0.8		
Histologic type	Ductal	82 (108)	18 (23)	1.0 (ref)		1.8		
	Lobular	92 (23)	8 (2)	0.41 (0.09-1.84)	0.243			
Grade	Low	88 (57)	12 (8)	1.0 (ref)		1.3		
	High	81 (74)	19 (17)	1.64 (0.66-4.06)	0.288			
ERα primary (%)		95 (1-100)[b]	50 (0-100)[b]	0.645 (0.554-0.751)*	<0.001	39.3	0.669 (0.573-0.781)*	0
PR primary (%)		90 (0-100)[b]	35 (0-90)[b]	0.82 (0.731-0.92)*	0.001	13	0.874 (0.760-1.005)*	0.059
HER2 primary	Negative	88 (114)	12 (15)	1.0 (ref)		9.6		
	Positive	63 (17)	37 (10)	4.47 (1.73-11.54)	0.002			
Hormonal therapy	No	83 (80)	17 (16)	1.0 (ref)		0.1		
	Yes	85 (51)	15 (9)	0.90 (0.37-2.20)	0.808			
Chemotherapy	No	84 (84)	16 (16)	1.0 (ref)		0.1		
	Yes	84 (47)	16 (9)	1.06 (0.43-2.61)	0.909			
Time to metastasis (months)		51.7 (0-264)[b]	33.4 (0-96)[b]	0.983 (0.969-0.997)	0.014	8.3		

Table 2b. Continued

Risk of conversion from HR positive in the primary to negative in the metastasis (1% threshold)

		No conversion (N=161)	Conversion (N=29)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2	OR (95%CI)	p-value
Age (years)		53.8 (12)[a]	52.1 (11.9)[a]	0.988 (0.956-1.022)	0.496	0.4		
	<=50 yrs	86 (71)[a]	14 (12)					
	>50 yrs	84 (90)	16 (17)					
Tumor size (cm)		2.5 (0.6-16)[b]	2.5 (0.9-15)[b]	1.053 (0.881-1.259)	0.569	0.5		
Histologic type	Ductal	83 (137)	17 (28)	1.0 (ref)		3.4		
	Lobular	96 (24)	4 (1)	0.20 (0.03-1.56)	0.126			
Grade	Low	89 (62)	11 (7)	1.0 (ref)		1.9		
	High	82 (99)	18 (22)	1.88 (0.71-4.97)	0.207			
ERα primary (%)		90 (0-100)[b]	1 (0-100)[b]	0.746 (0.669-0.831*	<0.001	28.9	0.810 (0.711-0.923)*	0.002
PR primary (%)		75 (0-100)[b]	5 (0-90)[b]	0.769 (0.678-0.872)*	<0.001	19.6	0.899 (0.772-1.049)*	0.176
HER2 primary	Negative	88 (133)	12 (18)	1.0 (ref)		5.1		
	Positive	72 (28)	28 (11)	2.90 (1.24-6.82)	0.014			
Hormonal therapy	No	80 (101)	20 (26)	1.0 (ref)		8		
	Yes	95 (60)	5 (3)	0.21 (0.06-0.72)	0.013			
Chemotherapy	No	89 (100)	11 (12)	1.0 (ref)		4		
	Yes	78 (61)	22 (17)	2.35 (1.02-5.44)	0.046			
Time to metastasis (months)		46 (0-264)[b]	18.1 (0-186.3)[b]	0.979 (0.965-0.993)	0.003	10.6	0.989 (0.974-1.004)	0.152

* Data are reported as OR (95%CI) per 10% increase. [a] Mean (±sd). [b] Median (range).
HR, hormone receptor; ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 3a. Association between clinicopathologic variables and negative to positive ERα receptor conversion from primary tumor to distant breast cancer metastases.

Risk of conversion from ERα negative in the primary to positive in the metastasis (10% threshold)

		No conversion (N=79)	Conversion (N=7)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2 (%)	OR (95%CI)	p-value
Age (years)		53.7 (11.7)[a]	48.7 (6.2)[a]	0.960 (0.89-1.031)	0.266	3.4		
	<=50 yrs	88 (30)	12 (4)					
	>50 yrs	94 (49)	6 (3)					
Tumor size (cm)		2.5 (0.7-15)[b]	3.5 (1.5-4.7)[b]	0.959 (0.682-1.347)	0.808	0.3		
Histologic type	Ductal	92 (78)	8 (7)					
	Lobular	100 (1)	0 (0)					
Grade	Low	78 (7)	22 (2)	1.0 (ref)		5.1		
	High	93 (72)	7 (5)	0.25 (0.04-1.58)	0.142			
ERα primary (%)		0 (0-5)[b]	0 (0-5)[b]	1.566 (0.010-256.991)*	0.863	0.1		
PR primary (%)		0 (0-90)[b]	0 (0-90)[b]	1.232 (0.899-1.689)*	0.194	3.7		
HER2 primary	Negative	95 (54)	5 (3)	1.0 (ref)		4.7		
	Positive	86 (25)	14 (4)	2.88 (0.60-13.85)	0.187			
Chemotherapy	No	96 (32)	4 (1)	1.0 (ref)		4.2		
	Yes	89 (47)	11 (6)	3.091 (0.34-28.48)	0.32			
Time to metastasis (months)		22.5 (0-186.3)[b]	62.7 (0-107)[b]	1.022 (1.001-1.044)	0.045	11	1.022 (1.001-1.044)	0.045

Table 3a. Continued

Risk of conversion from ERα negative in the primary to positive in the metastasis (1% threshold)

		No conversion (N=47)	Conversion (N=12)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2(%)	OR (95%CI)	p-value
Age (years)		53.6 (10.3)[a]	52.5 (6.9)[a]	0.988 (0.924-1.057)	0.724	0.3		
	<=50 yrs	77 (17)	23 (5)					
	>50 yrs	81 (30)	19 (7)					
Tumor size (cm)		2.6 (0.9-14)[b]	2.5 (1.4-7.1)[b]	0.918 (0.656-1.285)	0.618	1.3		
Histologic type	Ductal	79 (46)	21 (12)					
	Lobular	100 (1)	0 (0)					
Grade	Low	77 (3)	23 (1)	1.0 (ref)		0.2		
	High	80 (44)	20 (11)	0.80 (0.07-9.34)	0.855			
PR primary (%)		0 (0-90)[b]	0 (0-5)[b]	0.257 (0.003-25.176)*	0.562	3.3		
HER2 primary	Negative	87 (34)	13 (5)	1.0 (ref)		9.9	1.0 (ref)	
	Positive	65 (13)	35 (7)	3.66 (0.98-13.62)	0.053		3.66 (0.98-13.62)	0.053
Chemotherapy	No	85 (19)	15 (3)	1.0 (ref)		2.2		
	Yes	76 (28)	24 (9)	1.85 (0.43-8.01)	0.411			
Time to metastasis (months)		18.3 (0-186.3)[b]	17.9 (0-107)[b]	1.005 (0.987-1.025)	0.574	0.8		

* Data are reported as OR (95%CI) per 10% increase. [a] Mean (±sd)/ [b] Median (range).
ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 3b. Association between clinicopathologic variables and negative to positive HR receptor conversion from primary tumor to distant breast cancer metastases.

Risk of conversion from HR negative in the primary to positive in the metastasis (10% threshold)

		No onversion (N=69)	Conversion (N=8)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2 (%)	OR (95%CI)	p-value
Age (years)		53.7 (11.8)[a]	50.9 (6.1)[a]	0.977 (0.914-1.045)	0.501	1.2		
	<=50 yrs	90 (26)	10 (3)					
	>50 yrs	90 (43)	10 (5)					
Tumor size (cm)		2.5 (0.7-15)[b]	3 (1.5-4.7)b	0.915 (0.641-1.305)	0.623	0.9		
Histologic type	Ductal	89 (68)	11 (8)					
	Lobular	100 (1)	0 (0)					
Grade	Low	84 (5)	16 (1)	1.0 (ref)		0.6		
	High	90 (64)	10 (7)	0.57 (0.06-5.83)	0.638			
ERα primary (%)		0 (0-5)[b]	0 (0-5)[b]	1.301 (0.009-195.639)*	0.918	0		
PR primary (%)		0 (0-5)[b]	0 (0-5)[b]	4.119 (0.067-252.474)*	0.5	1.1		
HER2 primary	Negative	96 (50)	4 (2)	1.0 (ref)		17.5	1.0 (ref)	
	Positive	76 (19)	24 (6)	7.90 (1.46-42.58)	0.016		7.90 (1.46-42.58)	0.016
Chemotherapy	No	95 (26)	5 (1)	1.0 (ref)		4.6		
	Yes	87 (43)	13 (7)	3.22 (0.36-28.91)	0.297			
Time to metastasis (months)		21.6 (0-186.3)[b]	28.1 (0-107)[b]	1.014 (0.994-1.034)	0.182	4.2		

Table 3b Continued

Risk of conversion from HR negative in the primary to positive in the metastasis (1% threshold)

		No conversion (N=24)	Conversion (N=19)	Univariable analysis			Full model	
		% (N) or value		OR (95%CI)	p-value	R^2	OR (95%CI)	p-value
Age (years)		55.7 (11.3)[a]	52.9 (6.5)[a]	0.967 (0.903-1.035)	0.334	3		
	<=50 yrs	40 (6)	60 (9)					
	>50 yrs	64 (18)	36 (10)					
Tumor size (cm)		2.8 (1.1-8.5)[b]	2.6 (1.3-14)[b]	1.12 (0.866-1.449)	0.388	3.2		
Histologic type	Ductal	55 (23)	45 (19)					
	Lobular	100 (1)	0 (0)					
Grade	Low	50 (1)	50 (1)	1.0 (ref)		0.1		
	High	56 (23)	44 (18)	0.78 (0.05-13.39)	0.866			
HER2 primary	Negative	63 (19)	37 (11)	1.0 (ref)		6.9	1.0 (ref)	
	Positive	38 (5)	62 (8)	2.76 (0.72-10.57)	0.138		2.16(0.52-9.02)	0.289
Chemotherapy	No	71 (11)	29 (4)	1.0 (ref)		6.7	1.0 (ref)	
	Yes	48 (14)	52 (15)	2.67 (0.66-10.82)	0.169		2.11 (0.48-9.16)	0.321
Time to metastasis (months)		24.5 (0-56.2)[b]	15.9 (0-107) [b]	1.01 (0.983-1.038)	0.476	1.7		

* Data are reported as OR (95%CI) per 10% increase. [a] Mean (±sd). [b] Median (range).
HR, hormone receptor; ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 4a. Performance of prediction models for positive to negative ERα or HR conversion from primary breast cancer to distant metastases at 10% and 1% thresholds.

	10% threshold		1% threshold	
	ERα	HR	ERα	HR
Apparent results				
Overall performance				
Nagelkerke R^2 (%)	24 (5-43)	42 (23-62)	30 (12-49)	33 (16-49)
Discrimination				
AUC	0.833 (0.743-0.924)	0.882 (0.812-0.951)	0.835 (0.750-0.920)	0.840 (0.758-0.922)
Discrimination slope	0.17 (0.06-0.27)	0.35 (0.22-0.48)	0.22 (0.14-0.3)	0.23 (0.16-0.3)
Overoptimism-corrected results[a]				
Shrinkage factor	0.84	0.92	0.91	0.88
Overall performance				
Nagelkerke R^2 (%)	14 (0-33)[b]	35 (16-54)[b]	22 (4-41)[b]	23 (7-40)[b]
Discrimination				
AUC	0.795 (0.704-0.886)[b]	0.862 (0.792-0.931)[b]	0.812 (0.727-0.897)[b]	0.815 (0.733-0.897)[b]
Discrimination slope	0.13 (0.03-0.24)[b]	0.32 (0.20-0.45)[b]	0.20 (0.12-0.28)[b]	0.21 (0.14-0.28)[b]
Predictor selection stability[c]				
Age	0.6%	0.6%	1.2%	0.4%
Tumor size	7.6%	3.4%	1.4%	1.0%
Histologic Type	2.0%	3.2%	1.0%	1.8%
Grade	0.8%	3.4%	0.2%	0.4%
ERα primary	88.2%*	99.8%*	99.0%*	99.2%*
PR primary	47.8%*	63.8%*	83.8%*	89.2%*
HER2 primary	*18.2%*	*44.0%*	*0.2%*	*14.2%*
Hormonal therapy	*1.6%*	*1.0%*	*17.0%*	*20.4%*
Chemotherapy	*2.0%*	*0.4%*	*6.8%*	*6.2%*
Time to metastasis	*7.2%*	*30.6%*	*15.4%*	*50.6%**

[a] Following 500 bootstrap resamples. [b] Assuming same standard error as observed for model development. [c] Frequency of selection of predictors using the full forward AIC based selection procedure in 500 bootstrap resamples. * Predictor selected for full model.
ERα, estrogen receptor alpha; HR, hormone receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 4b. Performance of prediction models for negative to positive ERα or HR conversion from primary breast cancer to distant metastases at 10% and 1% thresholds.

	10% threshold		1% threshold	
	ERα	HR	ERα	HR
Apparent results				
Overall performance				
R^2 (%)	11 (0-39)	18 (0-46)	10 (0-32)	10 (0-33)
Discrimination				
AUC	0.714 (0.459-0.968)	0.737 (0.578-0.896)	0.653 (0.5-0.807)	0.651 (0.492-0.81)
Discrimination slope	0.06 (-0.01-0.13)	0.1 (0.03-0.17)	0.07 (0-0.14)	0.07 (-0.01-0.16)
Overoptimism-corrected results[a]				
Shrinkage factor	0.46	0.57	0.43	0.51
Overall performance				
R^2 (%)	0 (0-17)[b]	1 (0-29)[b]	0 (0-17)[b]	0 (0-13)[b]
Discrimination				
AUC	0.596 (0.341-0.85)[b]	0.650 (0.491-0.809)[b]	0.569 (0.416-0.723)[b]	0.553 (0.394-0.712)[b]
Discrimination slope	0.00 (-0.07-0.07)[b]	0.05 (-0.02-0.12)[b]	0.02 (-0.05-0.09)[b]	-0.01 (-0.09-0.07)[b]
Predictor selection stability[c]				
Age	7.6%	3.6%	6.6%	27.2%
Tumor size	0.0%	0.0%	11.2%	26.6%
Histologic Type	5.2%	7.8%	0.2%	20.2%
Grade	11.4%	1.8%	6.4%	15.0%
ER α primary (%)	3.8%	4.2%		
PR primary (%)	14.6%	7.2%	17.4%	
HER2 primary	14.0%	59.6%*	51.6%*	47.6%*
Hormonal therapy				
Chemotherapy	4.8%	7.6%	10.8%	39.0%*
Time to metastasis	44.6%*	17.8%	12.4%	17.2%

[a] Following 500 bootstrap resamples. [b] Assuming same standard error as observed for model development. [c] Frequency of selection of predictors using the full forward AIC based selection procedure in 500 bootstrap resamples. * Predictor selected for full model.
ERα, estrogen receptor alpha; HR, hormone receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 5. Positive to negative ERα or HR conversion risk classification in patients with metastatic breast cancer according to overoptimism-corrected full prediction models.

	10% threshold				1% threshold			
	ERα		HR		ERα		HR	
Predicted risk of conversion	Patients; N (% of total patients)	Conversion present; predicted N (% of category)	Patients; N (% of total patients)	Conversion present; predicted N (% of category)	Patients; N (% of total patients)	Conversion present; predicted N (% of category)	Patients; N (% of total patients)	Conversion present; predicted N (% of category)
<2.5%	-	-	-	-	-	-	13 (7%)	0 (2%)
2.5-5.0%	33 (22%)	2 (5%)	44 (28%)	2 (4%)	79 (45%)	3 (4%)	50 (26%)	2 (4%)
5.0-7.5%	50 (34%)	3 (6%)	39 (25%)	2 (6%)	18 (10%)	1 (6%)	32 (17%)	2 (6%)
7.5-10%	12 (8%)	1 (9%)	14 (9%)	1 (9%)	22 (13%)	2 (9%)	23 (12%)	2 (9%)
10-25%	38 (26%)	5 (14%)	33 (21%)	5 (14%)	23 (13%)	3 (15%)	25 (13%)	4 (15%)
25-50%	8 (5%)	3 (31%)	10 (6%)	4 (35%)	32 (18%)	14 (42%)	46 (24%)	18 (40%)
50-75%	6 (4%)	3 (56%)	9 (6%)	6 (67%)	-	-	2 (1%)	1 (50%)
75-90%	-	-	7 (4%)	6 (79%)	-	-	-	-
≥90%	-	-	-	-	-	-	-	-
Regression equation	0.779 - 0.0275 x %ERα primary - 0.0110 x %PR primary		1.653 - 0.0369 x %ERα primary - 0.0124 x %PR primary		-0.114 - 0.0214 x %ERα primary - 0.0119 x %PR primary		0.039 - 0.0185 x %ERα primary - 0.0093 x %PR primary - 0.118 x years from primary	

The predicted risk of conversion for an individual patient is estimated using the overoptimism-corrected logistic regression equations as follows: Risk of presence of conversion = 1/(1+exp-(regression equation)). Percentages have been rounded and my not equal to the correct numbers. ERα, estrogen receptor alpha; HR, hormone receptor.

Discussion

In this study we described the development of prediction models for hormone receptor conversion in metastatic breast cancer patients, using data of a retrospective study in 233 metastatic breast cancer patients and using easily available clinicopathological information. We showed that especially the absence of positive to negative hormone receptor conversion can be accurately predicted in a large subset of metastatic breast cancer patients, potentially obviating the need for a metastatic biopsy. The performance of models aimed at positive to negative ERα/HR conversion was very good – also following internal validation for overoptimism –, showing good calibration and discrimination. Unfortunately, the predictive performance of the models aimed at predicting negative to positive ERα/HR conversion was very low.

To value these findings, some strengths and limitations of our study need to be addressed. This is the first study evaluating readily available clinicopathologic variables for their potential of predicting hormone receptor conversion in breast cancer metastases. We were able to establish a large study of patients with rare biopsy material of both metastases and primary breast cancers by making use of the pathology archives of many hospitals and spanning a large number of years. Thorough pathological re-evaluation of full sections of this material by an expert breast pathologist – blinded to within patient metastasis/primary status –, and following re-staining for ERa, PR and HER2 ensured adequate assessment of receptor conversion and pathological predictor variables. All clinicopathologic variables used in the models are easy and inexpensive to measure. Nevertheless, and despite the relative large study population, the low absolute number of conversions in each clinically relevant subgroup did not allow extensive multivariable analysis. This limited the number of predictors we could include simultaneously in each model. As the literature on this topic is currently insufficient for knowledge-based candidate predictor pre-selection, we had to select predictors with a forward modeling selection approach based on the results of univariable analyses. Forward selection does not provide for a simultaneous assessment of the effects of all candidate predictors, and may lead to inappropriate selection and/or omission of predictors [26]. Nevertheless, the stability of predictor selection for all positive to negative conversion models was robust under internal validation by bootstrap, but less so for negative to positive conversion models. Then, the relatively scarce data also precluded the use of transformations or cubic splines to accommodate potential non-linear relations between continuous predictors and conversion risk. Assuming linearity can lead to misinterpretation of the influence of a predictor and to inaccurate predictions in new patients [27].

These drawbacks may all have contributed to the poor performance of the models evaluated for negative to positive conversion, and may have actually led to an underestimation of the true ability of the evaluated clinicopathological predictors to forecast positive to negative conversion, even though discrimination was already observed to be excellent with reasonable calibration.

In view of the limited sample size, we used multiple imputation of missing values (mainly for size of the primary tumor), thereby maximally conserving the effective sample size (and thus power), and actually also curbing the threat of bias [18, 19]. Although multiple imputation is the best way to address the issue of missing values, it should be noted that the final predictors used

for predicting positive to negative ERα/HR conversion did not contain any missing values. Finally, although we made the deliberate choice not to include bone metastases in our study to avoid false negative immunohistochemical results due to decalcification artifacts, the bone (marrow) is the preferential metastatic site of breast cancer, and omission of patients with bone metastases may have limited the generalizability of our study results.

The mechanisms underlying prediction of positive to negative hormone receptor conversion based on percentage ERα and PR positive cells in the primary tumor could be explained by intratumoral heterogeneity of breast cancer; since only a limited number of cells in a primary tumor has the potential to metastasize[12, 28]. When ERα and/or PR positive cells are very dominantly present in the primary tumor, they have a competitive advantage over other – negative- cells in the tissue, reducing the likelihood of occurrence of receptor conversion to negative in corresponding distant metastases, and vice versa.

Although biologically plausible, data concerning the role of adjuvant hormonal treatment on clonal selection are until now equivocal with regard to its effect on receptor conversion[2, 5, 29]. We showed that neither the use of adjuvant hormonal nor adjuvant chemotherapy predicts receptor conversion. Given that primary HER2 positivity was associated with an increased risk of hormone receptor conversion irrespective of the direction of conversion – although not always statistically significant in our data –, may show a possible association between heterogeneity in ERα and PR expression in breast carcinomas and HER2 positivity. Konecny et al. showed that in patients with tumors classified as HR-positive, HER-2/neu amplification/ overexpression was associated with lower tumor ERα and PR levels[30].

The prediction models can be applied in metastatic breast cancer patients. Within primary tumor receptor positive patients with a low probability of receptor conversion, hormonal therapy may perhaps be started without biopsy of and re-evaluating receptor status in metastatic tissue. Importantly, to identify the chance on receptor conversion in individual patients in clinical practice, the accurate percentage hormone receptor positive cells should be mentioned in pathology reports. In the future digital pathology may be an attractive alternative for conventional microscopic evaluation and more accurate estimation of percentage positive cells. It has proven to be an effective tool for achieving consistent interpretation by decreasing intraobserver and interobserver variability[31].

Although negative to positive receptor conversion has high clinical implications i.e. susceptibility for hormonal therapy, unfortunately, we were unable to develop prediction models for negative to positive ERα/HR conversion based on our data.

In conclusion, prediction of receptor conversion from positive to negative in metastatic breast cancer patients is feasible with routinely available pathological information is feasible. Information on the risk of conversion in metastatic breast cancer patients can be of great value in clinical practice. Identifying receptor positive patients with a low chance on receptor conversion to negative may allow clinicians to avoid unnecessary biopsies of the metastases in these patients. Expedited external validation of the prediction models in a large population would be very valuable to confirm our findings and ultimately save metastastatic biopsy in a substantial proportion of breast cancer patients.

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REFERENCES

1. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, et al: Receptor conversion in distant breast cancer metastases. Breast Cancer Res, 12:R75.
2. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, Oldfield M, Dranitsaris G, Tomlinson G, Laupacis A, et al: Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol, 30:587-592.
3. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, Purdie CA: Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res, 12:R92.
4. Amir E, Clemons M, Purdie CA, Miller N, Quinlan P, Geddie W, Coleman RE, Freedman OC, Jordan LB, Thompson AM: Tissue confirmation of disease recurrence in breast cancer patients: pooled analysis of multi-centre, multi-disciplinary prospective studies. Cancer Treat Rev, 38:708-714.
5. Lindstrom LS, Karlsson E, Wilking UM, Johansson U, Hartman J, Lidbrink EK, Hatschek T, Skoog L, Bergh J: Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol, 30:2601-2608.
6. Broom RJ, Tang PA, Simmons C, Bordeleau L, Mulligan AM, O'Malley FP, Miller N, Andrulis IL, Brenner DM, Clemons MJ: Changes in estrogen receptor, progesterone receptor and Her-2/neu status with time: discordance rates between primary and metastatic breast cancer. Anticancer Res 2009, 29:1557-1562.
7. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E: Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol, 23 Suppl 7:vii11-19.
8. National Comprehensive Cancer Network: Practice Guidelines in Oncology: Breast Cancer-V2.2012.
9. National Cancer Institute PDQ. Breast Cancer treatment. In;2011.
10. Munnink THO, Nagengast WB, Brouwers AH, Schröder CP, Hospers GA, Hooge MNL-d, Wall Evd, Diest PJv, Vries EGEd: Molecular imaging of breast cancer. The Breast 2009, 18:S66-S73.
11. Hospers GA, Helmond FA, de Vries EG, Dierckx RA, de Vries EF: PET imaging of steroid receptor expression in breast and prostate cancer. Curr Pharm Des 2008, 14:3020-3032.
12. Polyak K: Breast cancer: origins and evolution. J Clin Invest 2007, 117:3155-3163.
13. van Diest PJ: No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002, 325:648-651.
14. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, et al: American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol, 28:2784-2795.
15. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al.: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707-712.
16. Powell RD, Pettay JD, Powell WC, Roche PC, Grogan TM, Hainfeld JF, Tubbs RR: Metallographic in situ hybridization. Hum Pathol 2007, 38:1145-1159.
17. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, et al: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007, 25:118-145.
18. Janssen KJ, Donders AR, Harrell FE, Jr., Vergouwe Y, Chen Q, Grobbee DE, Moons KG: Missing covariate data in medical research: to impute is better than to ignore. J Clin Epidemiol, 63:721-727.
19. Janssen KJ, Vergouwe Y, Donders AR, Harrell FE, Jr., Chen Q, Grobbee DE, Moons KG: Dealing with missing predictor values when applying clinical prediction models. Clin Chem 2009, 55:994-1001.
20. Donders AR, van der Heijden GJ, Stijnen T, Moons KG: Review: a gentle introduction to imputation of missing values. J Clin Epidemiol 2006, 59:1087-1091.
21. Moons KG, Donders RA, Stijnen T, Harrell FE, Jr.: Using the outcome for imputation of missing predictor values was preferred. J Clin Epidemiol 2006, 59:1092-1101.
22. Rubin DB: Multiple Imputation for Nonresponse in Surveys. 1987.

23. Harel O: The estimation of R 2 and adjusted R 2 in incomplete data sets using multiple imputation. Journal of applied statistics 2009, 36:1109-1118.
24. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR: A simulation study of the number of events per variable in logistic regression analysis. J Clin Epidemiol 1996, 49:1373-1379.
25. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Moher D, Rennie D, de Vet HC, Lijmer JG: The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Ann Intern Med 2003, 138:W1-12.
26. Moons KG, Biesheuvel CJ, Grobbee DE: Test research versus diagnostic research. Clin Chem 2004, 50:473-476.
27. Royston P, Moons KG, Altman DG, Vergouwe Y: Prognosis and prognostic research: Developing a prognostic model. Bmj 2009, 338:b604.
28. Chambers AF, Groom AC, MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002, 2:563-572.
29. Li BD, Byskosh A, Molteni A, Duda RB: Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. J Surg Oncol 1994, 57:71-77.
30. Konecny G, Pauletti G, Pegram M, Untch M, Dandekar S, Aguilar Z, Wilson C, Rong HM, Bauerfeind I, Felber M, et al: Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Natl Cancer Inst 2003, 95:142-153.
31. Nassar A, Cohen C, Agersborg SS, Zhou W, Lynch KA, Barker EA, Vanderbilt BL, Thompson J, Heyman ER, Olson A, et al: A multisite performance study comparing the reading of immunohistochemical slides on a computer monitor with conventional manual microscopy for estrogen and progesterone receptor analysis. Am J Clin Pathol, 135:461-467.

SUPPLEMENTAL

Table S1a+b. Clinicopathologic characteristics of patient subgroups according to ER and HR status, and 1% and 10% threshold for receptor positivity.

		ER + 10%		ER - 10%		ER + 1%		ER - 1%	
Feature		Study population (N=147)	Missing	Study population (N=86)	Missing	Study population (N=174)	Missing	Study population (N=59)	Missing
Age at primary diagnosis (years)		54.0 (±11.7)[a]	0	53.3 (±11.4)[a]	0	53.8 (±12.1)[a]	0	53.4 (±9.7)[a]	0
Year of diagnosis		1997 (1985-2007)[b]	0	2000 (1988-2008)[b]	0	1997 (1985-2007)[b]	0	2000 (1988-2008)[b]	0
Tumor size (cm)		2.5 (0.6-16.0)[b]	24	2.5 (0.7-15.0)[b]	15	2.5 (0.6-16.0)[b]	28	2.4 (1.0-14.0)[b]	11
Histologic type	Ductal	83% (121)	1	99% (85)	0	86% (148)	1	98% (58)	0
	Lobular	17.% (25)		1% (1)		15% (25)		2% (1)	
Histologic grade	1	6% (8)	1	0% (0)	2	5% (8)	1	0% (0)	2
	2	36% (53)		10% (8)		34% (58)		5% (3)	
	3	58% (85)		91% (76)		62% (107)		95% (54)	
ERα primary	Negative								
	Positive								
PR primary	Negative	18% (27)	0	90% (77)	0	10% (17)	0	73% (43)	0
	Positive	82% (120)		11% (9)		90% (157)		27% (16)	
HR primary	Negative	0% (0)	0	90% (77)	0	0% (0)	0	73% (43)	0
	Positive	100% (147)		11% (9)		100% (174)		27% (16)	
HER2 primary	Negative	84% (124)	0	66% (57)	0	82% (142)	0	66% (39)	0
	Positive	16% (23)		34% (29)		18% (32)		34% (20)	
Adjuvant therapy	No therapy	38% (53)	8	29% (24)	4	36% (60)	9	30% (17)	3
	Chemotherapy	22% (30)		60% (49)		27% (45)		61% (34)	
	Hormonal therapy	26% (36)		9% (7)		24% (39)		7% (4)	
	Both chemo- and hormonal therapy	14% (20)		2% (2)		13% (21)		2% (1)	
Time to metastasis (months)		49(0-264)[b]	1	25(0-187)[b]	1	46(0-264)[b]	1	19(0-187)[b]	1

Percentages have been rounded and my not equal to 100%.
ERα, estrogen receptor alpha; PR, progesterone receptor; HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

Table S1a+b. *Continued*

Feature		HR + 10%		HR - 10%		HR + 1%		HR - 1%	
		Study population (N=156)	Missing	Study population (N=77)	Missing	Study population (N=190)	Missing	Study population (N=43)	Missing
Age at primary diagnosis (years)		53.9 (±11.7)[a]	0	53.4 (±11.3)[a]	0	53.6 (±12.0)[a]	0	54.5 (±9.5)[a]	0
Year of diagnosis		1997 (1985-2007)[b]	0	2000 (1988-2008)[b]	0	1997 (1985-2007)[b]	0	2001 (1988-2008)[b]	0
Tumor size (cm)		2.5 (0.6-16.0)[b]	25	2.5 (0.7-15.0)[b]	14	2.5 (0.6-16.0)[b]	32	2.4 (1.1-14.0)[b]	7
Histologic type	Ductal	84% (130)	1	99% (76)	0	87% (164)	1	98% (42)	0
	Lobular	16% (25)		1% (1)		13% (25)		2% (1)	
Histologic grade	1	5% (8)	1	0% (0)	2	4% (8)	3	0% (0)	0
	2	36% (56)		7% (5)		32% (59)		5% (2)	
	3	59% (91)		93% (70)		64% (120)		95% (41)	
ERα primary	Negative	6% (9)		100% (77)		8% (16)			
	Positive	94% (147)		0% (0)		92% (174)			
PR primary	Negative	17% (27)	0	100% (77)	0	9% (17)	0		0
	Positive	83% (129)		0% (0)		92% (173)			
HR primary	Negative								
	Positive								
HER2 primary	Negative	83% (129)	0	68% (52)	0	80% (151)	0	70% (30)	0
	Positive	17% (27)		33% (25)		21% (39)		30% (13)	
Adjuvant therapy	No therapy	39% (57)	8	27% (20)	4	37% (67)	10	24% (10)	2
	Chemotherapy	22% (33)		63% (46)		29% (52)		66% (27)	
	Hormonal therapy	26% (38)		7% (5)		22% (39)		10% (4)	
	Both chemo- and hormonal therapy	14% (20)		3% (2)		12% (22)		0 % (0)	
Time to metastasis (months)		47(0-264)b	1	23(0-187)[b]	1	43(0-264)b	1	20(0-108)[b]	1

Percentages have been rounded and my not equal to 100%.
ERα, estrogen receptor alpha; PR, progesterone receptor; HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

Table S2. Comparison of clinicopathologic characteristics between patients without and with missing data.

Feature		Missing	Patients without missing data (N=182)	Patients with ≥ 1 variable missing (N=51)	p-value
Age at primary diagnosis (years)		0	53.4 (±11.9)[a]	54.7 (±10.4)[a]	0.49[c]
Year of diagnosis		0	1998 (1988-2008)[b]	1995 (1985-2007)[b]	0.028[d]
Tumor size (cm)		39	2.5 (0.7-16.0)[b]	2.4 (0.6-15.0)[b]	0.94[d]
Histologic type	Ductal	1	90% (163)	86% (43)	0.48[e]
	Lobular		10% (19)	14%(7)	
Histologic grade	1	3	3% (5)	6% (3)	0.49[e]
	2		26% (48)	27% (13)	
	3		71% (129)	67% (32)	
ERα primary*	Negative	0	37%(67)	37% (19)	0.95[e]
	Positive		63% (115)	63% (32)	
PR primary*	Negative	0	45% (82)	43% (22)	0.81[e]
	Positive		55% (100)	57% (29)	
HR primary*	Negative	0	32% (59)	35% (18)	0.70[e]
	Positive		68% (123)	65% (33)	
HER2 primary	Negative	0	78% (142)	77% (39)	0.81[e]
	Positive		22% (40)	24% (12)	
Adjuvant therapy	No therapy	12	37%(68)	23% (9)	0.102[e]
	Chemotherapy		32% (59)	51% (20)	
	Hormonal therapy		19% (35)	21% (8)	
	Both chemo- and hormonal therapy		11% (20)	5% (2)	
Time to metastasis (months)		2	38 (0-186)[b]	43 (0-264)[b]	0.85[d]
ER receptor conversion*	No conversion		91% (165)	86% (44)	0.66[e]
	Conversion ER + → ER -		7% (12)	10% (5)	
	Conversion ER - → ER +		3% (5)	4% (2)	
HR receptor conversion*	No conversion		86% (157)	84% (43)	0.61[e]
	Conversion HR + → HR -		10% (18)	14% (7)	
	Conversion HR - → HR +		4% (7)	2% (1)	

Percentages have been rounded and my not equal to 100%. * 10% threshold. [a] Mean (±sd). [b] Median (range) [c] T-test. [d] Mann Whitney test. [e] Chi-square test.
ERα, estrogen receptor alpha; PR, progesterone receptor; HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

A.

Percent **estrogen receptor** positive primary tumor

Percent **progesterone receptor** positive primary tumor	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5
100	4.5	5.1	5.8	6.6	7.5	8.5	9.6	10.9	12.3	13.8	15.6	17.4	19.5	21.8	24.2	26.8	29.6	32.5	35.6	
95	4.7	5.3	6.1	6.9	7.9	8.9	10.1	11.4	12.9	14.5	16.3	18.2	20.4	22.7	25.2	27.9	30.7	33.7	36.9	
90	4.9	5.6	6.4	7.3	8.3	9.4	10.6	12.0	13.5	15.2	17.0	19.1	21.3	23.7	26.3	29.0	31.9	35.0	38.1	
85	5.2	5.9	6.7	7.7	8.7	9.8	11.1	12.6	14.2	15.9	17.8	19.9	22.2	24.7	27.3	30.1	33.1	36.2	39.5	
80	5.5	6.2	7.1	8.1	9.1	10.3	11.7	13.2	14.8	16.7	18.7	20.8	23.2	25.7	28.4	31.3	34.3	37.5	40.9	
75	5.8	6.6	7.5	8.5	9.6	10.9	12.3	13.8	15.5	17.4	19.5	21.8	24.2	26.8	29.6	32.5	35.6	38.8	42.1	
70	6.1	6.9	7.9	8.9	10.1	11.4	12.9	14.5	16.3	18.2	20.4	22.7	25.2	27.9	30.7	33.7	36.9	40.1	43.4	
65	6.4	7.3	8.3	9.4	10.6	12.0	13.5	15.2	17.0	19.1	21.3	23.7	26.2	29.0	31.9	35.0	38.1	41.4	44.8	
60	6.7	7.7	8.7	9.8	11.1	12.6	14.2	15.9	17.8	19.9	22.2	24.7	27.3	30.1	33.1	36.2	39.4	42.8	46.2	
55	7.1	8.1	9.1	10.3	11.7	13.2	14.8	16.7	18.6	20.8	23.2	25.7	28.4	31.3	34.3	37.5	40.8	44.1	47.5	
50	7.5	8.5	9.6	10.9	12.3	13.8	15.5	17.4	19.5	21.7	24.2	26.8	29.6	32.5	35.6	38.8	42.1	45.5	48.9	
45	7.9	8.9	10.1	11.4	12.9	14.5	16.3	18.2	20.4	22.7	25.2	27.9	30.7	33.7	36.8	40.1	43.4	46.8	50.3	
40	8.3	9.4	10.6	12.0	13.5	15.2	17.0	19.1	21.3	23.7	26.2	29.0	31.9	34.9	38.1	41.4	44.8	48.2	51.6	
35	8.7	9.8	11.1	12.6	14.1	15.9	17.8	19.9	22.2	24.7	27.3	30.1	33.1	36.2	39.4	42.8	46.1	49.6	53.0	
30	9.1	10.3	11.7	13.2	14.8	16.6	18.6	20.8	23.2	25.7	28.4	31.3	34.3	37.5	40.7	44.1	47.5	50.9	54.4	
25	9.6	10.9	12.3	13.8	15.5	17.4	19.5	21.7	24.2	26.8	29.5	32.5	35.6	38.8	42.1	45.5	48.9	52.3	55.7	
20	10.1	11.4	12.9	14.5	16.3	18.2	20.4	22.7	25.2	27.9	30.7	33.7	36.8	40.1	43.4	46.8	50.3	53.7	57.1	
15	10.6	12.0	13.5	15.2	17.0	19.1	21.3	23.7	26.2	29.0	31.9	34.9	38.1	41.4	44.8	48.2	51.6	55.0	58.4	
10	11.1	12.6	14.1	15.9	17.8	19.9	22.2	24.7	27.3	30.1	33.1	36.2	39.4	42.7	46.1	49.6	53.0	56.4	59.7	
5	11.7	13.2	14.8	16.6	18.6	20.8	23.2	25.7	28.4	31.3	34.3	37.5	40.7	44.1	47.5	50.9	54.4	57.7	61.0	

B.

Percent **estrogen receptor** positive primary tumor

Percent **progesterone receptor** positive primary tumor	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5
100	3.7	4.4	5.2	6.2	7.3	8.7	10.3	12.1	14.2	16.6	19.3	22.4	25.7	29.4	33.4	37.6	42.0	46.6	51.2	55.8
95	3.9	4.6	5.5	6.6	7.8	9.2	10.9	12.8	15.0	17.5	20.3	23.5	26.9	30.7	34.8	39.1	43.5	48.1	52.7	57.3
90	4.1	4.9	5.8	6.9	8.2	9.7	11.5	13.5	15.8	18.4	21.3	24.6	28.2	32.0	36.2	40.5	45.1	49.7	54.3	58.8
85	4.4	5.2	6.2	7.4	8.7	10.3	12.1	14.2	16.6	19.4	22.4	25.8	29.4	33.4	37.6	42.0	46.6	51.2	55.8	60.3
80	4.6	5.5	6.6	7.8	9.2	10.9	12.8	15.0	17.5	20.3	23.5	27.0	30.7	34.8	39.1	43.6	48.1	52.7	57.3	61.7
75	4.9	5.8	7.0	8.2	9.8	11.5	13.5	15.8	18.4	21.4	24.6	28.2	32.1	36.2	40.6	45.1	49.7	54.3	58.8	63.2
70	5.2	6.2	7.4	8.7	10.3	12.1	14.3	16.7	19.4	22.4	25.8	29.5	33.4	37.7	42.1	46.6	51.2	55.8	60.3	64.6
65	5.5	6.6	7.8	9.2	10.9	12.8	15.0	17.5	20.4	23.5	27.0	30.8	34.8	39.1	43.6	48.2	52.8	57.3	61.8	66.0
60	5.9	7.0	8.3	9.8	11.5	13.5	15.8	18.4	21.4	24.6	28.2	32.1	36.2	40.6	45.1	49.7	54.3	58.8	63.2	67.4
55	6.2	7.4	8.7	10.3	12.2	14.3	16.7	19.4	22.4	25.8	29.5	33.5	37.7	42.1	46.7	51.3	55.8	60.3	64.6	68.7
50	6.6	7.8	9.2	10.9	12.8	15.0	17.5	20.4	23.5	27.0	30.8	34.9	39.2	43.6	48.2	52.8	57.4	61.8	66.0	70.1
45	7.0	8.3	9.8	11.5	13.5	15.8	18.5	21.4	24.7	28.2	32.1	36.3	40.6	45.2	49.7	54.3	58.9	63.2	67.4	71.3
40	7.4	8.7	10.3	12.2	14.3	16.7	19.4	22.5	25.8	29.5	33.5	37.7	42.1	46.7	51.3	55.9	60.4	64.7	68.8	72.6
35	7.8	9.3	10.9	12.8	15.1	17.6	20.4	23.6	27.0	30.8	34.9	39.2	43.7	48.2	52.8	57.4	61.8	66.1	70.1	73.8
30	8.3	9.8	11.5	13.6	15.9	18.5	21.4	24.7	28.3	32.2	36.3	40.7	45.2	49.8	54.4	58.9	63.3	67.4	71.4	75.0
25	8.8	10.3	12.2	14.3	16.7	19.4	22.5	25.9	29.5	33.5	37.7	42.2	46.7	51.3	55.9	60.4	64.7	68.8	72.6	76.1
20	9.3	10.9	12.9	15.1	17.6	20.4	23.6	27.1	30.8	34.9	39.2	43.7	48.3	52.9	57.4	61.9	66.1	70.1	73.8	77.2
15	9.8	11.5	13.6	15.9	18.5	21.4	24.7	28.3	32.2	36.3	40.7	45.2	49.8	54.4	58.9	63.3	67.5	71.4	75.0	78.3
10	10.4	12.2	14.3	16.7	19.5	22.5	25.9	29.6	33.6	37.8	42.2	46.7	51.4	55.9	60.4	64.7	68.8	72.6	76.1	79.3
5	10.9	12.9	15.1	17.6	20.4	23.6	27.1	30.9	34.9	39.2	43.7	48.3	52.9	57.5	61.9	66.1	70.1	73.8	77.3	

C.

Percent **estrogen receptor** positive primary tumor

Percent **progesterone receptor** positive primary tumor	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5
100	3.1	3.4	3.8	4.2	4.7	5.1	5.7	6.3	7.0	7.7	8.5	9.3	10.3	11.3	12.4	13.7	15.0	16.4	17.9	19.5
95	3.3	3.6	4.0	4.4	4.9	5.4	6.0	6.7	7.4	8.1	9.0	9.9	10.9	11.9	13.1	14.4	15.8	17.2	18.8	20.5
90	3.5	3.8	4.2	4.7	5.2	5.8	6.4	7.0	7.8	8.6	9.5	10.4	11.5	12.6	13.8	15.1	16.6	18.1	19.7	21.5
85	3.7	4.1	4.5	5.0	5.5	6.1	6.7	7.4	8.2	9.1	10.0	11.0	12.1	13.3	14.5	15.9	17.4	19.0	20.7	22.5
80	3.9	4.3	4.8	5.3	5.8	6.4	7.1	7.9	8.7	9.6	10.5	11.6	12.7	14.0	15.3	16.7	18.3	19.9	21.7	23.6
75	4.1	4.6	5.0	5.6	6.2	6.8	7.5	8.3	9.2	10.1	11.1	12.2	13.4	14.7	16.1	17.6	19.2	20.9	22.7	24.7
70	4.4	4.8	5.3	5.9	6.5	7.2	8.0	8.8	9.7	10.6	11.7	12.9	14.1	15.5	16.9	18.5	20.1	21.9	23.8	25.8
65	4.6	5.1	5.6	6.2	6.9	7.6	8.4	9.3	10.2	11.2	12.3	13.5	14.8	16.3	17.8	19.4	21.1	22.9	24.9	26.9
60	4.9	5.4	6.0	6.6	7.3	8.0	8.9	9.8	10.8	11.8	13.0	14.3	15.6	17.1	18.7	20.3	22.1	24.0	26.0	28.1
55	5.2	5.7	6.3	7.0	7.7	8.5	9.4	10.3	11.4	12.5	13.7	15.0	16.4	17.9	19.6	21.3	23.2	25.1	27.2	29.4
50	5.5	6.0	6.7	7.4	8.1	9.0	9.9	10.9	12.0	13.1	14.4	15.8	17.3	18.8	20.5	22.3	24.2	26.3	28.4	30.6
45	5.8	6.4	7.1	7.8	8.6	9.5	10.4	11.5	12.6	13.8	15.2	16.6	18.1	19.8	21.5	23.4	25.4	27.4	29.6	31.9
40	6.1	6.8	7.5	8.2	9.1	10.0	11.0	12.1	13.3	14.6	16.0	17.4	19.0	20.7	22.6	24.5	26.5	28.6	30.9	33.2
35	6.5	7.1	7.9	8.7	9.6	10.6	11.6	12.8	14.0	15.3	16.8	18.3	20.0	21.7	23.6	25.6	27.7	29.9	32.2	34.5
30	6.8	7.5	8.3	9.2	10.1	11.1	12.2	13.4	14.7	16.1	17.6	19.2	20.9	22.8	24.7	26.8	28.9	31.1	33.5	35.9
25	7.2	8.0	8.8	9.7	10.7	11.7	12.9	14.1	15.5	16.9	18.5	20.2	22.0	23.8	25.8	27.9	30.1	32.4	34.8	37.3
20	7.6	8.4	9.3	10.2	11.3	12.4	13.6	14.9	16.3	17.8	19.4	21.2	23.0	24.9	27.0	29.2	31.4	33.8	36.2	38.7
15	8.1	8.9	9.8	10.8	11.9	13.0	14.3	15.7	17.1	18.7	20.4	22.2	24.1	26.1	28.2	30.4	32.7	35.1	37.6	40.1
10	8.5	9.4	10.3	11.4	12.5	13.7	15.0	16.5	18.0	19.6	21.4	23.2	25.2	27.2	29.4	31.7	34.0	36.5	39.0	41.6
5	9.0	9.9	10.9	12.0	13.2	14.4	15.8	17.3	18.9	20.6	22.4	24.3	26.3	28.4	30.7	33.0	35.4	37.9	40.4	43.0

D.

0 years from primary to metastasis

Percent **estrogen receptor** positive primary tumor

Percent **progesterone receptor** positive primary tumor	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5
100	6.1	6.6	7.2	7.9	8.5	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.5	22.1	23.7	25.4	27.2
95	6.3	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.5	14.6	15.8	17.0	18.4	19.8	21.3	22.9	24.6	26.3	28.2
90	6.6	7.2	7.9	8.6	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.5	22.1	23.7	25.4	27.2	29.1
85	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.5	14.6	15.8	17.0	18.4	19.8	21.3	22.9	24.6	26.3	28.2	30.1
80	7.2	7.9	8.6	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.7	25.5	27.3	29.1	31.1
75	7.5	8.2	8.9	9.7	10.5	11.5	12.4	13.5	14.6	15.8	17.0	18.4	19.8	21.3	22.9	24.6	26.3	28.2	30.1	32.1
70	7.9	8.6	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.7	25.5	27.3	29.1	31.1	33.1
65	8.2	8.9	9.7	10.6	11.5	12.4	13.5	14.6	15.8	17.0	18.4	19.8	21.3	22.9	24.6	26.4	28.2	30.1	32.1	34.1
60	8.6	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.8	25.5	27.3	29.1	31.1	33.1	35.2
55	8.9	9.7	10.6	11.5	12.4	13.5	14.6	15.8	17.1	18.4	19.8	21.3	22.9	24.6	26.4	28.2	30.1	32.1	34.1	36.2
50	9.3	10.1	11.0	11.9	12.9	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.8	25.5	27.3	29.2	31.1	33.1	35.2	37.3
45	9.7	10.6	11.5	12.4	13.5	14.6	15.8	17.1	18.4	19.8	21.3	22.9	24.6	26.4	28.2	30.1	32.1	34.1	36.3	38.4
40	10.1	11.0	11.9	13.0	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.8	25.5	27.3	29.2	31.1	33.1	35.2	37.3	39.5
35	10.6	11.5	12.4	13.5	14.6	15.8	17.1	18.4	19.8	21.4	23.0	24.6	26.4	28.2	30.1	32.1	34.2	36.3	38.4	40.6
30	11.0	12.0	13.0	14.0	15.2	16.4	17.7	19.1	20.6	22.1	23.8	25.5	27.3	29.2	31.1	33.1	35.2	37.4	39.5	41.8
25	11.5	12.4	13.5	14.6	15.8	17.1	18.4	19.9	21.4	23.0	24.6	26.4	28.2	30.1	32.1	34.2	36.3	38.4	40.7	42.9
20	12.0	13.0	14.0	15.2	16.4	17.7	19.1	20.6	22.2	23.8	25.5	27.3	29.2	31.1	33.1	35.2	37.4	39.5	41.8	44.0
15	12.5	13.5	14.6	15.8	17.1	18.4	19.9	21.4	23.0	24.6	26.4	28.2	30.1	32.1	34.2	36.3	38.5	40.7	42.9	45.2
10	13.0	14.1	15.2	16.4	17.7	19.1	20.6	22.2	23.8	25.5	27.3	29.2	31.1	33.2	35.2	37.4	39.6	41.8	44.1	46.3
5	13.5	14.6	15.8	17.1	18.4	19.9	21.4	23.0	24.7	26.4	28.2	30.2	32.1	34.2	36.3	38.5	40.7	42.9	45.2	47.5

2 years from primary to metastasis

Percent **estrogen receptor** positive primary tumor

Percent **progesterone receptor** positive primary tumor	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5
100	4.8	5.3	5.8	6.3	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	16.9	18.3	19.7	21.2	22.8
95	5.1	5.5	6.0	6.6	7.2	7.8	8.5	9.3	10.1	10.9	11.9	12.9	13.9	15.1	16.3	17.6	19.0	20.5	22.0	23.6
90	5.3	5.8	6.3	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.7	21.2	22.8	24.5
85	5.5	6.0	6.6	7.2	7.8	8.5	9.3	10.1	10.9	11.9	12.9	13.9	15.1	16.3	17.6	19.0	20.5	22.0	23.6	25.4
80	5.8	6.3	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.7	21.2	22.8	24.5	26.2
75	6.0	6.6	7.2	7.8	8.5	9.3	10.1	10.9	11.9	12.9	14.0	15.1	16.3	17.6	19.0	20.5	22.0	23.7	25.4	27.2
70	6.3	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.7	21.2	22.8	24.5	26.3	28.1
65	6.6	7.2	7.8	8.5	9.3	10.1	10.9	11.9	12.9	14.0	15.1	16.3	17.6	19.0	20.5	22.0	23.7	25.4	27.2	29.0
60	6.9	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.7	21.3	22.8	24.5	26.3	28.1	30.0
55	7.2	7.8	8.5	9.3	10.1	11.0	11.9	12.9	14.0	15.1	16.3	17.6	19.0	20.5	22.0	23.7	25.4	27.2	29.0	31.0
50	7.5	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.8	21.3	22.8	24.5	26.3	28.1	30.0	32.0
45	7.8	8.5	9.3	10.1	11.0	11.9	12.9	14.0	15.1	16.3	17.6	19.0	20.5	22.0	23.7	25.4	27.2	29.0	31.0	33.0
40	8.2	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.8	21.3	22.9	24.5	26.3	28.1	30.0	32.0	34.0
35	8.5	9.3	10.1	11.0	11.9	12.9	14.0	15.1	16.3	17.7	19.0	20.5	22.1	23.7	25.4	27.2	29.1	31.0	33.0	35.1
30	8.9	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.8	21.3	22.9	24.5	26.3	28.1	30.0	32.0	34.0	36.2
25	9.3	10.1	11.0	11.9	12.9	14.0	15.1	16.4	17.7	19.0	20.5	22.1	23.7	25.4	27.2	29.1	31.0	33.0	35.1	37.2
20	9.7	10.5	11.4	12.4	13.4	14.5	15.7	17.0	18.3	19.8	21.3	22.9	24.5	26.3	28.1	30.0	32.0	34.1	36.2	38.3
15	10.1	11.0	11.9	12.9	14.0	15.1	16.4	17.7	19.1	20.5	22.1	23.7	25.4	27.2	29.1	31.0	33.0	35.1	37.2	39.4
10	10.5	11.4	12.4	13.4	14.6	15.7	17.0	18.4	19.8	21.3	22.9	24.6	26.3	28.1	30.0	32.0	34.1	36.2	38.3	40.5
5	11.0	11.9	12.9	14.0	15.1	16.4	17.7	19.1	20.5	22.1	23.7	25.4	27.2	29.1	31.0	33.0	35.1	37.3	39.4	41.7

Figure S1. Easy to use score charts for positive to negative ERα or HR receptor conversion in metastatic breast cancer patients. (A) Predicted risk of primary ERα positive to negative metastasis conversion at 10% threshold. (B) Predicted risk of primary hormone receptor positive (i.e. ERα and/or PR positive) to negative metastasis conversion at 10% threshold. (C) Predicted risk of primary ERα positive to negative metastasis conversion at 1% threshold. (D) Predicted risk of primary hormone receptor positive (i.e. ERα and/or PR positive) to negative metastasis conversion at 1% threshold. Values in tables are absolute predicted risk of conversion based on overoptimism corrected logistic regression models. Color coding: green: <5% risk; yellow 5-10% risk; orange >10% risk.


Breast cancer
Metastases
metastases metastases

CHAPTER 5

Discordance in ERα, PR and HER2 receptor status across different distant breast cancer metastases within the same patient

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ABSTRACT

Background

We studied discordance in estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status between multiple distant metastases from the same breast cancer patient.

Methods

Multiple distant metastases from 55 female patients were stained for ERα, PR and HER2 by immunohistochemistry and *in situ* hybridization for confirmation of the HER2 status.

Results

Different metastatic sites within the same patient showed discordance in ERα receptor status in 7.3% or 10.9% of patients (using a 10% or 1% threshold for positivity, respectively). For PR, 29.1% or 30.9% of patients showed discordance. Taking ERα and PR together, 36.4% of cases (both thresholds) showed discrepancy between metastases. In 10.9% (10% threshold) or 14.5% of patients (1% threshold) such discordance could have clinical consequences with regard to hormonal treatment. For HER2, there was 3.6% discordance on the immunohistochemical level but 0% on the gene level.

Conclusion

In a significant proportion of metastatic breast cancer patients, discordance in ERα and PR receptor status between different metastatic sites was observed. This implies that multiple metastases may need to be biopsied to optimally reassess receptor.

Introduction

With 1,380,000 new cases causing 450,000 deaths, breast cancer is the leading cause of cancer death amongst females worldwide [1]. Despite early detection, optimal surgery and adjuvant therapy, about 30% of the patients will develop distant metastases which is the main cause of death in breast cancer [2]. At the occurrence of distant metastases, the choice of systemic treatment used to be based on the tissue characteristics of the primary tumor, refraining from routine biopsies of the metastatic lesions as part of the standard workup.

However, more recently we and others have shown that in a significant proportion of patients the expression of predictive tissuemarkers such as estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor (HER2 receptor) differ between the primary breast tumor and distant metastases. This could potentially lead to inappropriate systemic treatment when its choice would be based solely on the receptor status of the primary tumor [3-8]. Several guidelines therefore now advice to biopsy distant metastases to reassess hormone receptor and HER2 status whenever possible [9-12].

These previous studies have however been done on single distant metastases, while patients often develop multiple metastases. While heterogeneity is considered one of the hallmarks of cancer in general [13, 14], there is yet little information on discrepancy of ERα, PR and HER2 expression between different distant metastatic sites from the same patient, which may occur due to selective spread of positive and negative clones from the primary tumor or by genomic evolution [15]. Such discordance would have important consequences, since it would mean that multiple biopsies from different metastatic sites may be required to optimally reassess receptor status across different distant metastases within the same patient. Or this would rather imply an even more urgent need for molecular imaging that can functionally image receptor status [16].

So far, only a few published studies have addressed the receptor status across multiple metastases, which may be partly due to the rarity of such material [6, 17-19]. These previous studies are very small (10 and 17 patients) [17, 18], used original immunohistochemistry results from the pathology report instead of restaining [6] or did not analyze clinical consequences on the individual patient level [6].

We therefore aimed to study discordance of receptor status between different distant metastases from the same patient. This was done in a relatively large group by re-staining all primary tumors and metastases with current optimal immunohistochemical methods on full sections.

Materials and Methods

Patients

Tissues were identified through a search in the Dutch nationwide pathology database (PALGA). Laboratories that had relevant tissues were contacted directly by PALGA officials with a request to anonymously submit representative tissue blocks to the UMC Utrecht. In this way, formalin fixed paraffin embedded tissue of primary breast carcinomas and corresponding multiple (≥2)

distant metastases from 55 female patients were collected from the Departments of Pathology of the University Medical Center Utrecht, Isala klinieken Zwolle, Erasmus Medical Center Rotterdam, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Center, Gelre Hospital Apeldoorn, Laboratory Sazinon Hoogeveen, and the Laboratory for Pathology Oost Nederland, all in The Netherlands. Table 1 shows basic clinicopathological data. Supplementary Table 1 (online only) shows the time intervals (in months) between diagnoses of the primary tumors and occurrence of the different distant metastases. Fifty two patients had metachronous metastases, 2 patients had synchronous metastases, and in 2 cases the primary tumor was found after the patients had presented with a metastases.

Table 1. Clinicopathologic characteristics of 55 invasive breast cancer patients studied for discordance in receptor expression amongst 118 distant metastases.

Feature	Grouping	N or value	%
Tumor size (cm)	≤2	17	30.9
	>2 and ≤5	20	36.4
	>5	3	5.5
	Not available	15	27.3
Histologic type	Invasive ductal cancer	40	72.7
	Invasive lobular cancer	8	14.5
	Others	7	12.8
Histologic grade	1	5	9.1
	2	24	43.6
	3	26	43.7
MAI (per 2 mm^2)	≤12	29	52.7
	≥13	26	47.3
Lymph node status	Positive	19	34.5
	Negative	11	20
	Not available	25	45.5
Number of metastatic sites	2	48	87.3
	3	6	10.9
	4	1	1.8
Site of distant metastases	Brain	10	8.5
	Lung	15	12.7
	Liver	7	5.9
	Skin	15	12.7
	Bone (marrow)	34	28.8
	Gastro-intestinal	15	12.7
	Gynecologic tract	19	16.1
	Other	3	2.6

MAI, mitotic activity index.

All resection specimens were either immediately fixed in neutral buffered formaldehyde or transported fresh to the pathology laboratory within 1-2 hours of removal from the patient during which specimens were kept cool (known not to influence immunohistochemistry), while biopsies were immediately fixed. Decalcification was not regularly recorded in the pathology reports, but was likely applied to the majority of bone metastases.
Since we are using archival pathology material which does not interfere with patient care and does not involve physical involvement of the patient, no ethical approval is required according to Dutch legislation [20]. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients and therefore informed consent procedure was not required according to our institutional medical ethical review board, this has also been described by van Diest et al [21].
For each case, hematoxylin-eosin stained slides of the paraffin blocks were reviewed by a single pathologist (PJvD) to confirm the presence of malignancy in tumor samples. Workup for metastatic patients was standard and included imaging and pathology including immunohistochemistry to confirm the breast metastatic nature, and to exclude metastases from other sites.

Immunohistochemistry

Immunohistochemical analysis was carried out on full 4-µm sections as described before[3]. In short, mouse monoclonal antibodies used were against ERα (ID5, DAKO, Glostrup, Denmark), PR (PgR636, DAKO), and HER2 (SP3, Neomarkers, Lab Vision Corporation, Fremont, CA, USA). For detection of the primary antibodies, a poly-horseradish peroxidase antimouse/rabbit/rat immunoglobulin G (ready to use, Brightvision, Immunologic, Duiven, The Netherlands) was used. Appropriate controls were used throughout.
Scoring of IHC slides was carried out by an observer (PJvD) in random order, blinded to other data in the paired samples. For ERα and PR, the percentage of positively stained nuclei was estimated. In primary tumor samples, the adequacy of staining was checked by also evaluating the normal breast parenchyma when present. HER2 expression was scored using the DAKO scoring system as 0, 1+, 2+ and 3+ [22].
If HER2 status differed between primary tumor and metastases, or when either primary tumor or metastasis was shown IHC 2+ staining, silver *in situ* hybridization (SISH) analysis was carried out with a fully automated technique (INFORM, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's guidelines.

Scoring

Samples with 10% or more immunopositive malignant cells were classified as ERα or PR positive as usual. In order to also comply with the most recent ASCO guidelines [9], we also used the 1% threshold. HER2 expression was considered positive when the score was 3+. SISH results were evaluated by one observer (MJvdV) according to the manufacturer's instructions, blinded to other data in the paired samples and immunohistochemistry results. According to the ASCO/CAP guidelines, tumors with <6 HER2 copies/tumor cell nucleus were scored as HER2 non-amplified; and tumors with 6 or more HER2 copies/tumor cell nucleus were scored as HER2 amplified [23].

Percentages of patients with heterogeneity in receptor status were calculated. The clinical consequences of heterogeneity with regard to the indication for hormonal therapy were assessed as follows. If a patient has heterogeneity in hormone receptor expression between different distant metastases, a biopsy of a single metastasis with negative expression (for both ERα and PR) could lead to the clinical decision NOT to give hormonal therapy, while the other hormone receptor expressing metastases would be targeted by such hormonal therapy. Therefore, we calculated the percentage of cases with negative expression for both ERα and PR in at least one metastases while at least one other metastasis did express ERα or PR (using both the 1% and 10% thresholds).

Results

ERα and PR discordance

The percentages of nuclei expressing of ERa and PR across breast cancer metastases within the same patient are shown in Figure 1. Using a 10% threshold, 7.3% (4/55) of patients showed discordance in ERα receptor expression in tissue of different metastatic sites, whereas 74.5% (41/55) were uniformly receptor positive and 18.2% (10/55) receptor negative across metastases. Two out of 51 cases (3.9%) showed receptor conversion between the primary tumor and distant metastases but receptor status was stable across the different metastases. PR status differed between distant metastases in 29.1% (16/55) of patients, whereas 32.7% (18/55) were positive receptor and 38.2% (21/55) receptor negative across metastases. Eleven out of 39 cases (28.2%) did not show discordance in receptor expression between metastases, but did show receptor conversion from the primary tumor.

Taking ERα and PR together, 20 of 55 cases (36.4%) showed discordance in hormone receptor expression between metastases. In 6 out of 55 cases (10.9%) such discordance could have clinical consequences with regard to hormonal treatment (Table 2).

Using a 1% threshold, 10.9% (6/55) showed discordance in ERα receptor expression between metastases, whereas 78.2% (43/55) had stable positive and 10.9% (6/55) stable negative receptor status across metastases. Two out of 49 cases (4.1%) did not show discordance in receptor expression but the ERα receptor status differed from the primary tumor. For PR, 30.9% (17/55) showed discrepancy between different metastases, whereas 50.9% (28/55) had stable positive and 18.2% (10/55) stable negative status across metastases. Five out of 38 cases (13.2) did not show discordance but PR receptor status differed from the primary tumor. Taking ERα and PR together, 20 of 55 cases (36.4%) showed discordance in hormone receptor expression between metastases. In 8/55 cases (14.5%) such discordance could have important clinical consequences with regard to hormonal treatment (Table 3). Examples of discordance of hormone receptor expression between different metastases of the same patients are shown in Figure 2.

HER2 discordance

Of the 55 primary breast tumors, 53 cases were HER 2 negative (96.4%) (on the protein level), and 2 cases HER2 positive (3.6%). On the protein level, there was discordance in HER2 status

between metastases in 2 of the 55 cases (3.6%). However, by SISH, there was no difference in gene amplification status (Table 4). Two out of 53 cases (3.8%) did not show discrepancy by IHC, but differed from the primary tumor, although this could not confirmed by SISH analysis (Table 4).

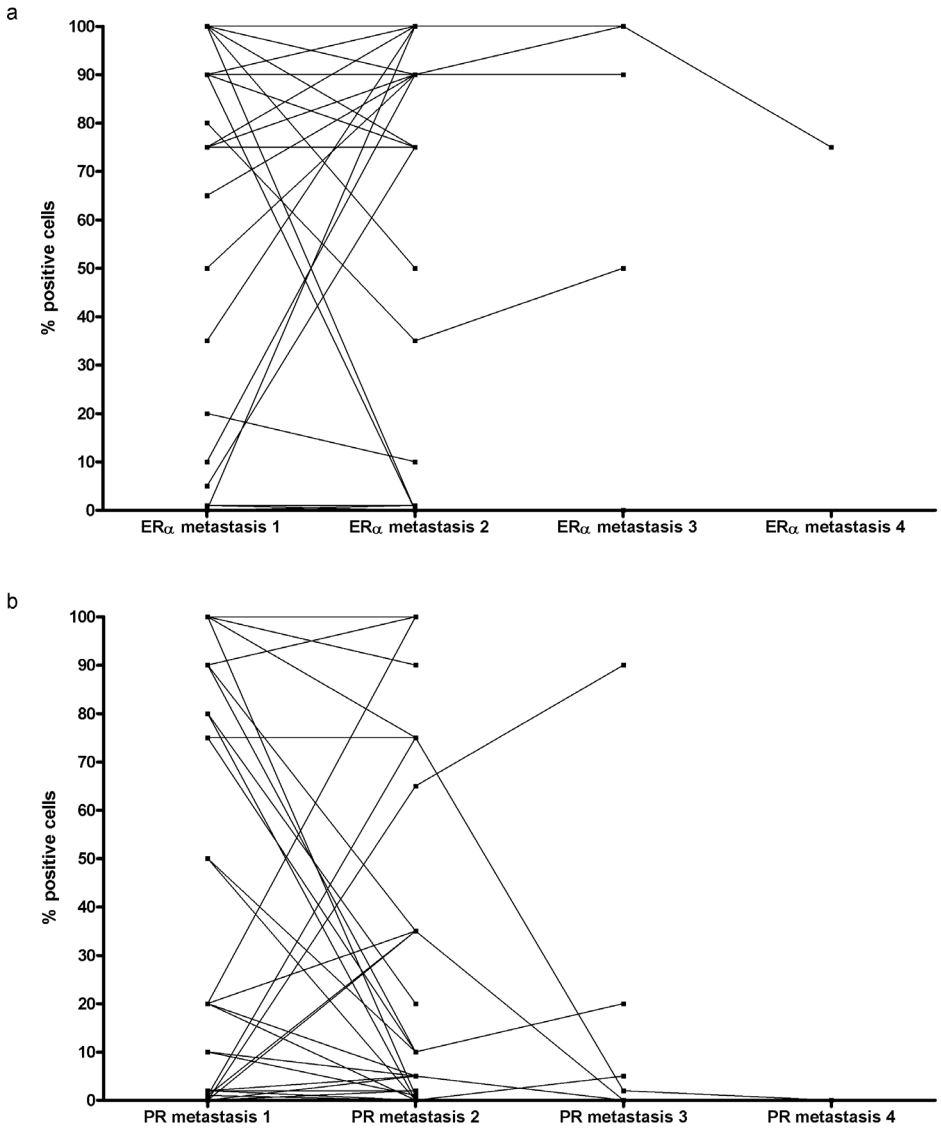

Figure 1. Immunophenotype for ERa (a) and PR (b) across different breast cancer metastases within the same patient.

Table 2. Overview of breast cancer cases with receptor conversion or discordance in hormone receptor status between different distant metastases using a 10% threshold for positivity.

	Primary		Metastasis 1			Metastasis 2			Metastasis 3			Metastasis 4		
	ERα	PR	ERα	PR	Site	ERα	PR	Site	ERα	PR	Site	ERα	PR	Site
1	+	+	+	+	skin	+	-	colon						
2	-	-	-	-	bone	+	-	skin						
3	+	+	+	+	skin	+	-	bone	+	-	ovary			
4	+	-	+	+	lung	+	-	skin						
5	+	+	-	-	ovary	+	-	bone						
6	+	+	+	+	lung	+	-	brain						
7	+	+	+	-	brain	+	+	lung						
8	+	-	+	+	intestine	+	-	stomach						
9	-	-	-	+	liver	-	-	lung						
10	+	+	+	+	bone	+	-	bone						
11	+	+	+	+	ovary	+	-	peritoneum						
12	+	+	+	-	skin	+	+	uterus						
13	+	-	+	-	uterus	+	+	ovary	+	-	bone			
14	+	-	-	+	lung	-	-	brain						
15	+	+	+	+	uterus	+	-	bone						
16	+	+	+	+	ovary	+	+	appendix	+	-	colon	+	-	bone
17	+	+	+	-	peritoneum	+	+	ovary	+	+	colon			
18	+	-	+	-	bone	-	-	bone						
19	+	+	+	-	bone	-	-	bone						
20	+	+	+	+	bone	+	-	stomach						

ERα, estrogen receptor alpha; PR, progesterone receptor.

Discussion

It has been well established that receptor conversion for ERα, PR and HER2 occurs between the primary breast cancer and corresponding distant metastases. Therefore, most guidelines now advice to biopsy a distant metastasis at presentation of metastatic disease in breast cancer patients. However, most patients develop metastases at multiple sites. In the current study we therefore studied ERα, PR and HER2 receptor status across different distant metastases from the same patient in a relatively large group by re-staining all primary tumors and metastases with current optimal immunohistochemical methods on full sections.

Irrespective of the 10% or 1% thresholds used, there was significant discordance in ERα status (7-11%) and PR status (29-31%) across different distant breast cancer metastases within the same patient. This could have important clinical implications when a patient would be denied hormonal therapy based on a biopsy from a single ERα-/PR- metastasis while other metastases do express ERα or PR, a situation observed in 11-15% of cases.

Table 3. Overview of breast cancer cases with receptor conversion or discordance in hormone receptor status between different distant metastases using a 1% threshold for positivity.

	Primary		Metastasis 1			Metastasis 2			Metastasis 3			Metastasis 4		
	ERα	**PR**	**ERα**	**PR**	**Site**	**ERα**	**PR**	**Site**	**ERα**	**PR**	**Site**	**ERα**	**PR**	**Site**
1	+	+	+	+	brain	+	-	lung						
2	+	+	+	+	skin	+	-	skin						
3	-	-	+	-	bone	+	+	skin						
4	+	+	+	+	skin	+	-	bone	+	+	ovary			
5	+	+	-	+	ovary	+	-	bone						
6	-	-	-	+	lung	-	-	bone						
7	+	+	-	-	lung	-	+	brain						
8	-	+	-	+	liver	-	-	lung						
9	+	+	+	+	bone	+	-	bone						
10	+	+	+	+	ovary	+	-	peritoneum						
11	+	-	+	-	uterus	+	+	ovary	+	-	bone			
12	+	-	+	-	lung	-	+	bone	-	-	lung			
13	+	+	+	+	uterus	+	-	bone						
14	+	+	+	+	ovary	+	+	appendix	+	+	colon	+	-	bone
15	+	+	+	-	peritoneum	+	+	ovary	+	+	colon			
16	+	+	+	-	bone	-	-	bone						
17	+	+	+	-	bone	-	-	bone						
18	-	-	+	+	bone	-	-	bone						
19	-	-	-	-	lung	+	-	brain						
20	+	+	+	+	lung	+	-	bone	+	-	bone			

ERα, estrogen receptor alpha; PR, progesterone receptor.

Our results are in line with those by Lindstrom et al. [6] who showed discordance between metastases in 33.6% for ERα status and in 32.0% for PR status. Although their series was much larger than ours, they used original immunohistochemistry results from the pathology report instead of restaining all cases in one laboratory with current optimal methods. In addition, they did not evaluate clinical consequences. Wu et al. [17] observed extensive heterogeneity in biomarker expression among multiple metastatic breast carcinomas from the same patient. However, for ERα and PR there was relative uniformity of expression between different metastases. The strength of their study was uniform specimen handling; warm autopsy material with different sites of metastatic disease sampled at the same time and processed together. However, they evaluated only 10 patients and used tissue microarrays, where focal expression may easily be missed. This may explain why we have found more heterogeneity in the current larger series using full sections.

There are several possible explanations for our findings. It may well be explained by phenomena such as genomic evolution during tumor progression [24], analytical variability associated with the assessment of these receptors [25], or clonal selection during the metastatic process.

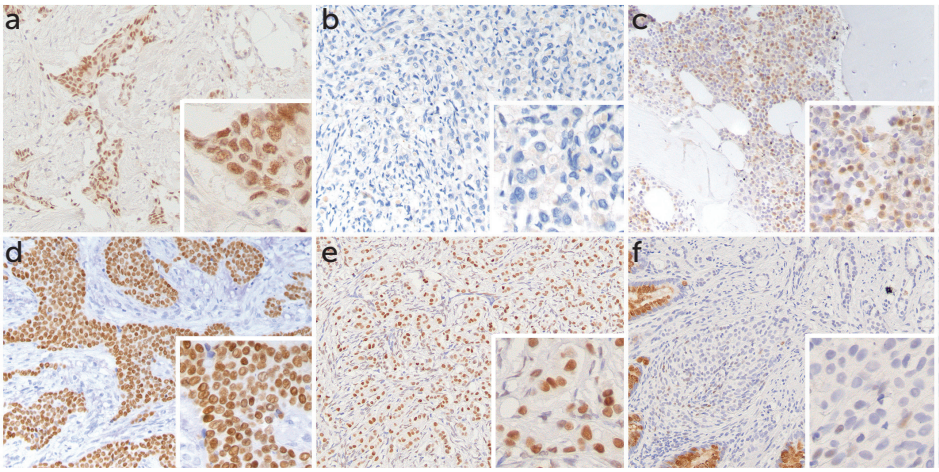

Figure 2. Examples of discordance in receptor status across different distant metastases of the same patient. ERα+ primary tumor (a) with ERα- ovary metastasis (b) and ERα+ bone metastasis tumor (c). PR+ primary tumor (d) with PR+ appendix metastasis (e) and PR- colon metastasis (f).

Data concerning the role of adjuvant hormonal treatment on clonal selection are until now not unanimous whereas effects on receptor status [5, 6, 26, 27]. Lindstrom et al. showed that the proportion of patients losing ERα in their metastases was highest in the group treated with hormonal therapy alone or combined with chemotherapy, lower in the group treated with chemotherapy alone, and lowest in patients that received no treatment ($p<.001$) [6]. However, others showed that there was no correlation between the use of adjuvant therapy and ERα or PR discordance between primary tumor and corresponding metastasis [5, 26]. Data on the role of interval hormonal therapy after the first metastases are yet lacking.

In contrast to these hormone receptors, there was little discrepancy across metastases for HER2: only 4% on the protein level and 0% on the gene level. The fact that scoring of HER2 SISH signals is more straightforward than interpretation of HER2 IHC may play a role here. These discrepancy rates are much lower than those reported by Ganceberg et al. [18] (18% by IHC and 19% by FISH) who analyzed HER-2 status in different metastatic lesions from 17 patients who had at least two metastatic sites. However, they included also lymph node metastases, which are not clinically relevant on deciding on the indication for HER2 targeted therapy. When omitting lymph node metastases, 1/12 cases (8%) showed discrepancy.

One potential confounder in the present study is the inclusion of bone metastases. The bone marrow is the most frequent metastatic site for breast cancer [28], and it has been reported that especially ER+ cancer metastasize to the bone [29]. However, the decalcification process may potentially compromise antigenicity and thereby lead to false negative results.

However, as shown in Tables 2 and 3, only 2/13 respectively 2/18 bone metastases were negative for ERα and PR, respectively, while the primary tumor did show ERα or PR expression. Second, we analyzed the percentage heterogeneity in cases with and without bone metastases. Using the 10% threshold, 10/27 with and 10/28 cases without bone metastases showed heterogeneity

Table 4. SISH results for breast cancer cases showing HER2 receptor conversion in distant metastases by IHC or 2+ scores by IHC in either the primary tumor or the metastases.

	Primary		Metastasis 1			Metastasis 2			Metastasis 3		
	IHC	SISH	IHC	SISH	Site	IHC	SISH	Site	IHC	SISH	Site
1	0	No amplification	0	No amplification	skin	2+	No signal	bone	2+	No amplification	ovary
2	0	No amplification	0	No amplification	skin	2+	No amplification	ovary			
3	0	No amplification	0	No signal	lung	2+	No amplification	brain			
4	1+	No amplification	2+	No amplification	lung	2+	No amplification	brain			
5	1+	No signal	2+	No signal	appendix	2+	No signal	skin			
6	2+	No signal	1+	No signal	liver	0	No signal	lung			
7	2+	No amplification	0	No amplification	lung	0	No amplification	brain			
8	2+	Low amplification	2+	Low amplification	liver	3+	Low amplification	bone			
9	2+	High amplification	2+	High amplification	bone	2+	No signal	bone			
10	2+	Low amplification	3+	High amplification	lung	3+	High amplification	brain			
11	2+	High amplification	3+	High amplification	bone	3+	High amplification	bone			
12	1+	No amplification	1+	No amplification	lung	1+	No amplification	bone	3+	No amplification	bone
13	0	No amplification	1+	No signal	bone	2+	No amplification	stomach			
14	1+	No amplification	1+	No amplification	stomach	2+	No amplification	uterus			

SISH, silver *in situ* hybridization; IHC, immunohistochemistry; HER2, human epidermal growth factor receptor 2

(n.s.). Using the 1% threshold, 13/27 with and 6/28 cases without bone metastases showed heterogeneity (p=0.074), while in 5/13 cases with heterogeneity the bone metastases was not causing heterogeneity. In addition, we observed HER2 expression in the bone metastases in similar rates as in the primary tumors, so we have at this stage insufficient arguments to assume that decalcification plays a significant role here and exclude results of bone metastases from the current analysis.

In total 9% (5 of 55 patients, 95% confidence interval 3-20%) had HER2 positive metastases. This is a slight under-representation of HER2 positive cases as would be expected in the metastatic breast cancer population. We have no likely explanation for this. Cases were identified through a search in the Dutch nationwide pathology database (PALGA), applying no selection criteria, so the current series should be a completely unselected one.

In conclusion, in a significant proportion of metastatic breast cancer patients, there was discordance in ERα and PR receptor status between different metastatic sites, with potential consequences for hormonal treatment in 11-15% of cases when only one random metastasis would be biopsied. This implies that multiple metastases may need to be biopsied to optimally reassess receptor status and set the optimal indication for hormonal therapy in these patients. For HER2, there seems to be only limited discordance on the protein level and even none on the gene level.

Having said this, one have to conclude that in the best interest of the patient it is adamant to stimulate the development of non-invasive assessments of the metastatic sites and their respective hormone receptor status, e.g. by molecular imaging [16]. In the current study we have specifically focused on the current most frequently used markers for breast cancer treatment, but with the evolving knowledge on molecular diagnostics it is likely that more markers will be found. These will contribute to an even greater discordance between primary tumors and their metastases and between the individual metastatic sites which makes the request for non-invasive molecular diagnostics even more pronounced.

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REFERENCES

1. Society AC. Global Cancer Facts & Figures 2nd Edition. 2011.
2. Rabbani SA, Mazar AP. Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer Metastasis Rev 2007; 26: 663-674.
3. Hoefnagel LD, van de Vijver MJ, van Slooten HJ et al. Receptor conversion in distant breast cancer metastases. Breast Cancer Res 12: R75.
4. Amir E, Clemons M, Purdie CA et al. Tissue confirmation of disease recurrence in breast cancer patients: pooled analysis of multi-centre, multi-disciplinary prospective studies. Cancer Treat Rev 38: 708-714.
5. Amir E, Miller N, Geddie W et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol 30: 587-592.
6. Lindstrom LS, Karlsson E, Wilking UM et al. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol 30: 2601-2608.
7. Niikura N, Liu J, Hayashi N et al. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. J Clin Oncol 30: 593-599.
8. Thompson AM, Jordan LB, Quinlan P et al. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res 12: R92.
9. Hammond ME, Hayes DF, Dowsett M et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol 28: 2784-2795.
10. National Comprehensive Cancer Network: Practice Guidelines in Oncology: Breast Cancer - V2.2012.
11. National Cancer Institute PDQ. Breast Cancer Treatment. In; 2011.
12. Cardoso F, Harbeck N, Fallowfield L et al. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 23 Suppl 7: vii11-19.
13. Russnes HG, Navin N, Hicks J, Borresen-Dale AL. Insight into the heterogeneity of breast cancer through next-generation sequencing. J Clin Invest 121: 3810-3818.
14. Gerlinger M, Rowan AJ, Horswell S et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366: 883-892.
15. Kuukasjarvi T, Karhu R, Tanner M et al. Genetic heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. Cancer Res 1997; 57: 1597-1604.
16. Munnink THO, Nagengast WB, Brouwers AH et al. Molecular imaging of breast cancer. The Breast 2009; 18: S66-S73.
17. Wu JM, Fackler MJ, Halushka MK et al. Heterogeneity of breast cancer metastases: comparison of therapeutic target expression and promoter methylation between primary tumors and their multifocal metastases. Clin Cancer Res 2008; 14: 1938-1946.
18. Gancberg D, Di Leo A, Cardoso F et al. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 2002; 13: 1036-1043.
19. Niehans GA, Singleton TP, Dykoski D, Kiang DT. Stability of HER-2/neu expression over time and at multiple metastatic sites. J Natl Cancer Inst 1993; 85: 1230-1235.
20. <http://www.ccmo-online.nl/main.asp?pid=10&sid=30&ssid=51>.
21. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002; 325: 648-651.
22. Slamon DJ, Godolphin W, Jones LA et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244: 707-712.
23. Wolff AC, Hammond ME, Schwartz JN et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007; 25: 118-145.
24. Polyak K. Breast cancer: origins and evolution. J Clin Invest 2007; 117: 3155-3163.
25. De Maeyer L, Van Limbergen E, De Nys K et al. Does estrogen receptor negative/progesterone receptor positive breast carcinoma exist? J Clin Oncol 2008; 26: 335-336; author reply 336-338.
26. Li BD, Byskosh A, Molteni A, Duda RB. Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. J Surg Oncol 1994; 57: 71-77.

27. Idirisinghe PK, Thike AA, Cheok PY et al. Hormone receptor and c-ERBB2 status in distant metastatic and locally recurrent breast cancer. Pathologic correlations and clinical significance. Am J Clin Pathol 133: 416-429.
28. Solomayer EF, Diel IJ, Meyberg GC et al. Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. Breast Cancer Res Treat 2000; 59: 271-278.
29. Largillier R, Ferrero JM, Doyen J et al. Prognostic factors in 1,038 women with metastatic breast cancer. Ann Oncol 2008; 19: 2012-2019.

Supplementary Table 1. Time intervals (months) between diagnosis of the primary tumors and different distant metastases for 55 invasive breast cancer patients studied for discordance in receptor expression amongst distant metastases.

	Time (months) to distant metastases			
Patient	first	second	third	fourth
1	53	54		
2	25	29		
3	17	42		
4	70	72		
5	62	64		
6	114	117		
7	49	57		
8	16	19		
9	20	23	24	
10	41	127		
11	45	45		
12	39	91		
13	40	52		
14	32	42		
15	31	67		
16	57	60		
17	66	123		
18	41	42		
19	35	40		
20	106	109		
21	95	95		
22	86	86		
23	90	123		
24	99	100		
25	24	25		
26	36	42		
27	86	90	110	
28	182	182		
29	50	74		
30	90	90		
31	0	6		
32	130	135		
33	21	21	25	
34	27	30		
35	62	76		
36	55	77		

Supplementary Table 1. *Continued*

	Time (months) to distant metastases			
Patient	first	second	third	fourth
37	162	170	179	
38	88	162		
39	11	11	22	141
40	112	112	112	
41	1	27		
42	18	25		
43	16	32		
44	23	26		
45	20	30		
46	13	15		
47	11	14		
48	15	26		
49	53	54		
50	0	0		
51	60	110	123	
52	-18	5		
53	-3	11		
54	3	27		
55	1	12		


Breast cancer
Metastases

CHAPTER 6

Phosporylated mTOR expression in primary and corresponding metastatic breast tumors after adjuvant endocrine therapy

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ABSTRACT

Background

Both preclinical and clinical data suggest that activation of the PI3K/Akt/mTOR pathway in response to hormonal therapy results in acquired endocrine therapy resistance.

Methods

We evaluated differences in activation of the PI3K/Akt/mTOR pathway in estrogen receptor α (ERα) positive primary and corresponding metastatic breast tumors tissue by scoring the proportion of tumor cells (0-100%) immunostained for phosphorylated mTOR (p-mTOR). The difference between p-mTOR in primary and metastatic tumor was calculated and tested for an association with adjuvant endocrine therapy.

Results

In patients who had received endocrine therapy (N=34), p-mTOR expression increased in metastatic tumor lesions compared to the primary tumor (median difference 45%), while in patients who had not received adjuvant endocrine therapy (N=37), no difference was found. In a multivariate analysis, adjuvant endocrine therapy was significantly associated with an increase in p-mTOR (p=0.01).

Conclusion

Compensatory activation of the PI3K/Akt/mTOR pathway might indeed be a clinically relevant resistance mechanism resulting in acquired endocrine therapy resistance.

Introduction

Until recently, interference with estrogen receptor alpha (ERα) and/or HER2 signaling were the only molecular targeted therapies clinically available for breast cancer patients. With the approval of everolimus for postmenopausal patients with ERα-positive metastatic breast cancer, targeting the PI3K/Akt/mTOR pathway has become a new therapeutic option in the disease. In breast cancer cell lines exposed to long term estrogen depletion, activation of the PI3K/Akt/mTOR pathway occurs as an adaptive change and results in hormone independent cell growth[1]. This escape from hormone dependency can be overcome by exposure of cells to inhibitors of the PI3K/AKT/mTOR pathway, like mTOR inhibitors[1, 2]. Metastatic breast cancer patients with previous exposure to endocrine therapy do derive substantial benefit from the addition of an mTOR inhibitor to endocrine therapy compared to endocrine therapy alone[3, 4]. This suggests that mTOR activation in response to anti-estrogens is indeed a clinically relevant mechanism, resulting in acquired endocrine therapy resistance. Nevertheless, the occurrence of compensatory activation of the PI3K/Akt/mTOR pathway in response to anti-estrogens has not yet been well established in clinical samples. Tumor biopsies from patients that progressed after treatment with anti-estrogens are not routinely taken in clinical practice. In addition, in prospective randomized trials, fresh biopsies from metastatic tumors are hardly ever mandatory. In a small series of primary breast tumors and their distant metastases, Akcakanat et al observed discordant expression of phosphorylated 4E-binding protein 1 (4E-BP1), a marker of mTOR activity[5]. Most of these primary tumors were ERα negative and thereby an association with adjuvant endocrine therapy could not be explored. Our aim was to evaluate changes in mTOR activation between ERα positive primary tumors and corresponding metastatic tumors and to test whether potential differences are associated with adjuvant endocrine therapy.

Materials and Methods

From a previously described series of 233 breast cancer patients from whom both primary tumor tissue as well as metachronous non-bone distant metastatic tumor tissue was collected[6], sufficient tumor material was available for 42 ERα positive patients who had received adjuvant endocrine therapy. In addition we selected a comparable amount of control patients (n=42) who had not received adjuvant endocrine therapy. The association between these two groups and known prognostics factors was calculated using Mann Whitney U or Fisher exact tests. Immunohistochemical analysis was carried out on 4-µm sections. ERα, progesterone receptor (PR) and HER2 status were determined as previously described[6]. Samples with 1% or more immunopositive ERα or PR malignant cells were classified according the new ASCO guidelines as positive[7]. Primary tumor tissue and corresponding metastatic tumor tissue were stained for the expression of p-mTOR using a standardized protocol on the Ventana Benchmark® Ultra system automatic immunostainer with a monoclonal antibody raised against p-mTOR(Ser2448) (Cell Signaling # 2976) (Supplementary Table 1). The percentage of tumor cells with submembranous staining was scored by one observer (JW), blinded to other data in the paired

samples. The difference in expression of p-mTOR between primary and metastatic tumor was calculated. We assessed whether this p-mTOR difference between primary and metastatic tumor was associated with known clinico-pathological factors (location of metastasis, lymph node status, T-stage, grade, HER2 status and PR status) or varied between patients who did and did not receive endocrine therapy, using Mann-Whitney tests. In addition we performed a multivariate linear regression model including the same clinico-pathological factors.

Results

Of the 84 selected patients, a total of 71 (34 from patients who had received adjuvant endocrine therapy and 37 from patients who had not received endocrine therapy) could be used for analysis after staining with p-mTOR (Supplementary Figure 1). Location of metastasis was predominantly skin (N=26) and liver (N=21). In addition, metastases were localized in brain (N=13), lung (N=7) or gastro-intestinal (N=4). Median time to metastasis was 54 months.

Table 1. Characteristics of patients who had not received endocrine therapy and who had received endocrine therapy.

			adjuvant endocrine therapy		
			No (37)	Yes (34)	
		Total (%)	N (%)	N (%)	p-value
Median time to metastasis (months)		54	53	54	0.46[a]
Site of distant metastasis	Skin	26 (37)	15 (41)	11 (32)	0.62[b]
	Other	45 (63)	22 (59)	23 (68)	
Histologic Grade	1-2	31 (44)	18 (49)	13 (38)	0.47[b]
	3	40 (56)	19 (51)	21 (62)	
T-stage	T 1-2	51 (72)	28 (76)	23 (68)	0.29[b]
	T 3-4	9 (13)	3 (8)	6 (18)	
	Missing	11 (15)	6 (16)	5 (15)	
Lymph node status	Negative	18 (25)	15 (41)	3 (9)	0.003[b]
	Positive	43 (61)	17 (46)	26 (76)	
	Missing	10 (14)	5 (14)	5 (15)	
PR	Negative	9 (13)	3 (8)	6 (18)	0.26[b]
	Positive	62 (87)	34 (92)	28 (82)	
HER2	Negative	64 (90)	36 (97)	28 (82)	0.05[b]
	Positive	7 (10)	1 (3)	6 (18)	
Chemotherapy	No	42 (59)	19 (51)	23 (68)	0.23[b]
	Yes	29 (41)	18 (49)	11 (32)	

[a] Mann Whitney U tests. [b] Fisher exact test (only cases without missing data were analyzed).
PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

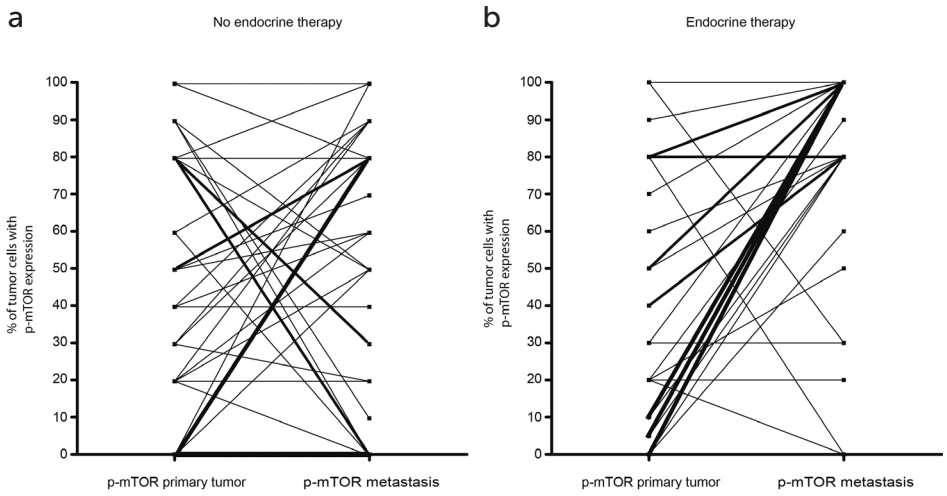

Figure 1. Changes in p-mTOR expression between primary and corresponding metastatic tumor tissue from patients who had not received adjuvant endocrine therapy (a) and who had received adjuvant endocrine therapy (b).

Patient characteristics of both endocrine-treated patients and patients who had not received endocrine therapy are shown in Table 1. Patients who had received endocrine therapy were more often lymph node positive compared to patients who had not received adjuvant endocrine therapy (p=0.002). In the total group of 71 patients, median p-mTOR expression in primary tumor tissue was 40% (41% mean), compared to 80% (65% mean) in tumor biopsies from metastatic sites. In our univariate analysis, none of the evaluated clinico-pathological factors was significantly associated with a differential p-mTOR change between primary and metastatic tumor tissue (Table 2). In patients who had received endocrine therapy we observed an increase in p-mTOR expression in metastatic tumor lesions compared to the primary tumor (median difference 45%) (Figure 1 and Supplementary figure S2b). This was significantly different from the change observed in patients who did not receive endocrine therapy (median difference 0 %) (Figure 1 and Supplementary figure S2a) p=0.003). The difference remained significant in the multivariate regression model (p=0.01) (Supplementary Table 2). The results did not substantially change when a cutoff of 10% was applied for ERα positivity. In addition, in multivariate analysis, a positive PR status was associated with an increase in p-mTOR (p=0.01).

Discussion

In this study, we showed that adjuvant endocrine therapy is significantly associated with an increase in p-mTOR expression in biopsies from subsequent metastatic tumor tissue compared to corresponding primary tumor tissue. This suggests that m-TOR activation in response to anti-estrogens results in acquired hormone resistance.

The observed increase in p-mTOR expression in metastatic tumor tissue was not only associated with adjuvant endocrine therapy, but in our multivariate analysis we did also observe an association with a positive progesterone receptor status. It is well known that PR expression is driven by estrogen receptor signaling[8]. We hypothesize that tumors that are highly dependent on ER-signaling are more likely to acquire activation of additional growth factor pathways compared to tumors that are not selectively dependent on ER-signaling.

A limitation of our study is the lack of a control group of patients that did not relapse. The analysis of mTOR activation in a control group of adjuvant endocrine treated patients who did not progress is however unfeasible. To analyze differences in mTOR activation between responders and non-responders, serial biopsies from patients treated with neo-adjuvant endocrine therapy would be suitable. Cavazonni et al[2] observed an increase in PI3K/AKT/mTOR related gene and protein expression in a small series of breast tumor biopsies from patients who progressed after neo-adjuvant endocrine therapy (N=9). Unfortunately, tumor biopsies

Table 2. Association between change in p-mTOR expression and clinico-pathological factors. Histologic grade was assessed according to the Nottingham modification of the Bloom-Richardson system, applying standardized mitotic counts [14].

		number	median difference between % p-mTOR expression metastatic and primary tumor tissue	p-value*
Site of distant metastasis	Skin	26	25%	0.76#
	Liver	21	20%	
	Brain	13	0%	
	Lung	7	60 %	
	Gastro-intestinal	4	40%	
Histologic grade	1-2	31	20 %	0.34
	3	40	30 %	
T-stage	T 1-2	62	30 %	0.71
	T 3-4	9	20 %	
Lymph node status	Negative	18	15 %	0.53
	Positive	53	30 %	
PR	Negative	9	0 %	0.16
	Positive	62	30 %	
HER2	Negative	64	25 %	0.56
	Positive	7	30 %	
Endocrine therapy	No	37	0%	0.003
	Yes	34	45 %	
Chemotherapy	No	42	30 %	0.25
	Yes	29	20 %	
All		71	40%	

* Mann-Whitney test, except for # Kruskall Wallis test.
PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

from patients who did not progress were not analyzed. Nevertheless, these and our results suggest that adaptive activation of the PI3K/Akt/mTOR pathway in response to adjuvant endocrine therapy is a clinically relevant mechanism resulting in acquired endocrine therapy resistance. An alternative explanation for the observed increase in mTOR activity could be that endocrine therapy results in clonal selection of tumor cells that are not selectively dependent on estrogen signaling. Although it is conceivable that small biopsies might have had better fixation and therefore would be more likely to stain for p-mTOR, the observed increase in p-mTOR expression in the group of endocrine treated patients was significantly different from the group of patients who did not receive endocrine therapy, and is therefore not likely to be simply explained by difference in fixation.

Activation of the PI3K/ AKT/mTOR pathway does not only result in endocrine therapy resistance, but may also cause trastuzumab resistance in HER2 positive breast cancer[9]. Similar to our results, Chandarlapaty et al observed a higher rate of PI3K/ AKT/mTOR pathway activation in metastatic breast tumors from patients with previous exposure to trastuzumab compared to trastuzumab naïve controls[10]. Considering these adaptive molecular changes in response to various treatments, it seems clear that the biological behavior of a metastatic tumor cannot always be predicted by the molecular make-up of the primary tumor. Consequently, for identification of companion diagnostics for molecular targeted drugs, analysis of primary tumor tissue is likely to fail to predict treatment response in the metastatic setting. As an example, biomarker analysis in metastatic breast cancer patients who participated in the Cleopatra trial (randomizing between Pertuzumab and placebo) failed to identify a subgroup of patients particularly benefitting from dual HER2 blockade[11]. The majority of analyzed tumor tissue in this study originated from primary tumors. Translational studies within the TAMRAD phase II trial were selectively performed in primary tumor tissues from a subset of 51 patients. In this small subset, only a trend towards increased benefit from the addition of everolimus to tamoxifen was observed for patients with high expression of p-4EBP1[12]. Biomarker analysis in primary tumor tissue from 227 patients who participated in the Bolero-2 trial showed that the benefit from everolimus added to exemestane was regardless of genetic alterations in the PI3K pathway[13]. Since inhibitors of the PI3K/Akt/mTOR are not without side effects, one would like to specifically identify those patients who will substantially benefit from these drugs. For identification of potential companion diagnostics for these drugs, it is of utmost importance to mandate biopsies from metastatic tumor lesions in future clinical trials. This will improve our knowledge about drug sensitivity and escape mechanisms and thereby accelerate developments that may eventually result in managing hormone receptor positive breast cancer as a chronic disease.

In conclusion, we observed a significant association between adjuvant endocrine therapy and mTOR activation in metastatic breast tumor tissue, suggesting that compensatory activation of the PI3K/Akt/mTOR pathway is a clinically relevant mechanism resulting in acquired hormone resistance. This stresses the importance of performing biopsies from metastatic tumor lesions, not only for predictive biomarker identification in clinical trials but also in general clinical practice.

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REFERENCES

1. Miller TW, Hennessy BT, Gonzalez-Angulo AM, Fox EM, Mills GB, Chen H, Higham C, Garcia-Echeverria C, Shyr Y, Arteaga CL: Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. J Clin Invest, 120:2406-2413.
2. Cavazzoni A, Bonelli MA, Fumarola C, La Monica S, Airoud K, Bertoni R, Alfieri RR, Galetti M, Tramonti S, Galvani E, et al: Overcoming acquired resistance to letrozole by targeting the PI3K/AKT/mTOR pathway in breast cancer cell clones. Cancer Lett, 323:77-87.
3. Bachelot T, Bourgier C, Cropet C, Ray-Coquard I, Ferrero JM, Freyer G, Abadie-Lacourtoisie S, Eymard JC, Debled M, Spaeth D, et al: Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. J Clin Oncol, 30:2718-2724.
4. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, et al: Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med, 366:520-529.
5. Akcakanat A, Sahin A, Shaye AN, Velasco MA, Meric-Bernstam F: Comparison of Akt/mTOR signaling in primary breast tumors and matched distant metastases. Cancer 2008, 112:2352-2358.
6. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, et al: Receptor conversion in distant breast cancer metastases. Breast Cancer Res, 12:R75.
7. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, et al: American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol, 28:2784-2795.
8. Osborne CK, Schiff R, Arpino G, Lee AS, Hilsenbeck VG: Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. Breast 2005, 14:458-465.
9. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, et al: A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 2007, 12:395-402.
10. Chandarlapaty S, Sakr RA, Giri D, Patil S, Heguy A, Morrow M, Modi S, Norton L, Rosen N, Hudis C, King TA: Frequent mutational activation of the PI3K-AKT pathway in trastuzumab-resistant breast cancer. Clin Cancer Res, 18:6784-6791.
11. J Baselga, J Cortes, S-A Im, E Clark, A Kiermaier, G Ross, SM Swain. Biomarker analyses in CLEOPATRA: A phase III, placebo-controlled study of pertuzumab in HER2-positive, first-line metastatic breast cancer (MBC) ., Cancer Res 24 (Suppl) 2012.
12. Treilleux I, Arnedos M, Cropet C, Ferrero JM, Lacourtoisie SA, Spaeth D, Levy C, Legouffe E, Pujade-Lauraine E, Wang Q, Bachelot T. Predictive markers of everolimus efficacy in hormone receptor positive (HR+) metastatic breast cancer (MBC): Final results of the TAMRAD trial translational study., J Clin Oncol, 31 (suppl), abstract 510, 2013.
13. Hortobagyi GN, Piccart-Gebhart MJ, Rugo HS, Burris HA, Campone M, Noguchi S, Perez A, Deleu I, Shtivelband m, Provencher L, Masuda N, Dakhil SR, et al. Correlation of molecular alterations with efficacy of everolimus in hormone receptor-positive, HER2-negative advanced breast cancer: Results from BOLERO-2., J Clin Oncol, 31 (suppl), abstract 509, 2013.
14. van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH, Kwee WS, . Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 1992 Jun;23(6):603-7.

SUPPLEMENTAL

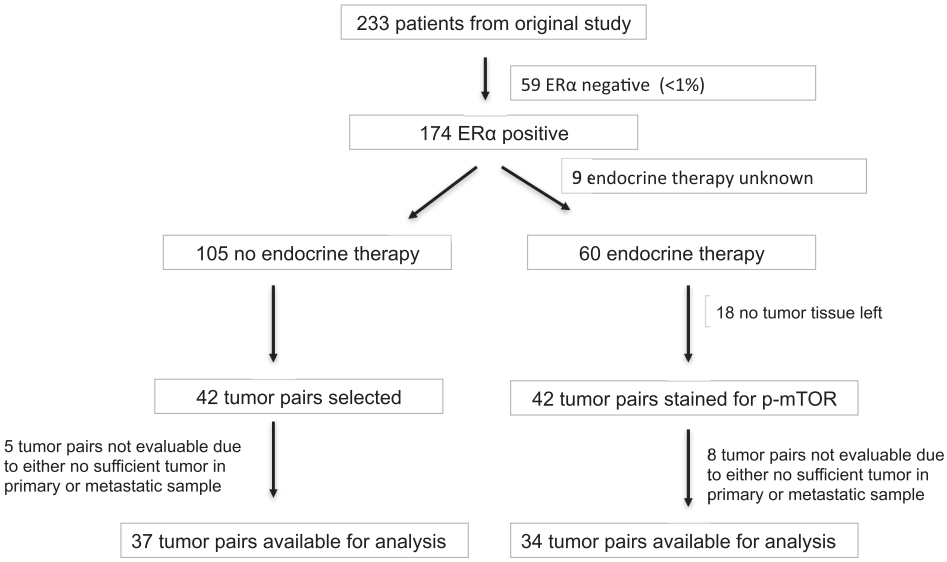

Figure S1. Data flow.

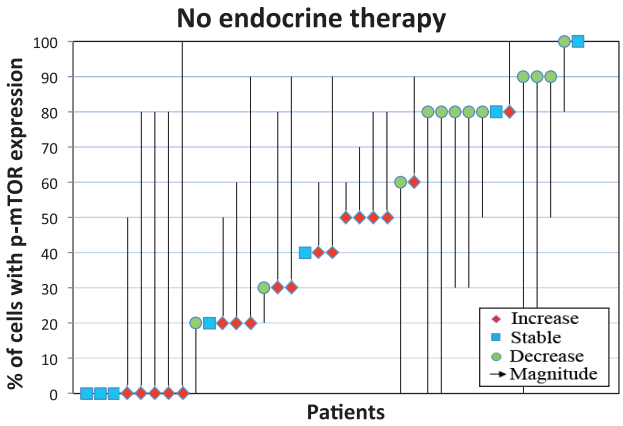

Figure S2a. Changes in p-mTOR expression between primary and metastatic tumor tissue from patients who did not receive endocrine therapy.

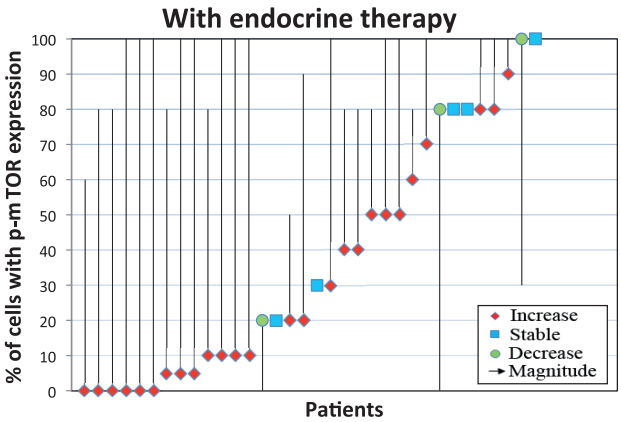

Figure S2b. Changes in p-mTOR expression between primary and metastatic tumor tissue from patients who did receive endocrine therapy.

Table S1. p-mTOR antibody and standardized protocol on the Ventana Benchmark® Ultra system.

Protein name	Clone	Company	Titer	Art. No.	Antigen retrieval	Primary
mTOR (phospho Ser2448)	49F9	Cell signaling	1/300	2976	36 min Cc1	1h

Table S2. Multivariate linear regression model for the difference in p-mTOR expression between metastatic and primary tumor tissue.

				95% CI for Beta	
		Beta	p-value	Lower Bound	Upper Bound
(Constant)		-29.68	0.16	-71.49	12.12
Localisation	Non-skin versus Skin	-3.80	0.76	-28.84	21.24
Histologic grade	Grade 3 versus Grade 1-2	16.20	0.18	-7.54	39.94
T-stage	T3-4 versus T1-2	9.69	0.61	-27.65	47.05
Lymph node status	Positive versus Negative	-11.17	0.55	-48.67	26.33
HER2 status	Positive versus Negative	3.75	0.86	-37.82	45.32
PR	Positive versus Negative	46.12	0.01	9.72	82.51
Endocrine therapy	Treated versus Untreated	37.75	0.01	9.11	66.40
Chemotherapy	Treated versus untreated	-14.87	0.34	-45.95	16.20

95% CI, 95% confidence interval; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.


Breast cancer
Metastases

CHAPTER 7

Influence of decalcification procedures on immunohistochemical receptor assessment of breast cancer

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Manuscript in preparation

ABSTRACT

Background

Discordance in estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status between primary breast cancers and distant metastases ("conversion") has been reported. Several of such series contained bone metastases, while it has been shown that decalcification procedures may negatively affect immunohistochemistry (IHC). We therefore re-evaluated the effect of several decalcification procedures on receptor IHC.

Methods

In ten prospectively collected breast cancer cases we compared receptor status of ERα, PR and HER2 by IHC in tissue routinely processed for diagnostic purposes and parallel tissue decalcified in Christensen's or EDTA buffer.

Results

The overall concordance for ERα expression was for both decalcifying procedures 100% (10/10, kappa = 1.000) and for PR 90%, (9/10), kappa = 0.800). For HER2 expression, concordance was 100% (10/10, kappa = 1.000) for EDTA and 90% for Christensen's (9/10, kappa = 0.615). For EDTA, there was no influence on therapeutic decision making with regard to hormonal therapy or trastuzumab.

Conclusion

Decalcification with EDTA does not seem to have a relevant impact on receptor IHC in breast cancer. This implies that receptor IHC can be reliably performed on EDTA decalcified bone metastases of breast cancer.

Introduction

At the occurrence of metastatic breast cancer, the choice of systemic treatment is traditionally based on the tissue characteristics of the primary tumor. Confirmatory biopsies of distant metastases are not routinely performed although several studies have shown that in a significant proportion of patients the expression of predictive tissuemarkers such as estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) may differ between the primary breast tumor and distant metastases ("receptor conversion")[1, 2]. This could potentially lead to inappropriate systemic treatment. Several guidelines therefore now advice to biopsy distant metastases to reassess hormone and HER2 receptor status by immunohistochemistry (IHC) whenever possible[3, 4].
Several mechanisms for receptor conversion have been proposed such as selective metastasis of infrequent clones with a different receptor profile from a heterogeneous tumor, genomic evolution in metastases and clonal selection under pressure of adjuvant hormonal therapy[5, 6]. Further, some receptor conversion studies included bone metastases, while it has been described that decalcification procedures, necessary to be able to cut sections from paraffin blocks containing bone, may cause decreased antigenicity[7-11]. In contrast, several other studies report that decalcifying methods can be applied without significant loss of immunoreactivity[12-20]. Only two small reports specifically describe whether ERα, PR and HER2 IHC can be reliably performed on decalcified tissue[20, 21]. Therefore, we set out to re-evaluate the influence of two routinely used decalcifying agents on assessment of hormone and HER2 receptors in breast cancer.

Materials and Methods

Material

Prospectively, tissue from ten random breast cancer cases were collected and processed according to routine procedures at the department of Pathology of the University Medical Center Utrecht, Utrecht, The Netherlands. All tissue samples were fixed in 4% buffered formaldehyde for 24-48 hours. After fixation, breast cancer tissue collected for diagnostic purposes was embedded in paraffin and processed according to a standard protocol. Extra tissue samples (0.4-0.8 cm^3 in size) of the tumors were processed in Christensen's buffer (containing 35% formic acid and 6.8% sodiumformate) or Ethylene Diamine Tetra acetic Acid disodium salt dehydrate (EDTA) buffer. Tissue was placed in Christensen's buffer overnight in a microwave at 37°C. Tissue was placed in EDTA buffer during the day in a microwave at 50°C. At the end of the day the tissue was washed thoroughly in running tap water and processed to paraffin. For each case, hematoxylin-eosin stained slides of the paraffin blocks were reviewed by a single pathologist (PJvD) to confirm the presence of malignancy.
Since we use left-over material to optimize diagnostic procedures, no ethical approval was required according to Dutch legislation. Use of anonymous or coded left over material for

scientific purposes is part of the standard treatment contract with patients and therefore informed consent was also not required[22].

Immunohistochemistry

IHC for ERα, PR and HER2 was carried out on full 4-µm sections with the automated Bondmax™ device (Leica Microsystems, Menarini, Florence, Italy) according to the manufacturer's instructions. For antigen retrieval ER2 (20 min) was used for ERα, ER1 (20 min) was used for PR and ER2 (30 min) for HER2. Mouse monoclonal antibodies used were against ERα (1:50, ID5, DAKO, Glostrup, Denmark), PR (1:100, PgR636; DAKO), and HER2 (1:100, SP3, Neomarkers, Lab Vision Corporation, Fremont, CA, USA). Appropriate controls were used throughout.

Scoring

Scoring of IHC slides was performed by 1 observer (PJvD) in random order, blinded to other data in the paired samples. For ERα and PR, the percentage of positively stained nuclei was estimated. The adequacy of staining was checked by also evaluating the normal breast parenchyma. HER2 expression was scored using the DAKO scoring system as 0, 1+, 2+ and 3+. Samples with 10% or more immunopositive malignant cells were classified as ERα or PR positive. HER2 expression was considering positive when 3+.

Statistical analysis

Results obtained with IHC were compared by cross tables, and the concordance percentages and weighted kappa-scores were calculated. In addition, median percentages were compared with Mann-Whitney test. P values below 0.05 were considered significant.

Results

Normal breast parenchyma, when present, showed usual expression of ERα and PR and lack of expression of HER2. No significant differences were found between median percentages of ER and PR positive cells between non-decalcified tissue on the one hand and Christensen's or EDTA treated tissue on the other. Table 1 shows expression of ERα, PR and HER2 by IHC in ten breast cancer cases without and with Christensen's or EDTA decalcification treatment. The ten breast cancer cases with and without decalcification were overall 90% ERα positive (9/10). Overall concordance for ERα was 100% (10/10) for both methods (Table 2).

For PR, breast cancer cases not undergoing decalcification were positive in 60% (6/10) of cases compared to 50% (5/10) of the Christensen's and EDTA treated cases. For PR expression the concordance of non-decalcified treated and Christensen's and EDTA treated tissue was 90% (kappa 0.800). The PR negative case after decalcification was heterogeneous in the original tumor material with 35% positive nuclei. Since the PR false negative case was still ERα positive, decalcification did not lead to clinically falsely stratifying patients for hormonal therapy.

For HER2, cases without decalcification and undergoing EDTA treatment were HER2 positive in 20% (2/10) of cases, while only one of the Christensen's treated breast cancer cases was

Table 1. Comparison of expression of ERα, PR and HER2 in split samples of 10 breast cancers routinely processed or undergoing decalcification in Christensen's buffer or EDTA.

	ERα (% positive cells)			PR (% positive cells)			HER2 *		
Sample	control	Christensen	EDTA	control	Christensen	EDTA	control	Christensen	EDTA
1	100	100	100	30	1	5	1	0	0
2	90	100	100	1	0	0	0	0	1
3	90	100	100	1	0	0	0	0	0
4	100	90	100	100	100	90	2	0	1
5	100	90	100	90	75	100	3	2	3
6	0	0	0	0	0	0	3	3	3
7	90	50	90	100	90	10	0	0	0
8	97	90	100	97	100	90	0	0	0
9	100	100	100	0	0	0	0	0	0
10	100	100	100	65	75	35	0	0	1

*HER2 expression was scored using the DAKO scoring system as 0, 1+, 2+ and 3+ according to standardized criteria. ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 2. Comparison of expression of ERα, PR and HER2 in routinely processed breast cancer tissue and parallel tissue undergoing decalcification in Chistensen's buffer or EDTA.

		ERα			
		Christensen		EDTA	
		-	+	-	+
ERα	-	1	0	1	0
	+	0	9	0	9
		PR			
		Christensen		EDTA	
		-	+	-	+
PR	-	4	0	4	0
	+	1	5	1	5
		HER2			
		Christensen		EDTA	
		-	+	-	+
HER2	-	8	0	8	0
	+	1	1	0	2

ERα, estrogen receptor alpha; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

HER2 positive (1/10, 10%). The false negative case was scored 2+ (Table 1). Concordance rates between the non-decalcified on the one hand and Christensen's and EDTA treated cases were 90% (kappa = 0.615) and 100%, respectively.

Discussion

It has been well established that receptor conversion for ERα, PR and HER2 occurs between the primary breast cancer and corresponding distant metastases. Therefore, most guidelines now advice to biopsy a distant metastasis at presentation of metastatic disease in breast cancer patients. However, several authors state that the decalcification process used for bone metastases diagnostics may potentially compromise antigenicity and thereby lead to false negative IHC results, although not all agree.

In the present re-evaluation we describe that ERα expression of the receptors is stable in non-decalcified and Christensen's or EDTA decalcified breast cancer tissue. PR expression was lost in 1 case after Christensen's and EDTA treated tissue compared to non-decalcified tissue, but this case was heterogeneous in the original tumor material (35% expression) and the additionally sampled pieces subjected to decalcification were smaller and one of them may have been derived from the negative tumor area. This PR "false negative" case was still ERα positive, so decalcification did not lead to clinically falsely stratifying patients for hormonal therapy. HER2 expression was lost in Christensen's treated tissue in one case, but not in EDTA treated tissue that allowed correctly stratifying all patients for trastuzumab therapy.

In a previous study using ten breast cancer samples decalcified in Decal Stat (containing hydrochloric acid and EDTA) for only 1 hour, which may in practice is probably too short for bone decalcification, it was described that decalcification of breast tumor tissue had an overall negative impact on receptor status based on the Allred scoring[23], but despite the overall drop in scores no hormone receptor positive cases were reclassified after decalcification[21]. The present HER2 results are in line with those by Zustin et al.[20] who also failed to demonstrate a detrimental effect of EDTA on HER2 IHC and FISH. Furthermore, we have studied the expression of ERα, PR and HER2 between different metastatic sites within the same patient and heterogeneity was not higher for patients with bone metastases, providing circumstantial evidence that decalcifying bone metastases has no detrimental influence of receptor assessments[24].

In conclusion, decalcification procedures based on Christensen's and EDTA in general seem to have little influence on ERα, PR and HER2 analysis by IHC. EDTA performed best since no patients where clinically falsely negatively stratified for hormonal treatment or trastuzumab. This implies that receptor IHC can be reliably performed on EDTA decalcified bone metastases of breast cancer.

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REFERENCES

1. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, et al: Receptor conversion in distant breast cancer metastases. Breast Cancer Res 2010, 12:R75.
2. van Diest PJ, Hoefnagel LD, van der Wall E: Testing for discordance at metastatic relapse of breast cancer matters. J Clin Oncol 2012, 30:3031; author reply 3031-3032, 3032-3033.
3. Hammond ME, Hayes DF, Wolff AC, Mangu PB, Temin S: American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Oncol Pract 2010, 6:195-197.
4. Carlson RW, Allred DC, Anderson BO, Burstein HJ, Edge SB, Farrar WB, Forero A, Giordano SH, Goldstein LJ, Gradishar WJ, et al: Metastatic breast cancer, version 1.2012: featured updates to the NCCN guidelines. J Natl Compr Canc Netw 2012, 10:821-829.
5. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, et al: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012, 366:883-892.
6. Russnes HG, Navin N, Hicks J, Borresen-Dale AL: Insight into the heterogeneity of breast cancer through next-generation sequencing. J Clin Invest 2011, 121:3810-3818.
7. Kamby C, Rasmussen BB, Kristensen B: Oestrogen receptor status of primary breast carcinomas and their metastases. Relation to pattern of spread and survival after recurrence. Br J Cancer 1989, 60:252-257.
8. Vignot S, Besse B, Andre F, Spano JP, Soria JC: Discrepancies between primary tumor and metastasis: a literature review on clinically established biomarkers. Crit Rev Oncol Hematol 2012, 84:301-313.
9. Linden HM, Dehdashti F: Novel methods and tracers for breast cancer imaging. Semin Nucl Med 2013, 43:324-329.
10. Sundararajan L, Linden HM, Link JM, Krohn KA, Mankoff DA: 18F-Fluoroestradiol. Semin Nucl Med 2007, 37:470-476.
11. Amir E, Clemons M: Should a biopsy be recommended to confirm metastatic disease in women with breast cancer? Lancet Oncol 2009, 10:933-935.
12. Bussolati G, Leonardo E: Technical pitfalls potentially affecting diagnoses in immunohistochemistry. J Clin Pathol 2008, 61:1184-1192.
13. Mukai K, Yoshimura S, Anzai M: Effects of decalcification on immunoperoxidase staining. Am J Surg Pathol 1986, 10:413-419.
14. Arber JM, Weiss LM, Chang KL, Battifora H, Arber DA: The effect of decalcification on in situ hybridization. Mod Pathol 1997, 10:1009-1014.
15. Matthews JB: Influence of decalcification on immunohistochemical staining of formalin-fixed paraffin-embedded tissue. J Clin Pathol 1982, 35:1392-1394.
16. Mullink H, Henzen-Logmans SC, Tadema TM, Mol JJ, Meijer CJ: Influence of fixation and decalcification on the immunohistochemical staining of cell-specific markers in paraffin-embedded human bone biopsies. J Histochem Cytochem 1985, 33:1103-1109.
17. Athanasou NA, Quinn J, Heryet A, Woods CG, McGee JO: Effect of decalcification agents on immunoreactivity of cellular antigens. J Clin Pathol 1987, 40:874-878.
18. Kremer M, Quintanilla-Martinez L, Nahrig J, von Schilling C, Fend F: Immunohistochemistry in bone marrow pathology: a useful adjunct for morphologic diagnosis. Virchows Arch 2005, 447:920-937.
19. Neves Jdos S, Omar NF, Narvaes EA, Gomes JR, Novaes PD: Influence of different decalcifying agents on EGF and EGFR immunostaining. Acta Histochem 2011, 113:484-488.
20. Zustin J, Boddin K, Tsourlakis MC, Burandt E, Mirlacher M, Jaenicke F, Izbicki J, Ruether W, Rueger JM, Bokemeyer C, et al: HER-2/neu analysis in breast cancer bone metastases. J Clin Pathol 2009, 62:542-546.
21. Darvishian F, Singh B, Krauter S, Chiriboga L, Gangi MD, Melamed J: Impact of decalcification on receptor status in breast cancer. Breast J 2011, 17:689-691.
22. van Diest PJ: No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002, 325:648-651.
23. Allred DC, Harvey JM, Berardo M, Clark GM: Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998, 11:155-168.

24. Hoefnagel LDC, van der Groep P, van de Vijver MJ, Boers JE, Wesseling P, Wesseling J, Dutch Distant Breast Cancer Metastases Consortium, van der Wall E, van Diest PJ. Discordance in ERα, PR and HER2 receptor status across different distant breast cancer metastases within the same patient. Annals of Oncology, in press


Breast cancer
Metastases
metastases metastases

CHAPTER 8

Genomic evolution from primary breast carcinoma to distant metastasis: few copy number changes of breast cancer related genes

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Submitted

ABSTRACT

Background

Cancer initiation and progression is characterized by (epi)genetic aberrations. However, little is known about the changes that occur during the metastatic process of breast cancer despite the fact that these metastases are the leading cause of death in breast cancer patients. We therefore aimed to investigate whether distant breast cancer metastases show progression in copy number changes compared to their primary tumors.

Methods

Multiplex ligation-dependent probe amplification (MLPA) was used to compare copy numbers of 21 established oncogenes and tumor suppressor genes between primary breast cancer samples and corresponding distant metastases in 55 patients.

Results

Overall, there was no significant difference in mean copy number between primary tumors and metastases (p=0.826), but on the individual gene level, mean copy numbers for PRDM14 (p=0,023), MED1 (p=0.001) and CCNE1 (p=0.039) were higher while TRAF4 (p=0.038) copy numbers were lower in the metastases. MTDH amplifications were more frequent in the primary tumors compared to the metastases (27.3% vs.14.6%, p=0.039), while more CDH1 losses were found in the metastases (23.6% vs. 9.1%, p=0.039). Primary tumor CCNE1 gain, in most cases coinciding with EGFR gain, was a marker of preferential brain metastasis.

Conclusion

For most of the 21 oncogenes and tumor suppressor genes investigated in this study, distant metastases of breast cancer generally show similar gene copy number aberrations compared to the corresponding primary tumors. The few genes that show copy number gain or loss in metastases are genes that may play a role in the development of therapy resistance.

Introduction

Despite advances in early diagnosis and treatment of breast cancer patients, still about 25% eventually die from distant metastases[1]. Little is known about the timing of genomic and other changes responsible for developing distant metastasis. Based on *in vitro* tumor cell cultures subsequently transplanted in mice[2-5], a model of metastasis was suggested proposed showing that the metastatic capacity is acquired late in tumorigenesis. Moreover, the metastases were thought to originate from particular subclones with a distinct "metastatic" profile. Others, however, have shown that metastases develop through stochastic events from primary tumor cells with an equal metastatic potential[6, 7]. This was confirmed by gene expression profiling where human primary breast tumors were strikingly similar to the distant metastasis of the same patient. These findings suggest that metastatic capability in breast cancer may be an inherent feature and is not based on clonal selection[8]. More evidence for this theory was provided by comparative genomic hybridization (CGH) studies showing, in a small number of cases, that metastases have similar aberration patterns to those found in the primary tumor[9, 10]. Furthermore, a complex 54-gene breast cancer set that marks and mediates breast cancer metastasis to the lungs[11] and a functionally diverse gene set that, when overexpressed, cooperatively promotes the metastasis of breast cancer cells to bone[12] also confirms that the metastatic potential exists already in the primary tumor. Other whole genome approaches have identified gene sets in primary tumors that can predict the occurrence of distant metastases but these genes were not analyzed in the tissue of the distant metastases as well[13, 14].

Only few high resolution studies have sequenced the complete genome of primary breast cancers as well as their distant metastases. Massive parallel DNA sequencing of a basal-like primary breast cancer and a brain metastasis of the same patient showed that the metastasis contained two *de novo* mutations and a large deletion not present in the primary tumor and was significantly enriched for 20 shared mutations. The differential mutation frequencies and structural variations patterns in the metastasis compared with the primary tumor indicate that secondary tumours may arise from a minority of cells within the primary tumor[15]. Similar findings were observed in another study, 19 of the 32 somatic non-synonymous coding mutations present in a metastasis were not detected in the primary lobular breast cancer, suggesting that mutational heterogeneity can be a property of low or intermediate grade primary breast cancers and that significant evolution can occur with disease progression.

Understanding the molecular background of the metastastic process is important to find new clues for prevention and therapy. Studies investigating metastatic profiles have been performed *in vitro*, *in vivo* and in primary tumors alone, but only few studies have compared primary tumors with distant metastases of the same patient. Gene copy number and/or expression studies allow the discovery of markers that can help to identify patients who are most likely to develop metastatic disease, and would therefore benefit from adjuvant chemotherapy. In addition, such studies might identify markers that are druggable or that can predict the site of metastasis. This might enable oncologists to start tailoring treatment for individual patients. However, analysis

of breast cancer metastases is often limited by the availability of tissue, especially fresh frozen tissue. We used in the present study multiplex ligation-dependent probe amplification (MLPA), an inexpensive and reliable high-throughput technique for the investigation of gene copy number changes in a small amount of DNA derived from more widely available paraffin embedded material[16-18], to compare copy number changes in 21 established breast cancer genes between 55 primary breast cancers and their corresponding distant metastases.

Materials and Methods

Tissue selection and DNA isolation

Fifty-five formalin fixed embedded primary breast cancer specimens and corresponding first biopsied distant metastases from different sites (11 brain, 12 lung, 10 liver and 22 skin) were obtained from the Departments of Pathology of the University Medical Center Utrecht (UMCU), Isala klinieken Zwolle, Erasmus Medical Center Rotterdam, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Center, Gelre Hospital Apeldoorn, Laboratory Sazinon Hoogeveen, and the Laboratory for Pathology Oost Nederland, all in The Netherlands. Table 1 shows basic clinicopathological characteristics of the primary tumors studied.

Use of anonymous or coded 'left over' material for scientific purposes does not require informed consent according to our institutional medical ethical review board and according to Dutch legislation [Medical Research Involving Human Subjects Act, http://www.ccmo-online.nl/main.asp?pid=10&sid=30&ssid=51] [19].

Haematoxylin-eosin stained slides were reviewed by an experienced pathologist (PvD) to confirm the presence of malignancy in tumor samples. Only samples with a tumor percentage of at least 80% were included in this study. Tumor tissue was scraped off from the marked tumor area on two 8 µm thick paraffin sections, and incubated for 1 hour in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56°C followed by boiling for 10 min. Areas with lymphocytic infiltrate or ductal carcinoma *in situ* were avoided.

Multiplex ligation-dependent Probe Amplification

Five µl of this DNA solution was, after centrifugation, used in the MLPA analysis according to the manufacturers' instructions, using the P078-B1 breast kit (MRC Holland, Amsterdam, The Netherlands), as before[20]. Table 2 shows the contents of this kit and includes chromosomal locations of all probes. The kit also contains a probe to the *AURKA* gene, but as the results for this gene were unstable, further analysis was omitted. All tests were performed in duplicate on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genescan (Applied Biosystems) and Coffalyser (version 7.0) software (MRC-Holland). Six negative reference samples (blood and normal breast) were taken along in each MLPA run to normalize MLPA ratios. For genes with more than one probe present in the kit, the mean of all the probe peaks of this gene in duplicate was calculated. A mean probe ratio

value below 0.7 was defined as loss, a value between 0.7 and 1.3 was defined as normal, 1.3–2.0 as gain/low-level amplification, and values >2.0 were defined as high-level amplification, as established previously[21]. Low-level and high-level amplifications were taken together as "amplifications".

Table 1. Baseline clinicopathological data.

		N	%
Site	Brain	11	20%
	Skin	23	42%
	Liver	10	18%
	Lung	11	20%
Histology	Ductal	45	82%
	Lobular	5	9%
	Other	5	9%
ER	Positive	33	60%
	Negative	22	40%
PR	Positive	32	58%
	Negative	23	42%
HER2	0/1+	47	85%
	2+	1	2%
	3+	7	13%
LN status	Positive	26	47%
	Negative	23	42%
Grade	1	1	2%
	2	14	25%
	3	40	73%
MAI	≥ 13	39	71%
	< 13	16	29%
Age	≥ 50	25	45%
	< 50	30	55%
	Mean (median)	53 (51)	
	Range	27-88	
Tumor size	≥ 2 cm	18	33%
	< 2 cm	31	56%
	Mean (median)	1.99 (1.5)	
	Range	0.2-9.5	
Time P-M	Mean (median)	46 (29)	
	Range	1-181	

LN status, lymph node status; MAI, mitotic activity index; Time P-M; time in months between primary tumor and first distant metastasis. LN status and tumor size were available for 49/55 patients (89%).

Table 2. Contents of the P078-B1 MLPA kit (MRC Holland, the Netherlands). For each gene the chromosomal position, Mapview distance from p-telomere, the number of probes present in the MLPA kit, and a proposed function of the transcript protein.

Gene	Chr	Mapview position	# probes	Functions
ESR1	06q25	06-152.423838 06-152.457215	2	Transcription factor
EGFR	07p11	07-055.191055 07-055.233957	2	Receptor tyrosine kinase involved in signal transduction
FGFR1	08p12	08-038.391533 08-038.434092	2	Receptor tyrosine kinase involved in signal transduction
ADAM9	08p11	08-038.998319	1	Metalloproteinase associated with protein metabolism
IKBKB	08p11	08-042.292902 08-042.302676	2	Serine/threonine kinase associated with signal transduction
PRDM14	08q13	08-071.130073	1	Transcription regulatory protein
MTDH	08q22	08-098.742504 08-098-788082	2	Metastasis promoting gene involved in chemoresistance
MYC	08q24	08-128.821796 08-128.822001 08-128.822151	3	Transcription factor involved in apoptosis and cell proliferation
CCND1	11q13	11-069.167779 11-069.175089	2	Cell cycle control protein involved in signal transduction
C11ORF30	11q13	11-075.902087 11-075.926543	2	Transcription regulatory protein
CDH1	16q22	16-067.328716 16-067.404826	2	Adhesion molecule associated with signal transduction
TRAF4	17q11	17-024.098403	1	Adaptor molecule involved in signal transduction, cell proliferation and apoptosis
CPD	17q11	17-025.795018	1	Carboxypeptidase involved in protein metabolism
MED1	17q12	17-034.840858	1	Transcription regulatory protein involved in signal transduction
ERBB2	17q12	17-035-118101 17-035.127183 17-035-133169 17-035.136344	4	Receptor tyrosine kinase associated with signal transduction
CDC6	17q21	17-035.699283	1	Cell cycle control protein involved in signal transduction
TOP2A	17q21	17-035.812698 17-035.816651 17-035.818297	3	DNA topoisomerase protein involved in regulation of the topological status of DNA
MAPT	17q21	17-041.423085	1	Structural protein involved in cell growth and/or maintainance
BIRC5	17q25	17-073.722036 17-073.722396 17-073.724340	3	Adapter molecule involved in signal transduction, cell communication and cell survival
CCNE1	19q12	19-035.000150 19-035.005214	2	Cell cycle control protein involved in signal transduction
AURKA	20q13	20-054.389980	1	Serine/threonine kinase involved in signal transduction

Statistical analysis

The mean copy number ratio including all 21 genes in all 55 patients was compared between primary tumor and metastases by Mann-Whitney test. We compared the mean copy number ratio of each individual gene between the pooled primary tumors on the one hand and the metastases on the other by paired T-test (normally distributed variables) or Wilcoxon test. Next, MLPA data were dichotomized as non-loss vs. loss (cut-off 0.7) and as non-amplified vs. gain/amplified (cut-off 1.3 or 2.0) and these gene dosage categories were compared between primary tumor and metastases by McNemar's test. Finally, the number of alterations (total amplifications and losses) between primary tumor and the different distant sites were compared by chi-square. Copy number differences according to site were calculated by ANOVA. All statistical analyses were conducted with SPSS 15.0 statistical software, regarding two-sided p-values below 0.05 as significant. Correction for multiple comparisons was performed by resetting the 0.05 threshold according to the Bonferroni-Holm approach.

Results

Comparison between primary tumors and metastases

No differences were observed in MLPA copy numbers between primary breast tumors from different intrinsic subtypes (luminal A, Luminal B, HER2-driven, triple-negative), except for the presence of higher *HER2*, *MED1* and *CPD* copy numbers in the HER2-driven subtype. The vast majority of aberrations detected in the primary tumors were retained in their metastases. Overall, the number of gains and amplifications was similar between primary tumors and metastases (p=0.68 and p=0.87 respectively), whereas the number of losses was significantly higher in metastases compared to primary tumors (p=0.01). Figure 1 illustrates the (total of low and high level) amplifications and losses seen for all analyzed genes in this study. Based on dichotomized MLPA data, more *CDH1* losses (ratio < 0.7) were found in the metastases compared to the paired primary tumors (23.6% vs. 9.1%, p=0.039, Figure 1) whereas more *MTDH* high-level amplifications were found in the primary tumors compared to the paired metastases (27.3% vs.14.6%, p=0.039).

Overall, there was no significant difference in mean MLPA copy numbers between primary tumors (1.26 +/- 0.6) and metastases (1.27 +/- 0.64) (p=0.826). Comparing the absolute MLPA ratio values between primary tumors and paired metastases, *PRDM14* (p=0.023), *MED1* (p=0.001) and *CCNE1* (p=0.039) copy number ratios were higher in the metastases, with respectively 32/55 (58%), 40/55 (73%) and 33/55 (60%) metastases showing higher copy numbers compared to their primary tumor. In 16/55 metastases (29%), all three genes showed higher copy numbers. In contrast, *TRAF4* copy number ratios were generally lower (p=0.038) in the metastases, with 36/55 metastases (65%) showing lower copy numbers than their primary tumor. However, after correction for multiple comparisons, only *MED1* copy number ratios were significantly higher in the metastases compared to the primaries.

Differences according to the site of the metastasis

Table 3 shows the frequency of amplifications and losses as detected by MLPA in primary tumors and their metastases, subdivided according to the site of metastasis. Note that the frequencies per metastatic site are similar for *MED1* and *HER2*, which is probably related to the fact that both genes on 17q12 are members of the same large amplicon. *CCNE1* gains were exclusively

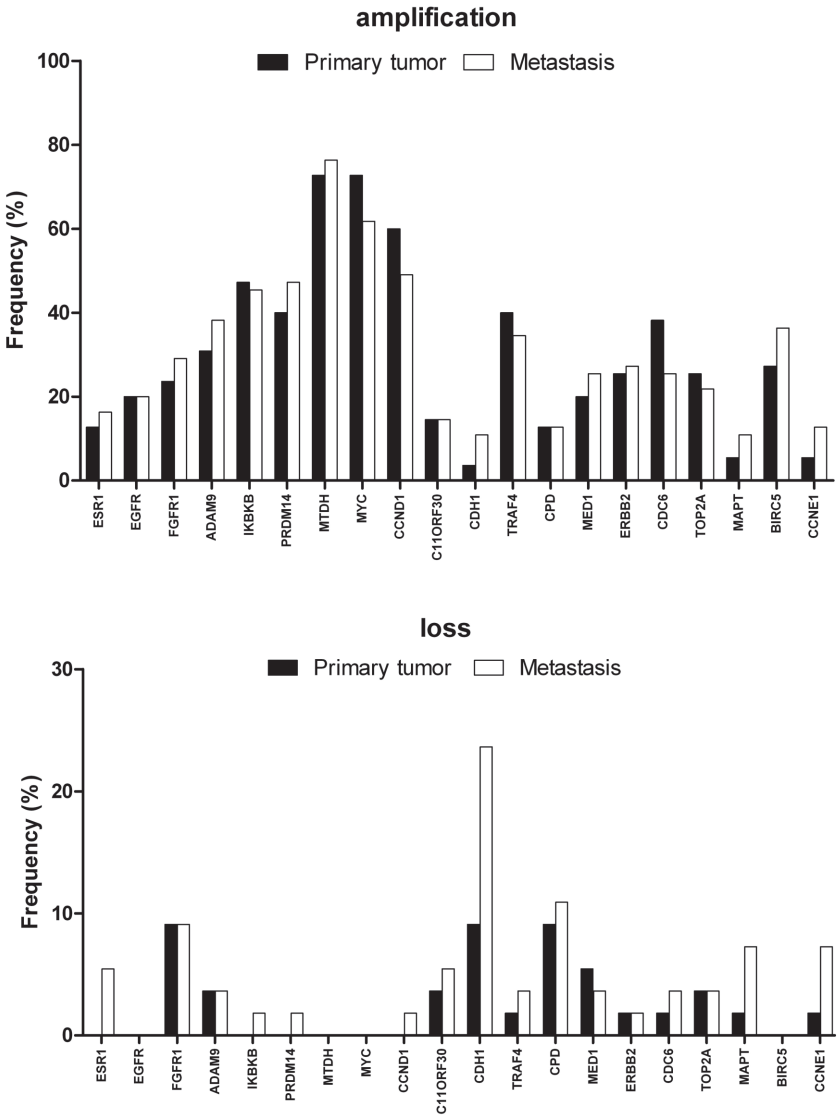

Figure 1. Frequency of (low and high level) amplifications (top) and loss (bottom) in primary breast tumors and corresponding metastases (n=55) analyzed by MLPA.

found in primary tumors leading to brain metastases. As a result, the absolute copy number of *CCNE1* gain was higher in primary tumors that lead to brain metastases, compared to primary tumors leading to skin metastases (p=0.005). *CDH1* loss was exclusively detected in primary tumors leading to liver and skin metastases, and there was a trend for higher frequency of *CDH1* loss in primary tumors with liver metastases (30%) than in primary tumors with brain (0%) and lung (0%) metastases (p=0.090 and p=0.078, respectively).

As for the primary tumors, brain metastases showed a higher *CCNE1* gain frequency and copy number than skin metastases (45% versus 0%, p=0.003 and p=0.003 respectively). The absolute *CCNE1* copy numbers also tended to be higher in brain metastases compared to lung metastases (p=0.061). In addition, several other copy number alterations including *FGFR1* losses, *PRDM14* high-level amplifications and *CDC6* high-level amplifications, showed different frequencies depending on the location of the metastasis with the highest frequency in brain metastases (p=0.026, p=0.037 and p=0.037 respectively). Lastly, *EGFR* gains were more frequent

Table 3. Frequencies of gene amplification by MLPA in 55 primary breast tumors and their distant metastases, subdivided according the site of metastasis (brain, liver, lung and skin). For *FGFR1* and *CDH1*, the number of losses is shown instead of the number of amplifications.

	% in primary tumor				% in metastasis			
	brain	liver	lung	skin	brain	liver	lung	skin
	n=11	n=10	n=12	n=22	n=11	n=10	n=12	n=22
ESR1	27	0	8	14	18	20	0	23
EGFR	18	30	25	14	36	10	33	9
FGFR1 [a]	18	0	8	9	27	20	0	0
ADAM9	36	20	33	32	36	40	42	36
IKBKB	64	40	33	50	45	20	50	55
PRDM14	64	30	33	36	64	50	58	32
MTDH	91	60	83	64	82	60	83	77
MYC	82	70	75	68	82	60	67	50
CCND1	55	50	83	55	55	40	58	45
C11ORF30	27	10	8	14	9	20	8	18
CDH1a	0	30	0	9	9	40	8	32
TRAF4	36	50	42	36	36	50	33	27
CPD	36	20	0	5	27	20	8	5
MED1	27	40	8	14	27	40	8	27
ERBB2	27	40	17	23	27	50	8	27
CDC6	36	30	25	50	27	20	17	32
TOP2A	36	20	8	32	18	30	8	27
MAPT	0	0	0	14	9	20	0	14
BIRC5	45	30	17	23	64	40	25	27
CCNE1	27	0	0	0	45	10	8	0

[a] number of losses is shown, instead of the number of amplifications

in brain (36%) and lung (33%) metastases compared to skin metastases (5%) (p=0.033 and p=0.042 respectively). Interestingly, *CCNE1* amplifications often coincided with EGFR amplifications in this study (in 2/3 primary tumors and 5/7 metastases).

Discussion

Previously, we showed receptor conversion in distant breast cancer metastases for estrogen receptor (ERα) and human epidermal growth factor receptor 2 (HER2) in 10.3% and 5.2% of patients, respectively, indicating that distant breast cancer metastases may differ from their primary tumor[22, 23]. In addition, we demonstrated the negative prognostic impact of ERα or PR receptor conversion from positive in the primary breast cancer to negative in the distant metastasis[23].

To identify copy number aberrations that may function as molecular markers for the metastatic potential of the primary breast tumor, we performed MLPA on both primary tumors and their corresponding metastases. MLPA is a relatively cheap and easy-to-perform PCR assay that allows simultaneous detection of multiple gene copy number aberrations in small amounts of fragmented DNA derived from formalin-fixed material. It is, however, a non-morphological method that requires proper tumor tissue selection guided by H&E sections since copy number ratios can be diluted by the presence of large amounts of lymphocytic infiltrate and normal tissue. We therefore avoided inflammatory areas and used only samples with a tumor percentage above 80%. To the best of our knowledge, this is the first study that systematically analyzed copy number changes of multiple genes (n=21) in primary breast carcinomas and their metachronal metastases at multiple sites.

The vast majority of copy number aberrations detected in primary tumors were retained in their corresponding metastases indicating that, on the copy number level of these genes, there is overall little genomic progression from primary breast tumors to their distant metastases. Previous studies using CGH reported similar findings in primary breast carcinomas and their corresponding lymph node metastases [9, 24-26], as did the few previous CGH studies comparing primary tumors with distant metastases[9, 10]. The latter publications included a maximum of 10 patients. Our study with 55 patients is thus the largest so far. In line with these previous reports, we also discovered some genetic differences between primary tumors and their metastases despite overall genetic similarity. However, these observed differences need to be interpreted with caution since, after correction for multiple comparisons, most of them were no longer significant. More metadherin (*MTDH*, or *AEG-1*) high-level amplifications were found in the primary tumors compared to the metastases. *MTDH* activation by 8q22 genomic gain has been shown to be an early event in breast cancer development [27], and to be correlated with mRNA and protein expression[28]. *MTDH* plays a role in numerous processes crucial for tumor development such as transformation, evasion of apoptosis, proliferation, cell survival, migration, invasion, metastasis and angiogenesis through the activation of several signaling pathways[29]. *MTDH* activation is known to promote chemoresistance and to induce metastasis of poor-prognosis breast cancer through epithelial to mesenchymal transition[30]. Its overexpression in

primary breast tumors was shown to be associated with high nuclear grade, absence of estrogen and progesterone receptor expression, high Ki-67 labelling index and shorter disease-free, distant metastasis-free and overall survival[28, 30]. It is therefore not surprising that *MTDH* gain (46%) and amplification (27%) were very frequent in the investigated primary (triple negative as well as luminal B) breast tumors. Our results also revealed more frequent E-cadherin (*CDH1*) loss in metastases (24%) compared to primary tumors (9%). This is in line with previous loss of heterozygosity (LOH) results in primary tumors and metastases of hereditary diffuse gastric cancer patients[31].

Comparing the absolute MLPA ratio values between primary tumors and paired metastases, *MED1*, *PRDM14* and *CCNE1* copy number ratios were generally higher in the metastases. Mediator subunit 1 (MED1) is a key ERα co-activator that plays a role in HER2-mediated tamoxifen resistance[33]. MED1 amplification at 17q12 is a frequent event in breast cancer[20] associated with ER and HER2 positivity but previous studies did not find an effect on survival[34]. *PRDM14*, a transcriptional regulator located on 8q13 is frequently amplified and overexpressed in breast tumors[20, 32]. Its amplification has been associated with a more aggressive phenotype (high grade, high mitotic index, HER2 overexpression) and its overexpression in cell lines increases growth and reduces sensitivity to chemotherapy[32]. Cyclin E1 (*CCNE1*) facilitates G1-S phase cell cycle transition. Amplification of *CCNE1* in breast cancer is rare[20] but is more frequent in triple-negative and basal-like breast tumors[35]. Overexpression of CCNE1 has been associated with a shorter distant metastasis-free survival[36] and with resistance to trastuzumab[37]. In contrast to *PRDM14*, *MED1* and *CCNE1*, tumor necrosis factor receptor associated factor 4 (*TRAF4*), an oncogene amplified and overexpressed in many cancer types including breast carcinomas[38], showed lower copy numbers in metastases. Its overexpression in breast cancer patients plays a role in destabilisation of p53 and has been associated with poor prognosis[39]. We have no obvious explanation for this paradoxical loss.

CCNE1 amplifications were exclusively seen in primary tumors leading to brain metastases. These amplifications were retained in the corresponding metastases, whereas in some cases only the metastasis harboured a *CCNE1* amplification. *CCNE1* amplifications were detected in 45% of the brain metastases. In contrast, *CCNE1* amplifications were present in only 10% and 8% of liver and lung metastases, respectively, and absent in skin metastases. These results suggest a preferential *CCNE1* copy number increase in primary tumors that are prone to metastasize to the brain. Interestingly, *CCNE1* amplifications almost always coincided with *EGFR* gains or amplifications in this study (in 2/3 primary tumors and 5/7 metastases) and both have been specifically associated with triple-negative and basal-like breast carcinomas[35, 40]. In line with these results, earlier reports showed high rates of brain metastases among basal-like (25%), triple-negative non-basal (22%) and HER2-enriched (29%) groups, whereas brain metastases were less frequently seen in the luminal/HER2 (15.4%) and other subgroups.[41] *EGFR* amplifications in our study were more frequent in brain and lung metastases compared to skin metastases. Wikman *et al.* analysed ten breast cancer samples that had metastasized to the brain by array CGH and reported gains of *EGFR* in 80% of the metastases but in only 13% of the primary tumors.[10] Although less striking, our results show the same trend: 36% of the brain metastases showed *EGFR* amplification whereas only 18% of their corresponding primary

tumors did. *CDH1* loss tended to be more frequent in primary tumors leading to liver and skin metastases compared to those resulting in brain or lung metastases. The same trend was observed in the metastases themselves. Loss of *CDH1* is a hallmark of lobular breast cancers, known to respond poorly to neoadjuvant chemotherapy[42], and absence of E-cadherin expression in breast tumors has been associated with the first distant metastases in the skin[43]. Despite the presence of ER and HER2 receptor conversion at the immunohistochemistry level[22, 23], there were no (significant) differences at the gene copy number level for *ESR1* and *HER2/ERBB2*. We previously showed that *ESR1* amplification detected by MLPA is rare in breast cancer. In a group of 135 patients we found only 2% amplification and 6% gains of the ESR1 gene[44]. Because HER2 receptor conversion is a relatively rare (5%) event, it might not have been picked up in this study due to the limited sample size.

In conclusion, we have shown that distant metastases generally present with similar gene copy number aberrations in common breast oncogenes and tumor suppressor genes compared to their primaries. However, copy numbers of several of these genes were elevated or decreased in the metastases. These differing genes are known to be involved in the development of chemoresistance (*MTDH, PRDM14*), tamoxifen resistance (*MED1*), trastuzumab resistance (*CCNE1*), and neoadjuvant chemotherapy resistance (*CHD1*). In addition, this study identified *CCNE1* gain, in most cases coinciding with *EGFR* gain, as a marker for preferential brain metastasis. After proper validation of these findings, determination of copy numbers in primary tumor and metastasis could potentially help the medical oncologist in deciding on the therapeutic strategy of metastatic breast cancer patients.

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REFERENCE LIST

1 Rabbani SA, Mazar AP. Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer Metastasis Rev 2007;26:663-674.
2 Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. Science 1977;197:893-895.
3 Poste G, Fidler IJ. The pathogenesis of cancer metastasis. Nature 1980;283:139-146.
4 Price JE, Carr D, Tarin D. Spontaneous and induced metastasis of naturally occurring tumors in mice: analysis of cell shedding into the blood. J Natl Cancer Inst 1984;73:1319-1326.
5 Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. Science 1982;217:998-1003.
6 Giavazzi R, Alessandri G, Spreafico F, Garattini S, Mantovani A. Metastasizing capacity of tumour cells from spontaneous metastases of transplanted murine tumours. Br J Cancer 1980;42:462-472.
7 Vaage J. Metastasizing potentials of mouse mammary tumors and their metastases. Int J Cancer 1988;41:855-858.
8 Weigelt B, Glas AM, Wessels LF, Witteveen AT, Peterse JL, van't Veer LJ. Gene expression profiles of primary breast tumors maintained in distant metastases. Proc Natl Acad Sci U S A 2003;100:15901-15905.
9 Nishizaki T, DeVries S, Chew K, et al. Genetic alterations in primary breast cancers and their metastases: direct comparison using modified comparative genomic hybridization. Genes Chromosomes Cancer 1997;19:267-272.
10 Wikman H, Lamszus K, Detels N, et al. Relevance of PTEN loss in brain metastasis formation in breast cancer patients. Breast Cancer Res 2012;14:R49.
11 Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518-524.
12 Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537-549.
13 Wang Y, Klijn JG, Zhang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 2005;365:671-679.
14 van ', V, Dai H, van d, V, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530-536.
15 Ding L, Ellis MJ, Li S, et al. Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature 2010;464:999-1005.
16 Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002;30:e57.
17 Moelans CB, de Weger RA, Ezendam C, van Diest PJ. HER-2/neu amplification testing in breast cancer by Multiplex Ligation-dependent Probe Amplification: influence of manual- and laser microdissection. BMC Cancer 2009;9:4.
18 Moelans CB, de Weger RA, van Blokland MT, et al. HER-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and in situ hybridization. Cell Oncol 2009;31:1-10.
19 van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002;325:648-651.
20 Moelans CB, de Weger RA, Monsuur HN, Vijzelaar R, van Diest PJ. Molecular profiling of invasive breast cancer by multiplex ligation-dependent probe amplification-based copy number analysis of tumor suppressor and oncogenes. Mod Pathol 2010;23:1029-1039.
21 Moelans CB, de Weger RA, van Diest PJ. Multiplex ligation-dependent probe amplification to detect HER2 amplification in breast cancer: new insights in optimal cut-off value. Cell Oncol 2010;32:311-312.
22 Hoefnagel LD, van d, V, van Slooten HJ, et al. Receptor conversion in distant breast cancer metastases. Breast Cancer Res 2010;12:R75.
23 Hoefnagel LD, Moelans CB, Meijer SL, et al. Prognostic value of estrogen receptor alpha and progesterone receptor conversion in distant breast cancer metastases. Cancer 2012;118:4929-4935.
24 Vecchi M, Confalonieri S, Nuciforo P, et al. Breast cancer metastases are molecularly distinct from their primary tumors. Oncogene 2008;27:2148-2158.

25 Poplawski AB, Jankowski M, Erickson SW, et al. Frequent genetic differences between matched primary and metastatic breast cancer provide an approach to identification of biomarkers for disease progression. Eur J Hum Genet 2010;18:560-568.
26 Desouki MM, Liao S, Conroy J, et al. The genomic relationship between primary breast carcinomas and their nodal metastases. Cancer Invest 2011;29:300-307.
27 Moelans CB, de Wegers RA, Monsuurs HN, Maess AH, van Diest PJ. Molecular differences between ductal carcinoma in situ and adjacent invasive breast carcinoma: a multiplex ligation-dependent probe amplification study. Cell Oncol (Dordr) 2011;34:475-482.
28 Tokunaga E, Nakashima Y, Yamashita N, et al. Overexpression of metadherin/MTDH is associated with an aggressive phenotype and a poor prognosis in invasive breast cancer. Breast Cancer 2012.
29 Wang C, Yang Q. Astrocyte elevated gene-1 and breast cancer (Review). Oncol Lett 2011;2:399-405.
30 Hu G, Chong RA, Yang Q, et al. MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. Cancer Cell 2009;15:9-20.
31 Oliveira C, Sousa S, Pinheiro H, et al. Quantification of epigenetic and genetic 2nd hits in CDH1 during hereditary diffuse gastric cancer syndrome progression. Gastroenterology 2009;136:2137-2148.
32 Nishikawa N, Toyota M, Suzuki H, et al. Gene amplification and overexpression of PRDM14 in breast cancers. Cancer Res 2007;67:9649-9657.
33 Cui J, Germer K, Wu T, et al. Cross-talk between HER2 and MED1 regulates tamoxifen resistance of human breast cancer cells. Cancer Res 2012;72:5625-5634.
34 Lamy PJ, Fina F, Bascoul-Mollevi C, et al. Quantification and clinical relevance of gene amplification at chromosome 17q12-q21 in human epidermal growth factor receptor 2-amplified breast cancers. Breast Cancer Res 2011;13:R15.
35 Agarwal R, Gonzalez-Angulo AM, Myhre S, et al. Integrative analysis of cyclin protein levels identifies cyclin b1 as a classifier and predictor of outcomes in breast cancer. Clin Cancer Res 2009;15:3654-3662.
36 Sieuwerts AM, Look MP, Meijer-van Gelder ME, et al. Which cyclin E prevails as prognostic marker for breast cancer? Results from a retrospective study involving 635 lymph node-negative breast cancer patients. Clin Cancer Res 2006;12:3319-3328.
37 Scaltriti M, Eichhorn PJ, Cortes J, et al. Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A 2011;108:3761-3766.
38 Camilleri-Broet S, Cremer I, Marmey B, et al. TRAF4 overexpression is a common characteristic of human carcinomas. Oncogene 2007;26:142-147.
39 Yi P, Xia W, Wu RC, Lonard DM, Hung MC, O'Malley BW. SRC-3 coactivator regulates cell resistance to cytotoxic stress via TRAF4-mediated p53 destabilization. Genes Dev 2013;27:274-287.
40 Valentin MD, da Silva SD, Privat M, aoui-Jamali M, Bignon YJ. Molecular insights on basal-like breast cancer. Breast Cancer Res Treat 2012;134:21-30.
41 Kennecke H, Yerushalmi R, Woods R, et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol 2010;28:3271-3277.
42 Delpech Y, Coutant C, Hsu L, et al. Clinical benefit from neoadjuvant chemotherapy in oestrogen receptor-positive invasive ductal and lobular carcinomas. Br J Cancer 2013;108:285-291.
43 Sihto H, Lundin J, Lundin M, et al. Breast cancer biological subtypes and protein expression predict for the preferential distant metastasis sites: a nationwide cohort study. Breast Cancer Res 2011;13:R87.
44 Moelans CB, Monsuur HN, de Pinth JH, Radersma RD, de Weger RA, van Diest PJ. ESR1 amplification is rare in breast cancer and is associated with high grade and high proliferation: a multiplex ligation-dependent probe amplification study. Cell Oncol (Dordr) 2011;34:489-494.


Breast cancer
Metastases
metastases metastases

CHAPTER 9

Summarizing discussion

The routine pathological work-up of breast cancer includes the evaluation of the estrogen receptor (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) which reveals biological information about the tumour (prognostic information) as well as provides predictive biomarkers regarding hormonal therapy or trastuzumab treatment. When distant metastases occur, the choice of systemic treatment used to be based on the receptor expression of the primary tumor, refraining from routine biopsies of the metastatic lesions as part of the standard workup.

Previous studies have shown that distant breast cancer metastases may show receptor conversion, which could result in not only withholding effective endocrine- or HER2-blocking agents from patients but could also result in unjust administration of these same agents. These previous studies however suffered from several limitations, not allowing to draw definite conclusions that could change clinical practice. In **chapter 2** we therefore re-evaluated receptor conversion in a large group of non-bone distant breast cancer metastases using optimal methodology. We found receptor conversion in 10.3% and 30% of patients for ERα and PR, respectively, when using a 10% threshold for positivity. When using the 1% ERα and PR thresholds according to the ASCO guidelines, conversion rates were even higher at 15.1% and 32.6%. HER2 conversion occurred in 5.2% by immunohistochemistry (IHC), and in 2.6% by in silver in situ hybridization (SISH). Methodological and technical defaults have been described as predominant causes for receptor discordances. However, we have ruled out the potential analytic features associated with the assessment of the receptors. We used whole sections, not tissue arrays which may introduce sampling bias. Fresh sections were cut and stained with current optimal, standardized staining methods to rule out differences in antigen retrieval, antibodies and antibody concentrations and detection methods. All sections were scored by one experienced pathologist to prevent interobserver variation. Of note, interlaboratory variation and interpretation variability in clinical practice may account for discordance in some patients. HER2 discrepancies between primary tumors and distant metastases were further evaluated by *in situ* hybridization, which may be more straightforward than interpretation of HER2 IHC. Using the 10% threshold, in 10.7% of patients conversion from ERα positive or PR positive to ERα negative/PR negative occurred, and in 3.4% of patients, conversion from ERα negative/PR negative to ERα positive or PR positive occurred, which is obviously relevant with respect to the decision on hormonal therapy. In 12.4% of patients, conversion from ERα positive or PR positive to ERα negative/PR negative occurred, and in 8.2% of patients, conversion from ERα negative/PR negative to ER positive or PR positive occurred, when using the 1% threshold. Together with a 2.6% conversion rate for HER2 by SISH (5.2% by IHC), receptor conversion would thereby have had direct consequences for the choice of the therapeutic regimen in 16.7-25.8% of women. These results are in line with those from the prospective study of Amir et al. who showed that biopsy of metastases is beneficial: one in seven women had a change in treatment as compared with the prebiopsy therapeutic plan[1].

In a retrospective follow-up study, **chapter 3**, we additionally showed that receptor conversion for ERα and PR from positive in the primary tumor to negative in the distant metastases has unfavorable prognostic impact.

In conclusion, therapy management of metastatic disease is suboptimal when only based on primary tumor characteristics. For this reason, receptor status should be reassessed on available biopsies from distant metastases and the tests results should be critically evaluated in conjunction with ER, PR and HER2 status of the primary tumor. Most contemporary guidelines indeed now advice to biopsy distant metastases to re-evaluate receptor status to guide treatment in the metastatic setting[2-4]. Despite the clear rationale for a metastatic breast cancer biopsy, this may not always be feasible because of limited lesion accessibility or potential complications. Non-invasive assessment of the receptor status by molecular imaging may be an attractive alternative. Nevertheless, radionuclide molecular imaging tests are costly and require complicated infrastructure and logistics.

Therefore, in **chapter 4**, we described the development of prediction models for hormone receptor conversion in metastatic breast cancer patients using widely available clinicopathological information. We showed that especially the absence of positive to negative hormone receptor conversion can be accurately predicted based on percentage ERα and PR positive cells of the primary tumor, which are easy and inexpensive to measure. The performance of models predicting positive to negative ERα and HR (ERα and PR) conversion was very good – also following internal validation for overoptimism –, showing good calibration and discrimination. Depending on ERα/HR and 1/10% threshold subgroups, 22 to 45% of all metastatic breast cancer patients of our study population with primary receptor positive disease had a predicted conversion risk below 5%, potentially obviating the need to biopsy. External validation of the prediction models in a large population would be very valuable. Although negative to positive receptor conversion has high clinical implications, i.e. eligibility for hormonal therapy, unfortunately, we were unable to develop prediction models for negative to positive ERα/HR conversion based on our data.

Previous studies have been done on single distant metastases, while patients often develop multiple metastases. We therefore aimed to study discordance of receptor status between different distant metastases from the same patient in **chapter 5**. We showed in a substantial proportion of 50 metastatic breast cancer patients discordance in ERα status (7-11%, depending on threshold used) and PR status (29-31%) across different distant breast cancer metastases within the same patient, with potential consequences for hormonal treatment in 11-15% of cases when only one random metastasis would be biopsied. For HER2, there seemed to be only limited discordance on the protein level and even none on the gene level. As a consequence, several metastases may need to be biopsied when possible to fully assess receptor status in case of multiple metastases, while molecular imaging with hormone receptor probes may be an alternative.

In light of the above, intratumoral heterogeneity of breast cancer may be an explanation for the occurrence of receptor conversion in distant breast cancer metastases; only a limited number of subpopulations of cells in the primary tumor has the potential to metastasize, such that within the same patient some metastases can be positive whereas others are negative for a specific phenotype. Furthermore, we showed in **chapter 4** that the *percentage* ERα and PR positive of the primary tumor cells are the strongest predictors of positive to negative receptor conversion.

In **chapter 6** we demonstrated that also other predictive biomarkers could differ between the primary tumor and corresponding metastasis. We showed an increase in phosphorylated mTOR (p-mTOR) expression in biopsies from metastatic tumor tissue compared to corresponding primary tumor tissue, which was associated with the use of adjuvant hormonal therapy. Considering these changes in response to treatment during tumor progression, the results re-emphasises the importance of performing biopsies from metastatic tumor lesions in clinical practice to guide therapy – here mTOR inhibitors.
Although we initially made the deliberate choice not to include bone metastases in our receptor conversion studies to avoid false negative immunohistochemical results due to potential decalcification artifacts, it may have limited the generalizability of our study results. Evidence for this was however limited. We therefore studied in **chapter 7** the influence of decalcification using Christensen's or EDTA buffer on receptor expression in breast cancer. The overall concordance for ER expression was for both decalcification procedures 100%, for PR 80%, and for HER2 expression concordance was 100% for EDTA and 90% for Christensen's. For EDTA, there was no influence on therapeutic decision making with regard to hormonal therapy or trastuzumab. This implies that receptor IHC can be reliable performed on EDTA decalcified bone metastases of breast cancer.
Finally, in **chapter 8**, we touch on the molecular background of distant breast cancer metastases formation by comparing copy number of common breast cancer genes by multiplex ligation-dependent probe amplification (MLPA), between primary breast carcinoma and distant metastases. The vast majority of copy number aberrations seen in primary tumors were retained in their corresponding metastases indicating that, on the copy number level of these genes there is overall little genomic progression from primary breast tumors to their distant metastases. Despite the presence of ERα and HER2 receptor conversion at the immunohistochemistry level (see **chapter 2**), there were no (significant) differences at the gene copy number level for *ESR1*.
Althogether, we hope to increase awareness that taking biopsies in metastatic breast cancer patients to guide therapy decisions should be done whenever possible. An alternative for taking biopsies could be molecular imaging methods like positron emission tomography (PET) and single photon emission computed tomography (SPECT), especially for metastases at inaccessible sites, also providing information on heterogeneity of receptor status between distant metastases[5].

Future perspectives

As previously suggested the best interest of the patient it is adamant to stimulate the development of non-invasive assessment of the metastatic sites and their respective hormone or HER2 receptor status, e.g. by molecular imaging. Linden et al. showed that [^{18}F]fluoroestradiol (FES) uptake in (PET) imaging was correlated with ER expression assayed by qualitative immunohistochemistry measurement[6]. The study suggested that 18F-FES PET is useful to predict treatment effect of hormonal therapy and may help guide treatment selection. Evaluation of the PET tracer ^{89}Zr-trastuzumab in metastatic breast cancer patients showed successful

detection and quantification of HER2 positive breast cancer metastases[7]. Apart from patient selection, these tracers might also be able to measure the functional effects of targeted agents in breast cancer (metastases)[8].

For optimal molecular imaging in the diagnosis of breast cancer metastases it is recommended to investigate the expression of new candidate biomarkers in metastases since no single current marker is completely specific and sensitive. Several proteins are expressed on primary breast cancers that are potentially useful as targets for imaging. These include the hypoxia related proteins CAIX and Glut-1, the epidermal growth factor receptor-1 (EGFR) and vascular endothelial growth factor (VEGF).

The main challenge for the future of cancer treatment is to provide every individual patient with the most effective drug tailored to their specific cancer ("perzonalized treatment"), instead of the traditional organ-related strategies we traditionally work with. The possibility to sequence large numbers of genes in a short time due to the introduction of next generation sequencing (NGS) technologies will improve personalized cancer treatment by selecting patients for specific treatment regimens[9]. In the era of DNA-guided personalized cancer treatment, it is important to be aware of which tumor tissue will best predict treatment response in (breast) cancer patients with metastatic cancer. This thesis showed that therapeutic decisions for these patients based on receptor status should be derived from real-time information from the metastasis rather than the primary tumor to ensure optimal treatment efficacy of targeted therapy. Vermaat et al. analyzed genetic differences between 21 primary colorectal adenocarcinomas and their corresponding hepatic metastasis[10]. On average, 83 potentially function-impairing variations were gained in the metastasis and 70 variations were lost. This indicates that also with regard to DNA status, systemic treatment should be based on the genomic profile of the metastases rather than that of the primary tumor. The expectation for the future is that a treatment plan will be offered to all (breast) cancer patient with metastatic cancer based on the genetic profile of the metastasis which may eventually result in managing cancer as a chronic disease. Since 2012 the high-tech DNA research in personalized cancer treatment is being conducted in The Netherlands by the Center for Personalized Cancer Treatment, to make this mission come true.

REFERENCES

1. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, Oldfield M, Dranitsaris G, Tomlinson G, Laupacis A, et al: Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol, 30:587-592.
2. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E: Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol, 23 Suppl 7:vii11-19.
3. National Cancer Institute PDQ. Breast Cancer Treatment. 2011.
4. National Comprehensive Cancer Network: Practice Guidelines in Oncology: Breast Cancer-V2.2012.
5. Bensch F, van Kruchten M, Lamberts LE, Schroder CP, Hospers GA, Brouwers AH, van Vugt MA, de Vries EG: Molecular imaging for monitoring treatment response in breast cancer patients. Eur J Pharmacol.
6. Linden HM, Stekhova SA, Link JM, Gralow JR, Livingston RB, Ellis GK, Petra PH, Peterson LM, Schubert EK, Dunnwald LK, et al: Quantitative fluoroestradiol positron emission tomography imaging predicts response to endocrine treatment in breast cancer. J Clin Oncol 2006, 24:2793-2799.
7. Dijkers EC, Kosterink JG, Rademaker AP, Perk LR, van Dongen GA, Bart J, de Jong JR, de Vries EG, Lub-de Hooge MN: Development and characterization of clinical-grade 89Zr-trastuzumab for HER2/neu immunoPET imaging. J Nucl Med 2009, 50:974-981.
8. de Vries EG, Oude Munnink TH, van Vugt MA, Nagengast WB: Toward molecular imaging-driven drug development in oncology. Cancer Discov, 1:25-28.
9. Gadellaa-van Hooijdonk CGM, Koudijs MJ, Cuppen EPJG, van Diest PJ, Martens JWM, Sleijfer S, Schellens JHM, Voest EE, Lolkema MJPK: Van DNA-afwijkingen tot therapie: selectie voor geindividualiseerde kankerbehandeling. NVTO 2011.
10. Vermaat JS, Nijman IJ, Koudijs MJ, Gerritse FL, Scherer SJ, Mokry M, Roessingh WM, Lansu N, de Bruijn E, van Hillegersberg R, et al: Primary colorectal cancers and their subsequent hepatic metastases are genetically different: implications for selection of patients for targeted treatment. Clin Cancer Res, 18:688-699.


Breast cancer
Metastases
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ADDENDUM

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NEDERLANDSE SAMENVATTING

(voor niet-ingewijden)

Borstkanker (ook wel mammacarcinoom genoemd) is wereldwijd de meest gediagnosticeerde vorm van kanker bij vrouwen. In Nederland ontwikkelt 1 op de 8 vrouwen borstkanker gedurende het leven. Borstkanker is een ongecontroleerde groei van cellen in de borst, als gevolg van veranderingen in het DNA. Als drager van erfelijke informatie bevat DNA alle gegevens die nodig zijn om de cel op het juiste moment te laten delen, en hem zijn specifieke functie te laten uitvoeren. Door fouten in het DNA kan de cel ongeremd gaan delen en ontstaat een tumor. De tumorcellen overwoekeren normale weefsels/organen met alle schadelijke gevolgen van dien. Daarnaast bestaat het risico dat losgeraakte borstkankercellen zich door het lichaam gaan verspreiden, zich hechten en uitgroeien tot nieuwe tumoren. Dit worden uitzaaiingen of metastasen genoemd. Uitzaaiingen kunnen zich via de lymfebanen verspreiden naar een of meer nabijgelegen lymfeklieren (regionale uitzaaiingen) of via de bloedbaan naar organen op afstand zoals de longen, lever of hersenen of naar de botten (uitzaaiingen op afstand). Ongeveer een derde van de borstkankerpatiënten ontwikkelt één of meer metastasen op afstand. De prognose van deze patiënten is erg ongunstig. Gemiddeld overlijden patiënten met metastasen binnen 18 to 24 maanden na diagnose. De behandeling van deze patiënten is gericht op levensverlenging met behoud van goede kwaliteit van leven.

Veel borstkankercellen zijn, net zoals het oorspronkelijke borstklierweefsel, gevoelig voor (vrouwelijke) hormonen. De borstkankercellen kunnen door deze hormonen (extra) gestimuleerd worden om te delen. De aanwezigheid van hormoonreceptoren in het tumorweefsel zegt iets over de hormoongevoeligheid van een tumor. Hormoonreceptoren zijn eiwitten die hormonen binden en vervolgens (groei)signalen doorgeven naar de cel. In het laboratorium kan met behulp van immunohistochemie (IHC), een techniek om eiwitten in weefsel zichtbaar te maken, de aanwezigheid van de hormoonreceptoren (oestrogeen receptor en progesteron receptor) in tumorweefsel bepaald worden. Ongeveer 70% van de borsttumoren bij vrouwen is hormoonreceptor-positief en komt in aanmerking voor (anti)hormonale therapie met tamoxifen of aromatase-remmers, waarbij de werking of de aanmaak van de vrouwelijke hormonen wordt belemmerd en de groei-stimulerende effecten zo worden tegengegaan. In een vroeg stadium van borstkanker kan hormonale therapie bijdragen aan betere overlevingskansen. Bij uitzaaiingen kan hormoontherapie het kankerproces (tijdelijk) vertragen. Mogelijke bijwerkingen van hormonale therapie zijn osteoporose, trombose en een licht verhoogd risico op baarmoederslijmvlieskanker.
Een andere belangrijke therapeutische en prognostische biomarker is het HER2 gen. Dit gen codeert voor Human Epidermal Growth Factor Receptor 2 (HER2), een eiwit dat een rol speelt in de regulatie van de normale celgroei. In een normale cel zijn 2 kopieën van het HER2-gen aanwezig, maar bij 10-20% van de mammacarcinomen zijn er meer kopieën van het gen aanwezig ("amplificatie"), wat doorgaans resulteert in te grote hoeveelheden receptoreiwit op de celmembraan ("overexpressie"). Amplificatie en overexpressie zijn geassocieerd met snellere vermeerdering van het aantal tumorcellen en een slechtere prognose voor de patiënt. Patiënten

met een HER2 amplificatie/overexpressie komen in aanmerking voor behandeling met een antilichaam (bijvoorbeeld trastuzumab of lapatinib) dat gericht is tegen de HER2 receptor en de werking hiervan blokkeert.

Als uitzaaiingen zich openbaren volgt opnieuw hormonale en/of antilichaamtherapie die traditioneel wordt gebaseerd op het receptor profiel van de oorspronkelijke tumor in de borst, de primaire tumor. Herevaluatie van aan– of afwezigheid van de verscheidene receptoren in het uitgezaaide weefsel wordt niet standaard uitgevoerd, aangezien niet vaak een weefselmonster (biopt) van de uitzaaiing wordt genomen. Dit komt enerzijds door de vaak beperkte bereikbaarheid van de uitzaaiingen, maar anderzijds ook omdat het niet noodzakelijk wordt geacht voor verdere therapiekeuze.
In dit proefschrift worden de aanwezige verschillen tussen primaire borsttumoren en corresponderende uitzaaiingen op afstand belicht. Met hulp van een groot aantal ziekenhuizen in Nederland hebben we het weefsel en de patiëntengegevens van 233 vrouwen met uitgezaaide borstkanker kunnen verzamelen. Aan de hand van immunohistochemische kleuringen werd per patiënte de receptor status van de primaire tumor en uitzaaiing bepaald en met elkaar vergeleken. Uit dit onderzoek is gebleken dat het receptor profiel van de uitzaaiingen kan verschillen van het receptor profiel van de oorspronkelijke borsttumor. Dit wordt receptor conversie genoemd. In **hoofdstuk 2** beschrijven we dat deze bevindingen belangrijke klinische consequenties kunnen hebben. Ten gevolge van deze receptor conversie zouden 19% van de patiënten in de groep niet de juiste behandeling krijgen indien gebaseerd op het receptor profiel van de primaire tumor. Wanneer een uitzaaiing in tegenstelling tot de oorspronkelijke borsttumor 'hormoonreceptor positief' is, kan alsnog een behandeling met hormoontherapie worden gestart. In **hoofdstuk 3** tonen we vervolgens aan dat patiënten met een veranderd receptor profiel een slechtere prognose hebben dan patiënten bij wie dit niet het geval is. Deze resultaten leiden tot de conclusie dat de uitzaaiingen wellicht gebiopteerd zouden moeten worden om de receptor status opnieuw te bepalen. Naar aanleiding van deze en vergelijkbare studies adviseren verschillende (inter)nationale richtlijnen voor borstkanker inmiddels het standaard biopteren van uitzaaiingen.
Biopteren van uitzaaiingen is echter niet altijd mogelijk door beperkte bereikbaarheid van uitzaaiingen, of gevaar voor complicaties. Het kunnen voorspellen van receptor conversie zou daarom erg waardevol zijn. In **hoofdstuk 4** evalueerden we de voorspellende waarde van verschillende kenmerken van de primaire tumor voor receptor conversie. Uit dit onderzoek blijkt dat de mate van *aanwezigheid* van de oestrogeen en progesteron receptoren in de primaire borsttumor de *afwezigheid* van receptor conversie in de metastasen kan voorspellen. De aanwezigheid van de hormoonreceptoren wordt door de patholoog bepaald als een percentage positieve cellen. Uit onze analyse is gebleken dat naar mate het percentage hormoonreceptoren in de primaire borsttumor hoger is, de kans op receptor conversie in de corresponderende metastase afneemt. Dit kan in een aantal patiënten van nut zijn omdat wanneer de kans op een hormoonreceptor-negatieve metastase zo klein is overwogen kan worden het biopt achterwege te laten.

Vrouwen met borstkanker ontwikkelen vaak meerdere uitzaaiingen door het hele lichaam. In **hoofstuk 5** hebben we aan de hand van IHC de hormoon- en HER2 receptor status bepaald van 55 borstkanker patiënten met 2 of meer uitzaaiingen. Na vergelijking is gebleken dat het receptorprofiel van verschillende uitzaaiingen van dezelfde patient ook niet altijd met elkaar overeen komt. Dit zou betekenen dat meerdere uitzaaingen gebiopteerd zouden moeten worden voor het bepalen het receptorprofiel om de juiste behandeling te starten, hetgeen belastend is voor de patient. Moleculaire beeldvormings technieken ("molecular imaging") kunnen in de toekomst een goed alternatief zijn voor het biopteren van meerdere metastasen. Dit is een niet-invasieve techniek waarbij moleculen (de hormoon- en HER2 receptoren) zichtbaar worden gemaakt met specifieke contrastmiddelen.

Hormoontherapie wordt gegeven aan vrouwen bij wie is aangetoond dat de tumor gevoelig is voor vrouwelijke hormonen. Ondanks deze behandeling, komt de ziekte bij veel patiënten terug en/of ontwikkelen er zich uitzaaiingen. Er is dan sprake van resistentie tegen de hormoontherapie. Eerdere studies hebben aangetoond dat een verhoogde expressie van mTOR (een van de vele eiwitten die betrokken is bij celgroei) ervoor zorgt dat de groei van de borstkankercel onafhankelijk is van oestrogenen. Een behandeling met een remmer van mTOR (een mTOR-inhibitor) kan deze resistentie teniet doen. In **hoofdstuk 6** hebben we beschreven dat de expressie van mTOR (ook) kan verschillen tussen de primaire tumor en corresponderende uitzaaiing. Dit leidt opnieuw tot de conclusie dat uitzaaiingen gebiopteerd moeten worden om de mTOR status te bepalen.

Bij borstkanker komen uitzaaiingen in het bot het meeste voor. Voorafgaand aan nader onderzoek van het een botbiopt moet het bot eerst voorbewerkt worden. Door de grote hoeveelheden calcium die in het bot zijn opgeslagen is het onmogelijk om bruikbare dunne plakjes weefsel (coupes) te snijden zonder het weefsel vooraf te ontkalken. Sommige studies poneren dat ontkalking de bepaling van de receptor status wel eens zou kunnen compromitteren. In **hoofdstuk 7** hebben we onderzocht wat het precieze effect is van verschillende ontkalkingsmethodes op de immunohistochemische kleuringen. Het bleek dat ontkalking niet veel invloed had op bepaling van receptor status, met name als de EDTA methode wordt gebruikt. Deze resultaten zijn van belang voor het analyseren van de hormoon- en HER2 receptor status van botmetastasen. Indien de EDTA methode wordt gebruikt kan de receptor status veilig worden bepaald op botmetastasen.

Om meer inzicht te krijgen in het proces van uitzaaiing en daarmee mogelijk nieuwe behandelopties op te sporen hebben we tot slot in **hoofdstuk 8** de genetische profielen van de primaire tumoren en uitzaaiing met elkaar vergeleken. Meer specifiek was de vraag of er toe- of afname is van 20 borstkanker gerelateerde genen bij metastasering. Hiervoor hebben we gebruik gemaakt van de techniek MLPA (Multiplex Ligation-dependent Probe Amplification). Deze techniek is gebaseerd op het vele malen vermenigvuldigen van kleine stukjes DNA, waardoor een mogelijke toe- of afname van een genkopie gemeten kan worden. Na analyse van 55 borsttumoren en corresponderende uitzaaiingen bleek er weinig verschil te zijn tussen

primaire tumor en uitzaaiing. Genetische profielen lijken dus overall niet veel te veranderen tijdens het proces van metastasering. Waarschijnlijk gaat het om een beperkt aantal genen die mogelijk met technieken waarbij alle chromosomen ("het genoom") bekeken worden opgespoord kunnen worden.

In dit proefschrift hebben we de aanwezige verschillen tussen primaire borsttumoren en corresponderende uitzaaiingen op afstand en de hieruit volgende klinische consequenties voor de patiënt beschreven. Omwille van therapeutische implicaties, leiden de resultaten tot de conclusie dat de uitzaaiingen in principe gebiopteerd zouden moeten worden om de receptor status (opnieuw) te bepalen.

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CURRICULUM VITAE

Laurien Danielle Célestine Hoefnagel was born on February 8, 1985 in 's-Hertogenbosch, The Netherlands. After graduating from the Theresia Lyceum (Gymnasium), she started in 2003 Medical School at Utrecht University. After obtaining her doctoral examination in Medicine in 2009, she started her PhD project under supervision of Prof. Paul van Diest en Prof. Elsken van der Wall, the results of which are described in this thesis. The pilot research she did was later awarded a full grant from the Dutch Cancer Society on which she continued working. In October 2009 she joined the "Sisters Hope 60 km walk; Raising money of breast cancer research.

In 2011 she started the postgraduate research master 'Epidemiology' at the Julius Center for Health Sciences and Primary Care, University of Utrecht. During this master program, she performed a one year research project about 'Predicting hormone receptor conversion in distant breast cancer metastases', at the department of (cancer) Epidemiology at the Julius Center, under direct supervision of Dr. Sjoerd G. Elias. The results obtained are part of this thesis (chapter 4). In 2013, Laurien obtained her Master of (health) Science.

LIST OF PUBLICATIONS

Hoefnagel LDC, van der Groep P, van de Vijver MJ, Boers JE, Wesseling P, Wesseling J, van der Wall E, van Diest PJ.
Discordance in ERα, PR and HER2 receptor status across different distant breast cancer metastases within the same patient.
Annals of Oncology, in press

Hoefnagel LDC, Moelans CB, Meijer SL, van Slooten H-J, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, van der Valk P, van Gils CH van der Wall E,van Diest PJ.
Prognostic value of ER and PR conversion in distant metastases of breast cancer patients.
Cancer 2012 Oct 15;118(20):4929-35

Ercan C, Vermeulen JF, **Hoefnagel LDC**, Bult P, van der Groep P, van der Wall E, van Diest PJ.
HIF-1α and NOTCH signaling in ductal and lobular carcinomas of the breast.
Cell Oncol. 2012 Dec;35(6):435-42.

van Diest PJ, **Hoefnagel LDC**, van der Wall E.
Testing for discordance at metastatic relapse of breast cancer matters.
J Clin Oncol. 2012 Aug 20;30(24):3031

Hoefnagel LDC, van de Vijver MJ, van Slooten H-J, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, van der Valk P, van der Groep P, de Vries EGE, van der Wall E, van Diest PJ.
Receptor conversion in distant breast cancer metastases.
Breast Cancer Res. 2010;12(5):R75

Moelans CB, de Weger RA, **Hoefnagel LDC**, van der Wall E, van Diest PJ.
Nieuwe ontwikkelingen in HER2- detectie bij het mammacarcinoom.
Nederlands Tijdschrift voor Oncologie 2010