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PRECLINICAL STUDY

The 70-gene signature as a response predictor for neoadjuvant chemotherapy in breast cancer

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Abstract The 70-gene signature (MammaPrint^{TM}) is a prognostic tool used to guide adjuvant treatment decisions. The aim of this study was to assess its value to predict chemosensitivity in the neoadjuvant setting. We obtained the 70-gene profile of stage II–III patients prior to neoadjuvant chemotherapy and classified the prognosis-signatures. Pathological complete remission (pCR) was used to measure chemosensitivity. Among 167 patients, 144 (86%) were having a poor and 23 (14%) a good prognosis-signature. None of the good prognosis-signature patients achieved a pCR (0/23), whereas 29/144 patients (20%) in the poor prognosis-signature group did ($P = 0.015$). All triple-negative tumors ($n = 38$) had a poor prognosis-signature. Within the non triple-negative subgroup, the response of the primary tumor remained associated with the classification of the prognosis-signature ($P = 0.023$). A pCR is unlikely to be achieved in tumors that have a good prognosis-signature. Tumors with a poor prognosis-signature are more sensitive to chemotherapy.

Keywords Breast cancer · Neoadjuvant · Chemosensitivity · Predictive · Gene expression signature

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Introduction

The mortality of breast cancer is decreasing in the developed part of the world. This is largely a result of effective adjuvant systemic therapy [1]. An important problem of adjuvant therapy is overtreatment, which consists of the administration of adjuvant therapy in patients for whom surgical resection of the tumor alone would be curative. These patients will experience toxicity without benefiting from the treatment. Currently, the selection of patients for adjuvant treatment is based on tumor and patients characteristics such as endocrine responsiveness, tumor grade, lymph node status and age. One strategy to reduce overtreatment is the use of prognostic biomarkers. Systematic analysis of gene-expression patterns using microarray technology has led to the discovery of prognostic gene-expression signatures, one of which is the 70-gene prognostic-signature (MammaPrint^{TM}) [2].

The prognostic value of 70-gene profile has been validated in a range of series of patients [3–6]. These studies

confirmed that the 70-gene signature accurately discriminates between patients at high risk of distant metastasis and death and patients with a favorable prognosis. Furthermore, it was shown that the 70-gene signature adds independent prognostic information to that provided by commonly used clinicopathological factors.

Node-negative patients with a good prognosis-signature who did not receive any adjuvant systemic therapy, had a 10-year overall survival of 89% (95%CI 0.81–0.94). These results suggest that adjuvant therapy could be limited to endocrine treatment for tumors with a good prognosis-signature. At present, the MINDACT trial is addressing this question. In this trial, treatment selection based on the 70-gene signature as compared to clinical risk assessment, may show that it does not compromise the overall outcome [7].

Virtually all (92–98%) of the good prognosis-signature tumors show a high expression of the estrogen receptor (ER). Furthermore, tumors with a good prognosis signature are usually those with lower proliferative rates. It is, therefore, often assumed that good prognosis-signature tumors may be less sensitive to chemotherapy than tumors with a poor prognosis-signature. If true, the 70-gene signature would have predictive power in addition to its prognostic value.

To analyze the predictive value of the 70-gene profile, we determined the 70-gene signature in tumors of patients treated with neoadjuvant chemotherapy at The Netherlands Cancer Institute. The objectives of this study were: (1) to analyze the association between the pathological complete response (pCR) rate and the results of the prognostic signature test, and (2) to assess the predictive value in different subgroups defined by the expression of hormone receptors and the amplification of the human epidermal growth factor receptor 2 (HER2) genes.

Methods

Patients

Fresh frozen tumor biopsies and clinical data were collected from a consecutive series of 171 patients who received neoadjuvant chemotherapy at The Netherlands Cancer Institute between 2000 and 2008. Patients received neoadjuvant chemotherapy in one of two clinical studies ongoing or received treatment according to the standard arm of these trials [8]. Patients with invasive breast cancer greater than 3 cm and/or involved lymph nodes were eligible for these studies. The clinical studies were approved by the institutional ethical committee and informed consent was obtained from all patients. Prior to neoadjuvant treatment, 14-gauge biopsies of the breast tumor were taken under ultrasound guidance. These biopsies were snap-frozen in liquid nitrogen and stored at −70°C. All patients of whom adequate RNA could be extracted from the tumor samples were included in the study, provided that they had undergone surgery to determine the pathological remission status.

Clinicopathological data

Clinical data were collected from medical records, blinded to the 70-gene prognosis-signature. Tumor size was assessed by MRI ($n = 155$) when available or by mammogram and ultrasound examination ($n = 16$). Nodal status prior to neoadjuvant chemotherapy was determined by ultrasound guided fine-needle aspiration or, when negative sentinel node biopsy. In 16 patients with inconclusive cytological assessment the pathological nodal status prior to neoadjuvant chemotherapy remained unknown. Tumor grading was defined according to the Elston and Ellis method [9]. Estrogen receptor (ER) status and progesterone receptor status were determined by immunohistochemistry and interpreted positive if more than 10% of the nuclei stained positive. HER2 status was assessed by scoring the intensity of membrane staining using immunohistochemistry. Tumors with a score of 3+ (strong homogeneous staining) were considered HER2-positive. In case of 2+ scores (moderate homogeneous staining) chromogenic in situ hybridization (CISH) was used to determine amplification [10]. Amplification was defined as a gene copy number of over five per cell. Tumors were classified in three subgroups according to their receptor status using immunohistochemical staining; (1) ER-positive and HER2-negative tumors, (2) triple negative tumors (ER-negative, PR-negative and HER2-negative) and (3) HER2-positive tumors.

The treatment regimen depended on the presence or absence of HER2 amplification. Preoperative chemotherapy for HER2-negative tumors employed one of the following regimens: AC (six cycles of doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2, q 3 weeks); dose dense (dd) AC (AC q 2 weeks with filgrastim) [11]; AD (six cycles of doxorubicin 50 mg/m^2 and docetaxel 75 mg/m^2) or DC (six cycles of docetaxel 75 mg/m^2 and capecitabine 2 × dd 1,000 mg/m^2 orally during 14 days, q 3 weeks) [12, 13]. For HER2-positive tumors, the regimens included ddAC and PTC (paclitaxel 80 mg/m^2 week^{-1}, trastuzumab 2 mg/kg and carboplatin AUC 2–3 mg/ml min times 6, q 8 weeks) after 2005 [14, 15]. After one or three cycles (depending on the specific protocol) the tumor response was evaluated by contrast enhanced MRI [16]. Chemotherapy regimens were changed to a presumably non-cross-resistant regimen when response failure was apparent upon radiological evaluation. After the last course of chemotherapy patients underwent mastectomy or breast conserving surgery. Three patients had progressive disease. In two patients, surgery was performed prior to the completion of chemotherapy and one

patient with mastitis carcinomatosa was treated with radiation therapy only. To prevent bias, these patients were kept in the analysis despite the fact that they underwent early or no surgery.

RNA extraction and gene expression analysis

RNA isolation and amplification were performed as previously described [17]. One 5-µm tissue section of the biopsy was hematoxylin and eosin stained to monitor the tumor cell percentage of the tissue. Only specimens with at least 50% tumor cells were further analyzed. To assess the mRNA expression level of the 70 genes, RNA was hybridized to a custom-designed array (MammaPrint^{TM}, FDA 510(K) cleared), blinded to clinical data, at Agendia's ISO17025-certfified and CLIA accredited laboratories. Tumors were classified as good prognosis-signature (low risk) or poor prognosis signature (high risk) as described previously [18].

Assessment of tumor response

Pathological complete response (pCR) was used as the outcome measure. It was defined as the absence of invasive carcinoma in both the breast and axilla at microscopic examination of the resection specimen, regardless of the presence of carcinoma in situ [19]. Furthermore, the response of the primary tumor in the breast was assessed separately. The response of the primary tumor was defined as a pCR when no residual tumor cells were seen at microscopy or as a 'near pCR' (npCR) when small numbers of scattered tumor cells or tumor cells in an area of less than 2 mm in diameter were present.

Statistical analysis

Analyses were performed using SPSS version 15.1 (SPSS Inc, Chicago, IL). The differences in patients and tumor characteristics between the 70-gene poor and good prognosis signature were tested using Fisher's Exact test and students t test. We used the Fisher's Exact test to assess the association between the response of the tumor and the outcome of the 70-gene profile. Disease-free survival curves were calculated using the Kaplan–Meier method and compared using the log-rank test. *P*-values reported are two sided.

Results

The 70-gene profile was analyzed in 171 patients who received neoadjuvant chemotherapy. Three of these 171 patients were excluded due to clinical reasons. Two patients did not undergo surgery of the primary tumor and in one patient the treatment with chemotherapy was discontinued early due to major toxicity. In one patient the RNA quality was insufficient to perform gene profiling. Thus, the predictive value of the 70-gene prognostic signature could be evaluated in 167 patients. (Fig. 1).

Patient and tumor characteristics are presented in Table 1. Among the 167 patients, 23 (14%) had a good prognosis-signature, whereas 144 (86%) patients had a poor prognosis-signature. Tumors with a poor prognosis-signature were of higher grade, and were more often classified as triple-negative or HER2 positive tumors. Consequently, more patients in the poor prognosis-signature group were treated with a trastuzumab based regimen.

The overall pCR rate (absence of invasive tumor in both breast and axilla) was 17% (29/167). None of the patients with a good prognosis-signature achieved a pCR (0/23). The pCR rate in the poor prognosis-signature group was

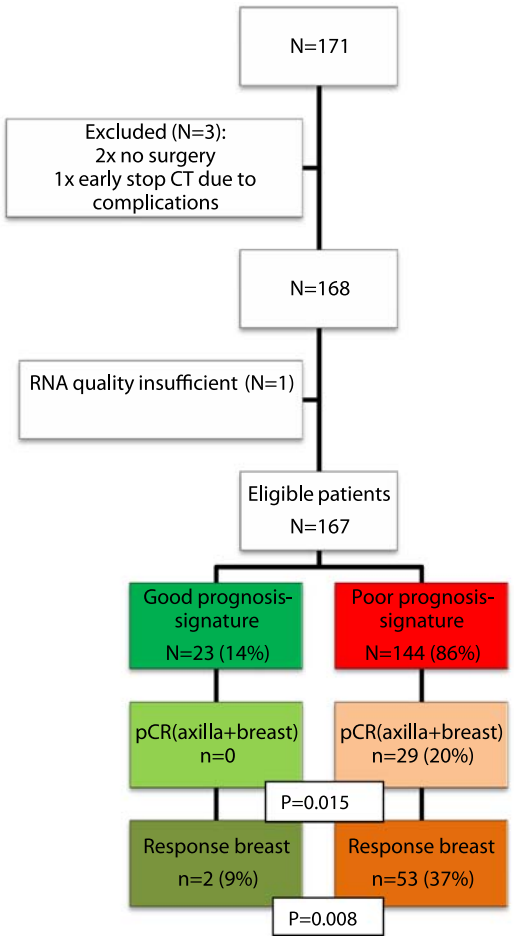

Fig. 1 The patients selection and the classification of the 70-gene signature. The pathological response in breast and axilla is determined for both risk groups. A poor prognosis profile was significantly associated with the pathological complete remission (pCR) of both the breast and axilla ($P = 0.015$) and with response (near pCR or pCR) of the primary tumor separately ($P = 0.008$)

Table 1 The patient and tumor characteristics and the association with the 70-gene signature

	Total $n = 167$ No. (%)	Good prognosis $n = 23$ No. (%)	Poor prognosis $n = 144$ No. (%)	P value
Mean age	46	46	46	NS
Range	23–68	31–58	23–68	
Menopausal status				
Pre-menopausal	119	18 (78)	101 (71)	NS
Post-menopausal	39	5 (22)	34 (24)	
Unknown	9	0 (0)	9 (5)	
T stage (prior to CT)				
T1	9	2(9)	7 (5)	NS
T2	87	13(57)	74(51)	
T3	62	7(30)	55 (38)	
T4	9	1(4)	8(6)	
pN stage (prior to CT)				
pN0 (SNB-)	30	5 (22)	25 (17)	NS
pN1 (SNB+/FNA+)	110	15 (65)	95(66)	
pN3 (sub/supraclavicular)	11	0 (0)	11 (8)	
pNX (cN0)	16	3 (13)	13 (19)	
Histology				
Ductal	131	14 (61)	117 (81)	0.008
Lobular	20	8 (35)	12 (8)	
Others	16	1 (4)	15 (11)	
Grade				
I	7	3 (13)	6 (3)	0.011
II	88	16 (70)	72(50)	
III	66	3 (13)	63 (44)	
missing	6	1(4)	5 (3)	
Subtype (based on receptor status)				
ER+ (ER+ , HER2-)	88	21 (91)	67 (47)	<0.001
TN (ER-, PR-, HER2-)	38	0 (0)	38 (26)	
HER2+	41	2 (9)	39(27)	
Primary systemic therapy				
Antracycline-like	85	10(43)	75 (52)	0.036
Antracycline-taxane	31	9 (39)	22 (15)	
Capectabine/docetaxel	14	2(9)	12 (8)	
PTC	37	2(9)	35 (25)	
Surgery breast				
No surgery[a]	1	0	1 (1)	NS
Mastectomy	81	10 (44)	71 (49)	
BCS	85	13 (56)	72 (50)	

The nodal status was determined prior to chemotherapy (CT) with ultrasound guided fine-needle aspiration (FNA) and sentinel node biopsy (SNB). pNX: no pathological nodal status

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor; NS, not significant ($P > 0.05$)

Antracycline-like: cyclophosphamide doxorubicine (AC). Antracycline-taxane: AC→ capecitabine docetaxel (CD) or cyclophosphamide/ docetaxel (AD). PTC: paclitaxel/trastuzumab/ carboplatin. BCT: breast conserving surgery

[a] Due to progression mastitis carcinomatosa only radiation therapy

20% (29/144; $P = 0.015$; Fig. 1). Furthermore, we assessed chemosensitivity by separately analyzing the pathological response of the primary tumor in the breast (pCR and near-pCR). In the good prognosis-signature group, 2 of the 23 patients achieved a near-pCR of the primary tumor (9%). In the poor prognosis-signature group the pathological response was 37% (53/144; $P = 0.008$). Figure 2 shows the relation between the classification of the 70-gene profile as a continuous variable and pCR. Patients with a low MammaPrint Index have a higher probability to achieve a pCR.

The pathological response (breast and axilla) was also analyzed in subgroups that were characterized according to receptor status of the tumor (Table 2). In ER-positive (HER2-), triple-negative and HER2-positive tumors the pCR rates were different; 3% (3/88), 34% (13/38) and 32%

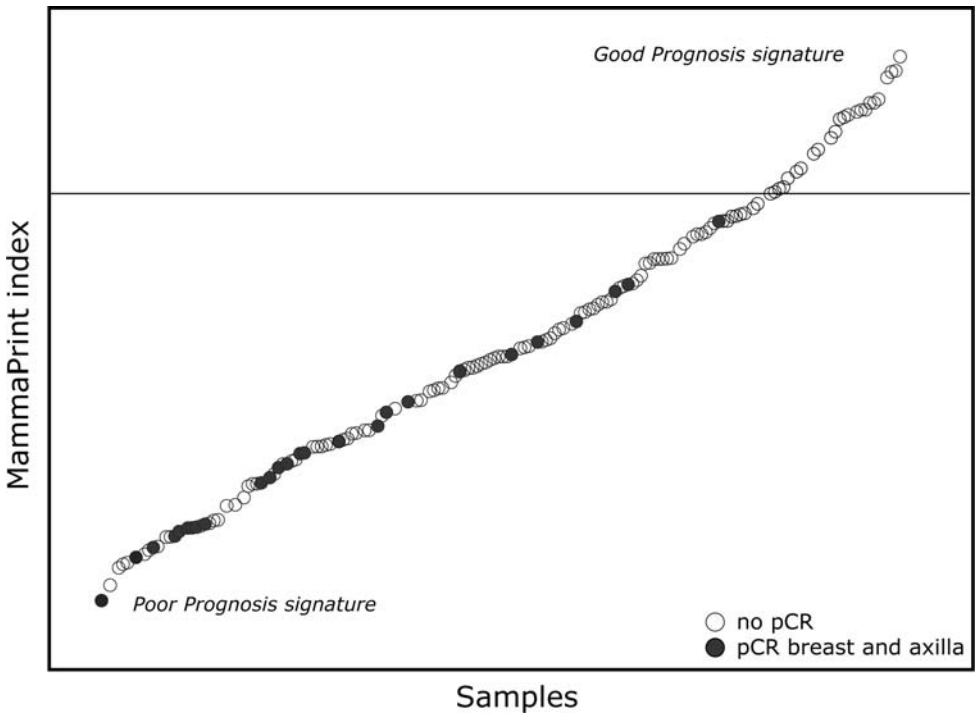

Fig. 2 The association between the classification of the 70-gene prognostic signature and the pathological response. Classification of the 70 gene prognosis signature of each sample is plotted. Tumors are ordered by their correlation to the average profile of the good prognosis group. Patients with a pCR are indicated by *black dots* and patients without a pCR are indicated by *white dots*

Table 2 The response in three subtypes characterized by receptor status

	Subtype			
	ER+/HER2- ($n = 88$) No. (%)	TN- ($n = 38$) No. (%)	HER2+[a] ($n = 41$) No. (%)	Total ($n = 167$) No. (%)
Response breast + axilla				
pCR	3 (3)	13 (34)	13 (32)	29 (17)
Response breast				
pCR	6 (7)	17 (45)	17 (42)	40 (24)
npCR	7 (8)	2 (5)	6 (15)	15 (9)

The response rate differed significantly between the subtypes. ($P < 0.001$)

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; TN, triple negative (estrogen, progesterone and HER2 negative); pCR, pathological complete remission; npCR, near pCR

[a] Four patients did not receive trastuzumab

(13/41), respectively, ($P < 0.001$). Among the ER-positive (HER2-) patients, 21/88 (24%) were classified as having a good prognosis-signature and 67/88 patients (76%) were classified as having a poor prognosis-signature (Fig. 3). None of the ER-positive patients with a good prognosis-signature achieved a pCR and all three patients who did, had a tumor with a poor prognosis-signature. Of the HER2-positive patients, 2/41 (5%) were classified as good prognosis-signature and 39/42 (95%) as having a poor prognosis-signature. Both HER2-positive patients with a good prognosis-signature were also ER-positive and both did not achieve a pCR. Among the HER2-positive patients with a poor prognosis-signature the pCR rate was 33% (13/39). None of the triple negative tumors in this study ($n = 38$) had a good prognosis-signature. We, therefore separately analyzed the predictive power of the 70 gene signature in the subgroup comprising all ER-positive and/or HER2-positive tumors. Thus, we excluded the patients with triple negative tumors which all had a poor prognosis-signature. In this non-triple negative subgroup the pCR rate in good and poor prognosis-signature tumors was 0% (0/23) and 15% (16/106), respectively, ($P = 0.07$). When the response of the primary tumor was assessed separately, a significant association between the response and the result of the 70-gene profile could be shown. Pathological response, pCR or near-pCR of the primary tumor, was achieved in 9% (2/23) of the patients with a good prognosis-signature tumor and in 32% (34/106) of the patients having a poor prognosis-signature tumor ($P = 0.023$).

After a median follow up of 25 months (range: 5–91), 17 patients had a relapse. These included local recurrences in 2 patients and distant metastases in 15 patients. None of the patients with a good prognosis-signature had a relapse. The disease-free survivals are shown in Fig. 4 ($P = 0.066$).

Discussion

To prospectively assess the predictive value of a prognostic marker, large and logistically challenging clinical trials are required, that—in case of node-negative breast

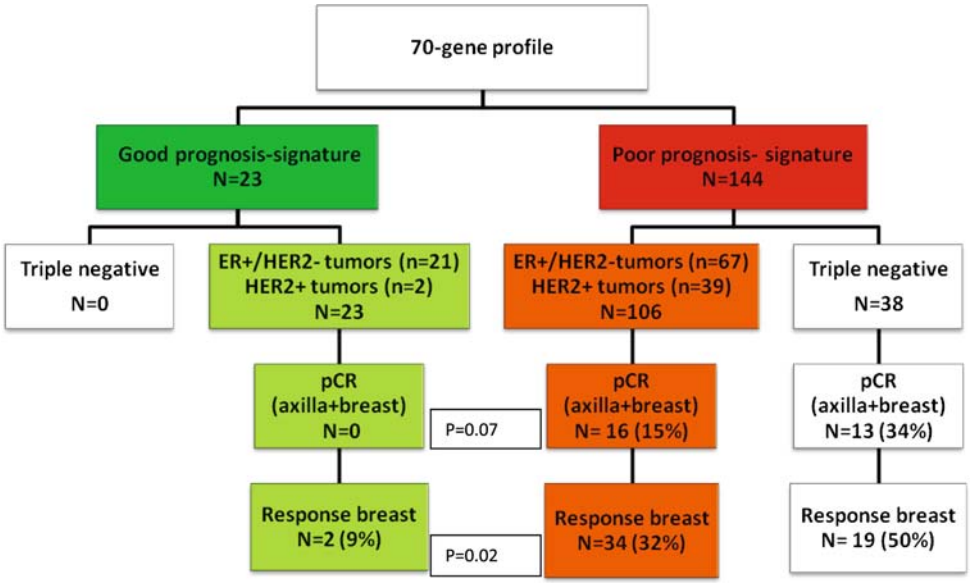

Fig. 3 The distribution of the three different subtypes characterized by receptor status within good and poor prognosis patients. All patients with triple negative tumors had a tumor with a poor prognosis-signature. We, therefore, separately analyzed the predictive power of the 70 gene signature in the subgroup including ER-positive and HER2-positive tumors. Response of the breast is defined pathological complete remission (pCR) or near pCR

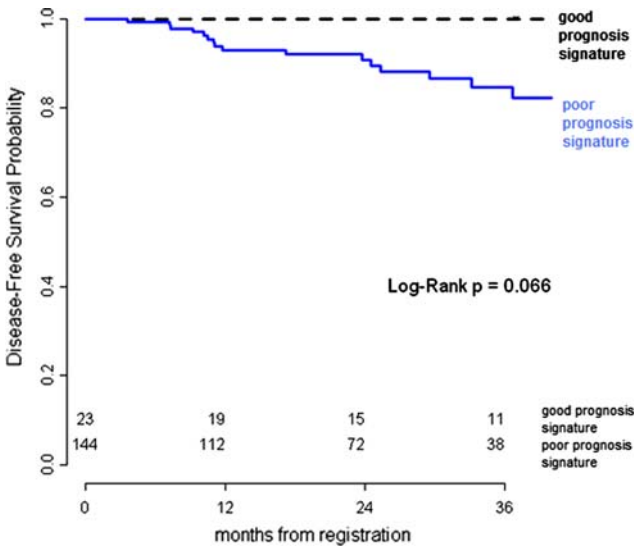

Fig. 4 The disease-free survival of the good and poor prognosis patients separately. The median follow up was 25 months and 17 relapses occurred in the poor prognosis group

cancer—may take decades to accumulate sufficient events for a useful analysis. An alternative and more rapid approach is to evaluate the predictive value of a prognostic marker for chemosensitivity in the neoadjuvant setting. Here, the pathological response can be used as endpoint, since the achievement of pCR has gained wide acceptance as a predictor of a good long-term prognosis [20, 21].

In this report a series of 167 patients with stage II and III primary invasive breast cancer who received neoadjuvant chemotherapy is described. Although several types of chemotherapy were used, most patients were treated with an anthracycline-based regimen. The proportion of tumors with a good 70-gene prognosis-signature was 14%, which is less than the percentage (38 and 41%) reported in earlier series of node-positive patients. This is likely the result of the inclusion criteria which intentionally selected clinically higher-risk patients, with larger tumors and/or axillary lymph node involvement, for neoadjuvant chemotherapy.

We observed that tumors with a poor prognostic 70-gene signature are more likely to achieve a complete response than those with a good prognostic signature. Even if the triple-negative tumor subgroup was excluded, which is usually associated with a relatively high pCR rate and in this current study with an invariably poor prognosis 70-gene profile, the response to chemotherapy remained significantly higher in the poor prognosis-signature tumors. This strongly suggests that the 70-gene profile has predictive power with respect to chemosensitivity.

The absence of ER expression and poor differentiation, tumor characteristics more often seen in the poor signature group, are generally believed to be associated with a higher likelihood of response to chemotherapy [1]. Molecular subtypes such as luminal, basal, ERBB2 and normal-like subtypes, differ markedly with respect to prognosis, with a basal-like subtype having a worse prognosis than a luminal subtype [22]. To a degree, these molecular subtypes can be also be distinguished using immunohistochemistry [23]. Carey et al. [24] classified tumors of a series of patients undergoing neoadjuvant therapy in basal-like, luminal and ERBB2-like molecular subtypes using immunohistochemical staining of the hormone and HER2 receptors. A significantly different response to neoadjuvant chemotherapy was observed in these subtypes. Basal-like tumors (i.e. triple negative) had a higher pCR rate compared to luminal subtypes which express the estrogen receptor. Our study confirms these findings, with a higher pCR rate in triple negative tumors as compared to ER-positive tumors. Since

all triple negative tumors had a poor prognosis-signature, it was expected that the pCR rate would be higher in the poor signature group. Nevertheless, the predictive value remained after exclusion of the triple negative tumors, suggesting additional predictive value for the 70-gene signature.

Another prognostic gene expression test, the 21-gene recurrence score (Oncotype DX® assay) has been correlated with pCR in the neoadjuvant setting [25]. Subsequently, Paik et al. [26] retrospectively assessed its predictive value for ER-positive patients in the adjuvant setting. They showed that the neoadjuvant result was confirmed in the adjuvant setting, as a high recurrence score was associated with relatively greater benefit from adjuvant cyclophosphamide, methotrexate, 5-flourouracil chemotherapy in patients with ER-positive, lymph node-negative disease.

Despite the proven predictive value of pCR in neoadjuvant trials, there is no consensus on the measurement of this important endpoint. Absence of tumor cells in both the axilla and breast is the most stringent definition and was therefore primarily used in this study. On the other hand, we believe that a reduction of tumor load of the primary tumor to single scattered tumor cells may also imply chemosensitivity. To gain more insight in chemosensitivity we separately assessed the responses of the primary tumors according to this frequently used definition. However, longer follow-up will be required to confirm that the eradication of a large number of tumor cells is associated with long-term survival.

In conclusion, this study shows that tumors with a poor 70-gene signature are more likely to achieve a pCR, whereas tumors with a good prognosis-signature are not. This finding has several important clinical implications. Currently, the 70 gene profile is used to select patients for adjuvant chemotherapy whereby chemotherapy is frequently withheld for tumors with a good prognosis-signature [27]. Our results add justification to this policy; not only is the absolute risk of relapse lower in these patients, but their tumors are also less sensitive to chemotherapy. For the good prognosis signature group, this may lead to a significantly lower proportional risk reduction than suggested in the Oxford overview or in the frequently used Adjuvant! Online program [28]. We also believe that the stratification of subjects according to the 70-gene profile could be helpful in controlled trials investigating the effectiveness of new drugs or new combinations of drugs.

Conflicts of interest This study was financially supported by Agendia, the commercial company that markets the 70-gene signature as Mammaprint. L. van 't Veer is a shareholder in and (part time) employed by Agendia. A.M. Glas is employed by Agendia.

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