Prognostic factors in breast cancer: one fits all?

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Mook, S. (2011). *Prognostic factors in breast cancer: one fits all?* [Thesis, fully internal, Universiteit van Amsterdam].

Chapter 5

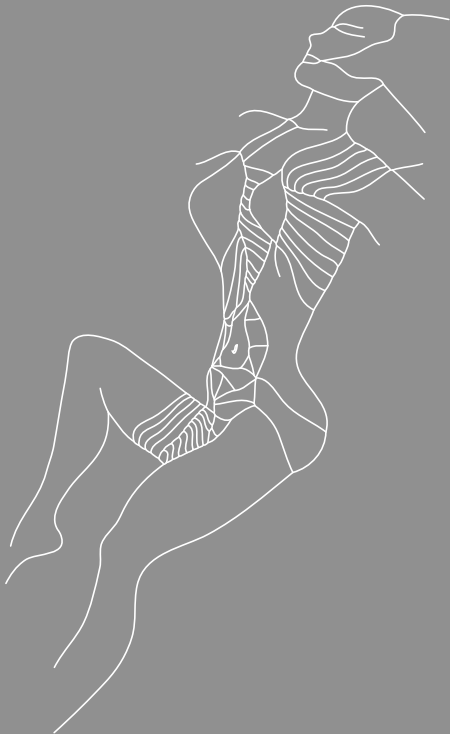# The 70-gene prognosis-signature predicts disease outcome in breast cancer patients with 1-3 positive lymph nodes in an independent validation study

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Abstract

Purpose

The 70-gene prognosis signature has shown to be a valid prognostic tool in node-negative breast cancer. Although axillary lymph node status is considered to be one of the most important prognostic factors, still 25–30% of node-positive breast cancer patients will remain free of distant metastases, even without adjuvant systemic therapy. We therefore investigated whether the 70-gene prognosis signature can accurately identify patients with 1-3 positive lymph nodes who have an excellent disease outcome.

Methods

Frozen tumor samples from 241 patients with operable T1-3 breast cancer, and 1-3 positive axillary lymph nodes, with a median follow-up of 7.8 years, were selected from 2 institutes. Using a customized microarray, tumor samples were analyzed for the 70-gene tumor expression signature. In addition, we reanalyzed part of a previously described cohort (n = 106) with extended follow-up.

Results

The 10-year distant metastasis-free (DMFS) and breast cancer specific survival (BCSS) probabilities were 91% (SE 4%) and 96% (SE 2%), respectively for the good prognosis signature group (99 patients), and 76% (SE 4%) and 76% (SE 4%), respectively for the poor prognosis signature group (142 patients). The 70-gene signature was significantly superior to the traditional prognostic factors in predicting BCSS with a multivariate hazard ratio (HR) of 7.17 (95% CI 1.81 to 28.43; $p = 0.005$).

Conclusions

The 70-gene prognosis signature outperforms traditional prognostic factors in predicting disease outcome in patients with 1-3 positive nodes. Moreover, the signature can accurately identify patients with an excellent disease outcome in node-positive breast cancer, who may be safely spared adjuvant chemotherapy.

Introduction

Axillary lymph node status is historically one of the most important prognostic factors in breast cancer, with deterioration in disease outcome as the number of positive nodes increases.[1-3] Consequently, patients with axillary lymph node metastases are considered as having a poor prognosis and hence are most likely to benefit from adjuvant chemotherapy, with an absolute benefit of 6-15% at 5 years.[4] However, up to 25-30% of node-positive patients will remain free of distant metastases even without adjuvant systemic therapy.[4,5] Thus, adjuvant treatment decision-making based on nodal status is only moderately accurate and results in overtreatment, with unnecessary exposure to treatment toxicity. Identifying robust and reliable prognostic factors that can select those node-positive patients who do not require adjuvant chemotherapy is essential to reduce overtreatment. One of the new prognostic markers which has been validated for lymph node-negative breast cancer is the 70-gene prognosis signature (MammaPrint™).[6-8] The original retrospective validation study demonstrated that the signature was also a significant prognostic factor in 144 node-positive patients.[8] The aim of this study is to further substantiate the prognostic value of the 70-gene signature in patients with 1-3 positive nodes in a new independent dataset, and to assess its relation to standard prognostic markers. Specifically, we investigated whether the 70-gene signature can select patients with 1-3 positive nodes with an excellent survival, who might be safely spared adjuvant chemotherapy.

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Methods

Patients

Patients were selected from the Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital (NKI-AVL), Amsterdam, The Netherlands (n = 213, consecutive series) and the European Institute of Oncology (EIO), Milan, Italy (n = 79, consecutive series), according to the following criteria: unilateral T1, T2 or operable T3 invasive breast carcinoma, with metastases in 1-3 axillary lymph nodes; frozen tumor tissue available; no prior malignancies, no bilateral synchronous breast tumors, and no neoadjuvant therapy. Micrometastases (tumor deposits > 0.2 and ≤ 2.0 mm) were considered as positive lymph nodes. Patients were diagnosed between 1994 and 2001 and were under the age of 71 years at diagnosis. Patients were treated with mastectomy or breast-conserving surgery, including dissection of the axillary lymph nodes (ALND), followed by radiotherapy and adjuvant systemic therapy if indicated. Adjuvant systemic therapy was administered according to national guidelines, taking into account patients' preferences and consent (*Table 1*). The proportion of adjuvant systemic therapy in our study was similar to all patients at NKI who fulfilled

the above mentioned selection criteria except for the availability of frozen tumor tissue in the same time period (data not shown). The study received approval of the medical ethical committee of NKI-AVL.

To allow more extensive analyses, follow-up data of all patients with 1-3 positive nodes from the previously described series by Van de Vijver,[8] were updated, blinded to the 70-gene prognosis signature.[9]

Tumor samples, RNA extraction and gene expression analysis

Frozen tumor samples were processed in Agendia's laboratories (Amsterdam, the Netherlands), for RNA isolation, amplification and labeling as previously described.[7,10] Samples were available for RNA isolation if they contained at least 30% tumor cells on hematoxylin/eosin stained sections. Of the 292 samples processed, 10 were rejected on the basis of RNA quality and 41 because of insufficient tumor cells. The 51 rejected samples were obtained from slightly smaller tumors than the 241 samples that were hybridized (mean tumor size 19 mm *versus* 23 mm; $p = 0.04$). However, there were no differences in age, tumor grade, ER status, systemic treatment and proportion alive after 10 years.

To assess the mRNA expression level of the 70 genes, RNA was hybridized to a custom-designed array (MammaPrint™) blinded to clinical data, at Agendia's ISO17025-certified and CLIA accredited laboratories. Tumors were classified as 70-gene good or poor prognosis signature as described previously.[6-8,10]

Clinicopathological data

Clinical data were retrieved from medical records, blinded to the 70-gene prognosis signature. Endpoints considered were time from surgery to distant metastasis as first event (DMFS), and breast cancer specific survival (BCSS), defined as time from surgery to breast cancer-related death. For the analysis of distant metastasis-free survival (DMFS) we considered distant metastases as first event as failure; patients were censored on date of local or regional recurrence, development of second primary including contralateral breast cancer, death from any cause or date of last follow-up visit. Tumor grading was defined according to the Bloom-Richardson method. Estrogen receptor (ER) status and progesterone receptor (PR) status were determined by immunohistochemistry and interpreted positive if more than 10% of the cells stained. For patients treated at NKI-AVL, HER2/NEU immunohistochemistry status was retrieved from the original pathology report. For patients treated at EIO, HER2/NEU status was determined by immunohistochemistry; in case of 2+ scores FISH analyses were used to determine amplification (ratio ≥ 2.2).

Clinical risk assessment by Adjuvant!

To assess the 70-gene prognosis signature in a clinical context, it was compared with the clinicopathological risk as predicted by Adjuvant! The Adjuvant! Software version 8.0 (available at www.adjuvantonline.com) calculated 10-year survival probability based on patient's age, co-morbidities, tumor size, tumor grade, ER-status and number of positive axillary lymph nodes.[11,12] Patients were considered as having low clinical risk when the 10-year BCSS as predicted by Adjuvant! was more than 88% for ER-positive tumors, and more than 92% for ER-negative tumors, respectively.[6]

Statistical analyses

Analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL) and EPICURE (Epicure release 2.0.Seattle: HiroSoft International Corporation, 1996). Kaplan-Meier survival plots and log-rank tests were used to assess the difference in DMFS and BCSS of the predicted good and poor prognosis groups. Cox proportional hazards regression analyses were used to calculate uni- and multivariate hazard ratios (HR) and their 95% confidence intervals (95% CI). In multivariate Cox regression analyses traditional clinicopathological variables were used. An interaction term of gene signature and chemotherapy, within a multivariate Cox regression model was tested for significance by the likelihood ratio test. *P*-values are two-sided.

Results

The 70-gene prognosis signature (MammaPrint™) was assessed in tumor tissue of an independent series of 241 invasive breast cancer patients with 1-3 positive lymph nodes.
Among the 241 patients, 99 (41%) were classified as good prognosis signature, whereas 142 (59%) patients were classified as poor prognosis signature. Patients with a poor prognosis signature were more frequently diagnosed at EIO, and had more often received adjuvant chemotherapy and less often received endocrine therapy. Moreover, tumors classified as poor prognosis signature were larger and more often poorly differentiated, ER- and PR negative, and HER2/NEU receptor positive (*Table 1*).
After a median follow-up of 7.8 years (range, 0.01-12.3) 66 patients had at least one event, including 13 local recurrences, 9 regional recurrences, 6 contralateral breast cancers, 9 second primary cancers, 43 distant metastases, including 35 distant metastases as first event, and 39 deaths of which 33 breast cancer-related deaths.

Table 1. Association between clinicopathological characteristics and the 70-gene prognosis signature for the new validation series (n = 241).

Characteristics	70-gene prognosis signature				P value*
	Good prognosis signature (n=99)		Poor prognosis signature (n=142)		
	No.	%	No.	%	
Hospital					< 0.001
NKI-AVL	84	84.8	90	63.4	
EIO	15	15.2	52	36.6	
Age (years)					0.18
<40	6	6.1	17	12.0	
40 - 49	41	41.4	61	43.0	
50 - 59	39	39.4	47	33.0	
60 - 70	13	13.1	17	12.0	
Surgery					0.17
BCT	54	54.5	90	63.4	
Mastectomy	45	45.5	52	36.6	
Axillary procedure					0.42
ALND	62	62.6	96	67.6	
SLNP & ALND	37	37.4	46	32.4	
Nodal status					0.93
1 positive node	49	49.5	74	52.1	
2 positive nodes	35	35.4	42	29.6	
3 positive nodes	15	15.1	26	18.3	
Tumor size (pTNM)					0.01
pT1 (≤ 20 mm)	58	58.6	59	41.5	
pT2 (> 20-50 mm)	40	40.4	81	57.1	
pT3 (> 50 mm)	1	1.0	2	1.4	
Histological tumor type					< 0.001
Ductal	72	72.8	132	93.0	
Lobular	12	12.1	3	2.1	
Mixed	14	14.1	3	2.1	
Other	1	1.0	4	2.8	
Histological grade					< 0.001
Good	45	46.4	12	8.5	
Moderate	46	47.4	53	37.3	
Poor	6	6.2	77	54.2	
Unknown	2		0		

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Characteristics	70-gene prognosis signature					P value*
	Good prognosis signature (n=99)		Poor prognosis signature (n=142)			
	No.	%	No.	%		
Estrogen-receptor status						< 0.001
Negative	4	4.0	46	32.4		
Positive	95	96.0	96	67.6		
Progesterone-receptor status						< 0.001
Negative	16	16.5	72	50.7		
Positive	81	83.5	70	49.3		
Unknown	2		0			
HER2/NEU receptor status						< 0.001
Negative	95	97.9	103	74.6		
Positive	2	2.1	35	25.4		
Unknown	2		4			
Adjuvant systemic treatment						0.41
None	7	7.3	3	2.3		
Chemotherapy only	10	10.4	43	32.3		
Endocrine therapy only	50	52.1	41	30.8		
Both	29	30.2	46	34.6		
Unknown	3		9			
Adjuvant chemotherapy						< 0.001
No	57	59.4	44	33.1		
Yes	39	40.6	89	66.9		
Unknown	3		9			
Adjuvant endocrine therapy						0.005
No	17	17.7	46	34.6		
Yes	79	82.3	87	65.4		
Unknown	3		9			

Abbreviations: NKI-AVL, Netherlands Cancer Institute–Antoni van Leeuwenhoek hospital; EIO, European Institute of Oncology; BCT, breast-conserving therapy;

ALND, axillary lymph node dissection; SLNP, sentinel lymph node procedure.

* Missing values were not used for calculation of p –values.
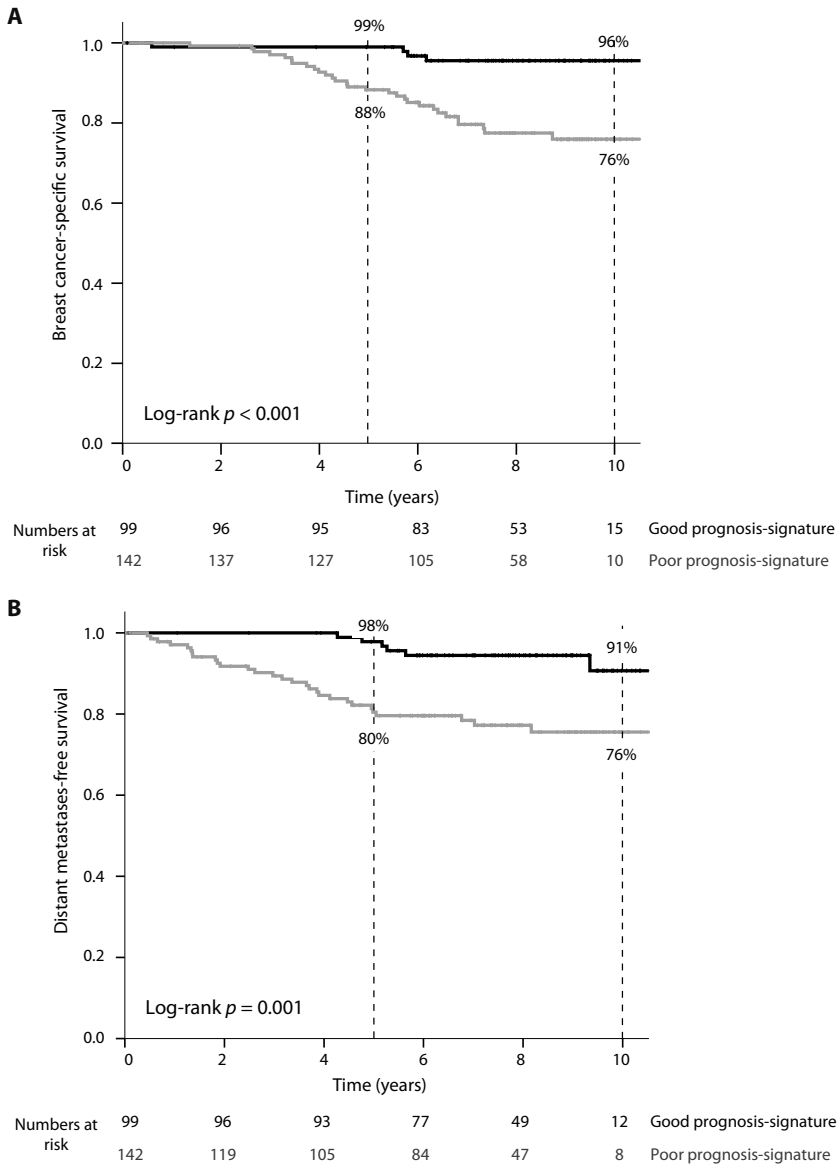
A

B

Figure 1. Kaplan-Meier curves by 70-gene prognosis signature among the 241 patients.

A) Breast cancer specific survival.

B) Distant metastasis-free survival (distant metastasis as first event).

BCSS and DMFS were significantly better in the good prognosis signature compared to the poor prognosis signature group (*Figure 1A and B*). The 5- and 10-year BCSS probabilities were 99% (SE 1%) and 96% (SE 2%), respectively for the good prognosis signature, and 88% (SE 3%) and 76% (SE 4%), respectively for the poor prognosis signature group. A poor prognosis signature was associated with shorter BCSS, with a HR of 5.70 (95% CI 2.01-16.23; *p* = 0.001) (*Figure 1A and Table 2A*). The HR for overall survival was 5.40 (95% CI 2.11-13.80; *p* < 0.001). The probabilities of remaining free of distant metastases at 5- and 10-years were 98% (SE 2%) and 91% (SE 4%), respectively for the good prognosis signature, and 80% (SE 4%) and 76% (SE 4%), respectively for the poor prognosis signature group, with a HR of 4.13 (95% CI 1.72-9.96; *p* = 0.002) (*Figure 1B and Table 2C*).

Table 2. Univariate and multivariate Cox-regression analysis for the new validation series (n=241).

a. Univariate analysis for breast cancer specific survival (BCSS)	P Value	Hazard Ratio (95% CI)
Age (years)	0.81	1.0 (0.96 – 1.04)
No. of positive nodes	< 0.001	
2 *versus* 1	0.18	0.47 (0.15 – 1.43)
3 *versus* 1	0.01	3.52 (1.70 – 7.29)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.09	1.85 (0.91 – 3.76)
Histological grade	< 0.001	
Moderate *versus* good	0.79	0.84 (0.24 – 2.97)
Poor *versus* good	0.009	4.17 (1.44 – 12.11)
Estrogen-receptor status	0.003	0.34 (0.17 – 0.69)
HER2/NEU receptor status	0.007	2.80 (1.33 – 5.89)
Surgery (mastectomy *versus* BCT)	0.62	1.19 (0.60 – 2.37)
Chemotherapy	0.29	1.47 (0.72 – 2.98)
Endocrine therapy	0.02	0.43 (0.21 – 0.85)
Prognosis-signature (poor *versus* good)	0.001	5.70 (2.01 – 16.23)

b. Multivariate analysis for BCSS*	P Value	Hazard Ratio (95% CI)
Age (years)	0.88	1.00 (0.96 – 1.05)
No. of positive nodes	< 0.001	
2 *versus* 1	0.18	0.46 (0.15 – 1.43)
3 *versus* 1	0.002	4.09 (1.71 – 9.80)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.28	1.61 (0.68 – 3.78)
Histological grade	0.13	
Moderate *versus* good	0.15	0.38 (0.10 – 1.43)
Poor *versus* good	0.99	1.00 (0.27 – 3.75)
Estrogen-receptor status	0.34	1.63 (0.61 – 4.38)
HER2/NEU receptor status	0.84	0.91 (0.35 – 2.32)
Surgery (mastectomy *versus* BCT)	0.50	1.30 (0.61 – 2.76)
Chemotherapy	0.64	0.80 (0.32 – 2.04)
Endocrine therapy	0.04	0.36 (0.13 – 0.96)
Prognosis-signature (poor *versus* good)	0.005	7.17 (1.81 – 28.43)

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Table 2. Continued

c. Univariate analysis for distant metastases as first event	P Value	Hazard Ratio (95% CI)
Age (years)	0.49	0.99 (0.95 – 1.03)
No. of positive nodes	0.02	
2 *versus* 1	0.10	0.44 (0.17 – 1.18)
3 *versus* 1	0.08	1.96 (0.93 – 4.12)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.02	2.36 (1.16 – 4.82)
Histological grade	< 0.001	
Moderate *versus* good	0.41	1.74 (0.47 – 6.41)
Poor *versus* good	0.002	6.45 (1.93 – 21.48)
Estrogen-receptor status	0.04	0.47 (0.23 – 0.96)
HER2/NEU receptor status	0.02	2.41 (1.13 – 5.14)
Surgery (mastectomy *versus* BCT)	0.16	1.60 (0.83 – 3.11)
Chemotherapy	0.25	1.51 (0.75 – 3.03)
Endocrine therapy	0.007	0.40 (0.20 – 0.78)
Prognosis-signature (poor- *versus* good)	0.002	4.13 (1.72 – 9.96)

d. Multivariate analysis for distant metastases as first event*	P Value	Hazard Ratio (95% CI)
Age (years)	0.48	0.98 (0.94 – 1.03)
No. of positive nodes	0.01	
2 *versus* 1	0.13	0.46 (0.17 – 1.27)
3 *versus* 1	0.05	2.29 (0.99 – 5.29)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.07	2.14 (0.95 – 4.81)
Histological grade	0.05	
Moderate *versus* good	0.90	1.09 (0.28 – 4.21)
Poor *versus* good	0.10	3.21 (0.79 – 13.07)
Estrogen-receptor status	0.07	2.40 (0.92 – 6.28)
HER2/NEU receptor status	0.91	0.95 (0.40 – 2.29)
Surgery (mastectomy *versus* BCT)	0.66	1.17 (0.58 – 2.39)
Chemotherapy	0.37	0.64 (0.25 – 1.69)
Endocrine therapy	0.02	0.31 (0.12 - 0.80)
Prognosis-signature (poor *versus* good)	0.05	2.99 (0.996 – 8.99)

Abbreviations: CI, confidence interval; BCT, breast-conserving therapy; ALND, axillary lymph node dissection.
[a] Multivariate models included 222 patients due to missing values in 19 patients.

Number of positive nodes (3 *versus* 1), tumor grade (poor *versus* good), ER status, HER2/ NEU status, endocrine treatment, and the 70-gene prognosis signature were significantly predictive for BCSS (*Table 2A*). In the Cox multivariate analysis (*Table 2B*), the 70-gene signature was the most powerful independent predictor for BCSS, with a HR of 7.17 (95% CI 1.81-28.43; $p = 0.005$). In addition to the signature, number of positive nodes (3 *versus* 1), and endocrine treatment were independent predictors for BCSS, with HRs of 4.09 (95% CI 1.71-9.80; $p = 0.002$) and 0.36 (95% CI 0.13–0.96; $p = 0.04$), respectively. In a multivariate model for DMFS (as first event) (*Table 2D*), only endocrine therapy was an independent prognostic

factor with an HR of 0.31 (95% CI 0.12-0.80; *p* = 0.02); the 70-gene signature and number of positive nodes (3 *versus* 1) tended to be prognostic factors with HRs of 2.99 (95% CI 0.996-8.99; *p* = 0.051) and 2.29 (95% CI 0.99-5.29; *p* = 0.053), respectively.

Adjuvant! classified 32 patients (13%) as clinical low risk and 209 patients (87%) as clinical high risk, using the pre-defined cut-off (See methods). The clinical risk assessment was discordant with the genomic risk by the 70-gene prognosis signature for 77 patients (32%); 5 were classified as clinical low risk and poor prognosis signature; 72 were classified as clinical high risk and good prognosis signature. Remarkably, in the 27 patients defined as both 70-gene good prognosis and clinical low risk none of the patients developed distant metastases nor died (*Figure 2*). Moreover, when the clinical high risk group (n = 209) was stratified by signature risk, the 10-year BCSS probability was 94% (SE 3%) for the good prognosis signature group and 76% (SE 4%) for the poor prognosis signature group, respectively [HR of 4.12 (95% CI 1.45-11.76; *p* = 0.008)]. This shows the additional value of the 70-gene prognosis signature up to and above the Adjuvant! risk assessment.

Interestingly, the 70-gene signature was also predictive for BCSS in the 101 chemotherapy naïve patients (HR 7.33; 95% CI 1.61-33.49; *p* = 0.01), 128 chemotherapy-treated patients (HR 4.70; 95% CI 1.09-20.17; *p* = 0.04) (*Supplementary Figure 3*), 63 endocrine therapy naïve patients (HR ∞ (infinity); 95% CI 2.97-∞; *p* = 0.001), and 166 endocrine therapy-treated patients (HR 3.63; 95% CI 1.21-10.94; *p* = 0.02). Moreover, the 70-gene signature accurately predicted BCSS in the 191 patients with ER-positive tumors (HR 9.75; 95% CI 2.26-42.01; *p* = 0.002). The group of 50 ER-negative patients of whom 4 were classified as good prognosis signature, and the 10 adjuvant untreated patients, were too small to analyze separately.

Among the 241 patients, 29 had solely micrometastatic axillary lymph node involvement (22 patients in 1 node, 6 in 2 nodes, and 1 in 3 nodes, respectively) and 18 patients had micrometastatic involvement in addition to macrometastases. The 70-gene signature maintained its prognostic value when nodes with micrometastases were excluded (multivariate HR for BCSS 6.68; 95% CI 1.65-27.08; *p* = 0.008).

The previously described validation of the 70-gene signature by Van de Vijver *et al.*, included 144 node-positive patients with no restriction to number of positive nodes.[8] To be able to do more extensive analyses we selected all patients with only 1-3 positive nodes from this series (n = 106).[8] Follow-up was updated from a median of 7.4 years to 10.3 years (range, 1.6 to 21.2 years).[9] This patient series was significantly different from our here described new series, with regard to age (median age 45 *versus* 50 years, respectively; *p* < 0.001), axillary procedure (all ALND), adjuvant systemic therapy and survival probabilities (*Supplementary Tables 3 and 4*). Most differences can be attributed to the fact that these patients were selected to be younger than 53 years and were diagnosed at earlier calendar years (before 1995) when sentinel lymph node procedure was not available, and adjuvant systemic treatment guidelines were not as comprehensive as today. The 10-year BCSS probability was 98% (SE 2%) for the good prognosis profile (43 patients), and 64% (SE 6%) for the poor prognosis profile group (63 patients), respectively. In this series a poor prognosis signature was also

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associated with shorter BCSS, with a univariate HR of 6.60 (95% CI 1.97-22.10; $p = 0.002$) and a multivariate HR (adjusted for the same variables as listed in *table 2*) of 3.63 (95% CI 0.88-14.96; $p = 0.07$).
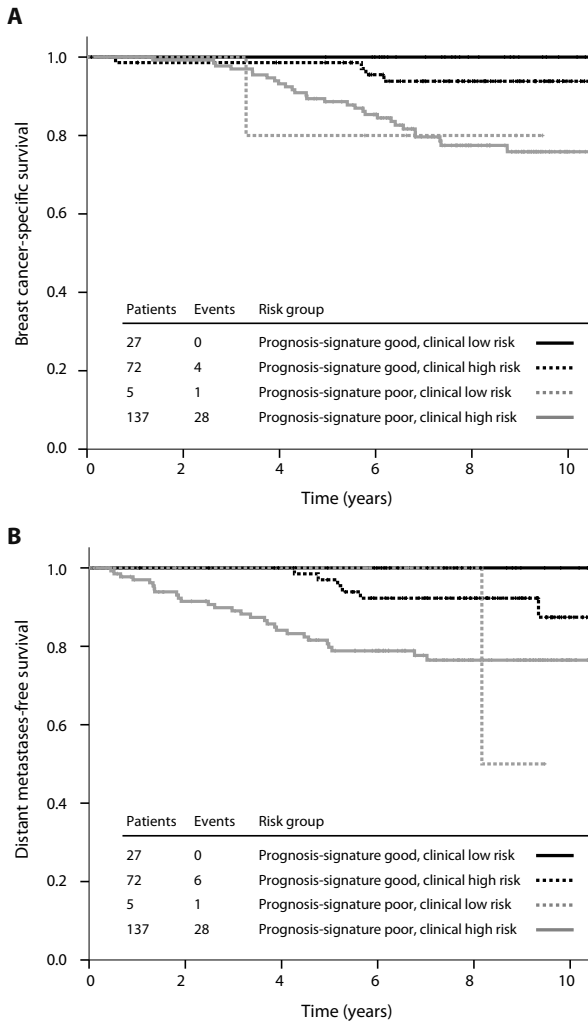
A

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Figure 2. Kaplan-Meier curves by 70-gene prognosis signature and clinical risk groups among the 241 patients. A) Breast cancer-specific survival. B) Distant metastasis-free survival (distant metastasis as first event).

Discussion

The present study demonstrates that molecular diagnostics can identify a group of low risk patients within node-positive breast cancer patients who are traditionally viewed as high risk for recurrence by conventional histopathological evaluation. As such, this study underscores the added value of molecular diagnostics and more specifically of the 70-gene prognosis signature in the tailoring of treatment for the individual patient.

The 70-gene prognosis signature, which was developed using tumors of lymph node-negative patients, first demonstrated its prognostic power in node-positive breast cancer in the paper by Van de Vijver *et al.*.[8] In this study, patients with one up to any number of positive nodes were included. Nevertheless, our present results are in good agreement with this previous publication: the HR for DMFS of 4.13 (95% CI 1.72-9.96; $p = 0.002$) in our series is similar to the prognostic value of the signature in the 151 node-positive patients from the Van de Vijver study (HR for DMFS 4.5; 95% CI 2.0-10.2; $p < 0.001$).[8]

In our new independent validation series both the 70-gene prognosis signature and traditional clinicopathological factors were predictive for BCSS. However, the multivariate analyses clearly demonstrate that the 70-gene signature remained the most powerful predictor for BCSS, even after adjustment for the clinicopathological factors, showing the added value of the signature.

The signature performed as a significant prognostic factor for DMFS (DM as first event) in the univariate analysis and retained this capacity at borderline significance when adjusted for clinicopathological variables. For DMFS with distant metastasis as any event the signature remained a strong independent predictor (HR 3.83; 95% CI 1.40-10.47; $p = 0.009$). In addition, in a pooled multivariate analysis of our new independent series and the 106 patients from the Van de Vijver study with extended follow-up, the HR for DMFS (as first event) for the signature remained consistent at 2.79 (95% CI 1.29-6.02; $p = 0.009$) (*Supplementary Table 5A*).

As a consequence of adjuvant treatment guidelines, a substantial proportion of patients in this validation series (128 of 241 patients) received adjuvant chemotherapy, with or without hormonal therapy. Patients classified as poor prognosis by the 70-gene signature more often received adjuvant chemotherapy (67% *versus* 41%, respectively; $p < 0.001$). Tumor characteristics in the poor signature group, *i.e.* more ER-negative and poorly differentiated, are generally believed to be associated with a higher likelihood of response to chemotherapy.[4] Moreover, Albain *et al.* recently presented data on the 21-gene recurrence score (RS) in lymph node-positive patients, showing that node-positive patients classified as high RS have more benefit from chemotherapy in addition to tamoxifen.[13] The larger efficacy of chemotherapy in combination with the larger proportion of chemotherapy-treated patients in the poor prognosis signature group would imply that the prognostic value of the 70-gene signature would potentially be higher in an untreated group. To further investigate this, we performed subgroup analyses in the chemotherapy-treated

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and untreated group, and confirmed similar prognostic power in each subgroup (HRs 4.85 and 5.99, respectively). To determine potential heterogeneity of the prognostic value of the signature among the chemotherapy-treated and untreated group, we also performed a multivariate analysis including an interaction variable between the signature and chemotherapy. In this multivariate analysis of our series and the 106 patients from the Van de Vijver study combined, the 70-gene prognosis signature maintained its prognostic value for BCSS (HR 5.50; 95% CI 1. 47-20.62; $p = 0.01$), while the interaction term did not reach significance ($p = 0.95$), showing no signal of potential difference in prognostic value in the two groups (*Supplementary Table 5B*).

The clinical utility of the 70-gene signature depends on its potential value in addition to traditional prognostic factors. Therefore, we compared the signature to clinicopathological risk assessment, by Adjuvant!.[11,12] As anticipated, Adjuvant! classified the majority of these node-positive patients as high clinical risk (87%). Interestingly, the 70-gene prognosis signature classified 72 (34%) clinical high risk patients as good prognosis and indeed the disease outcome in this discordant group (clinical high risk, good prognosis signature) was remarkably good, with a 10-year BCSS of 94%, indicating that the use of this signature could result in a substantial reduction of patients who would be recommended for chemotherapy, without jeopardizing outcome.

Although several prognostic markers have been studied in breast cancer, the majority of these markers have not been studied in node-positive breast cancer,[14,15] or lack prognostic value in node-positive disease.[16] Some previously identified markers do have prognostic value in node-positive breast cancer, however, since they do not identify a substantial group of patients with an excellent disease outcome, the clinical relevance as prognostic marker for this node-positive patients' group seems to be limited.[13,17,18] The only other signature that could identify a low risk group with a sufficiently good outcome within node-positive patients was the wound signature.[9] Since this wound signature is not available as a diagnostic test, its value for clinical practice seems to be limited at this moment.

The strong prognostic power of the signature with respect to distant metastases (hematogenous spread), regardless of nodal involvement, suggests that the molecular mechanism of hematogenous metastases leading to distant metastases is different from that of lymphogenic metastases leading to regional metastases.[19] As stated by Fisher 'lymph node metastases seem to be only "indicators" and not "instigators" of metastatic disease'.[20] With the strong prognostic information provided by the 70-gene signature, axillary staging might become less important for guiding adjuvant treatment. Since the signature accurately classifies as many as 41% of patients with 1-3 positive nodes as good prognosis, application of the 70-gene prognosis signature could result in a safe reduction of chemotherapy treatment in up to 41% of these patients. The distant relapse rate of 3% at 10 years in chemotherapy-untreated patients who were classified as good prognosis by the 70-gene signature (data not shown), further substantiate that withholding chemotherapy in this group seems justified, and implies a major change in the treatment of node-positive breast cancer.

This independent retrospective validation study provides additional strong evidence that the 70-gene signature is a powerful predictor of disease outcome in patients with 1-3 positive nodes, both in chemotherapy-treated and untreated patients. Based on the results of this study the inclusion criteria of the MINDACT trial (EORTC 10041 BIG 3-04), which is currently prospectively validating the 70-gene signature in node-negative patients, will be enlarged to include patients with 1-3 positive nodes.[21] Furthermore, our validation study shows that the signature adds independent prognostic information to that provided by traditional clinicopathological factors and can accurately identify patients with node-positive breast cancer and an excellent disease outcome, which would allow a more tailored approach for adjuvant systemic therapy in this patient group.

Acknowledgements

Conflicts of Interest

Laura J Van 't Veer is a named inventor on a patent application for MammaPrint™ and reports holding equity in Agendia B.V. Arno Floore and Annuska M Glas are employees of Agendia B.V..

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Supplements Chapter 5

Supplementary table 3. Clinical and pathological characteristics of new validation series (NKI-EIO; n=241) and of the 106 patients selected from the Van de Vijver study (Ref. Van de Vijver et al, *NEJM* 2002).

Characteristics	NKI-EIO n = 241		Van de Vijver n = 106		*P* value*
	No.	%	No.	%	
Age (years)					< 0.001
Mean (SD)	50 (7)		45 (5)		
<40	23	9.5	16	15.0	
40 - 49	102	42.4	68	64.2	
50 - 59	86	35.7	22	20.8	
60 - 70	30	12.4	0	.0	
Surgery					0.48
BCT	144	59.8	59	55.7	
Mastectomy	97	40.2	47	44.3	
Axillary procedure					< 0.001
ALND	158	65.6	106	100.0	
SLNP & ALND	83	34.4	0	.0	
Nodal status					0.78
1 positive node	123	51.0	58	54.7	
2 positive nodes	77	32.0	27	25.5	
3 positive nodes	41	17.0	21	19.8	
Tumor size (pTNM)					0.23
pT1 (≤ 20mm)	117	48.5	65	61.3	
pT2 (> 20 - 50mm)	121	50.3	41	38.7	
pT3 (> 50mm)	3	1.2	0	.0	
Histological tumor type					NA
Ductal	204	84.6	0	.0	
Lobular	15	6.2	0	.0	
Mixed	17	7.1	0	.0	
Other	5	2.1	0	.0	
Unknown	0		106	100.0	
Histological grade					0.07
Good	57	23.8	35	33.0	
Moderate	99	41.5	41	38.7	
Poor	83	34.7	30	28.3	
Unknown	2		0		
Estrogen-receptor status					0.56
Negative	50	20.7	18	18.0	
Positive	191	79.3	82	82.0	
Unknown	0		6		

Supplementary table 3. Continued

Progesterone-receptor status					0.40
Negative	88	36.8	32	32.0	
Positive	151	63.2	68	68.0	
Unknown	2		6		
HER2/NEU receptor status					0.92
Negative	198	84.3	83	83.8	
Positive	37	15.7	16	16.2	
Unknown	6		7		
Adjuvant systemic treatment					< 0.001
None	10	4.4	19	17.9	
Chemotherapy only	53	23.1	63	59.4	
Endocrine therapy only	91	39.7	13	12.3	
Both	75	32.8	11	10.4	
Unknown	12		0		
Adjuvant chemotherapy					0.02
No	101	44.1	32	30.2	
Yes	128	55.9	74	69.8	
Unknown	12		0		
Adjuvant endocrine therapy					< 0.001
No	63	27.5	82	77.4	
Yes	166	72.5	24	22.6	
Unknown	12		0		
Prognosis-signature					0.93
Good prognosis-signature	99	41.1	43	40.6	
Poor prognosis-signature	142	58.9	63	59.4	

Abbreviations: BCT, breast-conserving therapy; ALND, axillary lymph node dissection; SLNP, sentinel lymph node procedure.

* Missing values were not used for calculation of p-values.

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Supplementary table 4. Association between clinicopathological characteristics and the 70-gene prognosis-signature for 106 patients selected from the Van de Vijver study (Ref. Van de Vijver *NEJM* 2002).

| Characteristics | 70-gene prognosis-signature | | | | *P* value* |
| | Good prognosis signature (n=43) | | Poor prognosis signature (n=63) | | |
	No.	%	No.	%	
Age (years)					0.89
<40	4	9.3	12	19.1	
40 - 49	32	74.4	36	57.1	
50 - 55	7	16.3	15	23.8	
Surgery					0.98
BCT	24	55.8	35	55.6	
Mastectomy	19	44.2	28	44.4	
Axillary procedure					NA
ALND	43	100.0	63	100.0	
SLNP & ALND	0	.0	0	.0	
Nodal status					0.82
1 positive node	24	55.8	34	54.0	
2 positive nodes	11	25.6	16	25.4	
3 positive nodes	8	18.6	13	20.6	
Tumor size (pTNM)					0.14
pT1 (≤ 20mm)	30	69.8	35	55.6	
pT2 (> 20 - 50mm)	13	30.2	28	44.4	
Histological tumor type					NA
Unknown	43	100.0	63	100.0	
Histological grade					< 0.001
Good	24	55.8	11	17.5	
Moderate	18	41.9	23	36.5	
Poor	1	2.3	29	46.0	
Estrogen-receptor status					0.03
Negative	3	7.5	15	25.0	
Positive	37	92.5	45	75.0	
Unknown	3		3		
Progesterone-receptor status					< 0.001
Negative	4	10.0	28	46.7	
Positive	36	90.0	32	53.3	
Unknown	3		3		
HER2/NEU receptor status					0.27
Negative	34	89.5	49	80.3	
Positive	4	10.5	12	19.7	
Unknown	5		2		

Supplementary table 4. Continued

	70-gene prognosis-signature				
	Good prognosis signature (n=43)		Poor prognosis signature (n=63)		*P* value*
Adjuvant systemic treatment					0.28
None	6	14.0	13	20.6	
Chemotherapy only	26	60.4	37	58.8	
Endocrine therapy only	4	9.3	9	14.3	
Both	7	16.3	4	6.3	
Adjuvant chemotherapy					0.20
No	10	23.3	22	34.9	
Yes	33	76.7	41	65.1	
Adjuvant endocrine therapy					0.55
No	32	74.4	50	79.4	
Yes	11	25.6	13	20.6	

Abbreviations: BCT, breast-conserving therapy; ALND, axillary lymph node dissection; SLNP, sentinel lymph node procedure.

* Missing values were not used for calculation of *p*- values.

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Supplementary table 5. Multivariate Cox-regression analyses for new validation series and 106 patients of Van de Vijver study combined; n=320.

a. Multivariate analysis for DM as first event		n=320*
Variable	***P* Value**	**Hazard Ratio (95% CI)**
Age (years)	0.11	0.97 (0.93 - 1.01)
No. of positive nodes	0.05	
2 *versus* 1	0.48	0.77 (0.38 – 1.58)
3 *versus* 1	0.05	1.88 (1.01 – 3.49)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.16	1.52 (0.85 – 2.73)
Histological grade	0.14	
Moderate *versus* good	0.83	1.10 (0.46 – 2.62)
Poor *versus* good	0.13	2.08 (0.81 – 5.35)
Estrogen-receptor status	0.60	1.19 (0.62 – 2.30)
HER2/NEU receptor status	0.98	0.99 (0.51 – 1.92)
Surgery (mastectomy *versus* BCT)	0.33	1.31 (0.76 – 2.26)
Chemotherapy	0.03	0.50 (0.27 – 0.94)
Endocrine therapy	0.001	0.36 (0.19 – 0.67)
Prognosis-signature (poor *versus* good)	0.009	2.79 (1.29 – 6.02)

b. Multivariate analysis for BCSS including an interaction term		n=320*
Variable	***P* Value**	**Hazard Ratio (95% CI)**
Age (years)	0.35	0.98 (0.94 – 1.02)
No. of positive nodes	0.002	
2 *versus* 1	0.28	0.65 (0.29 – 1.42)
3 *versus* 1	0.006	2.34 (1.28 – 4.27)
Tumor size (> 20 mm *versus* ≤ 20 mm)	0.90	1.04 (0.57 – 1.91)
Histological grade	0.14	
Moderate *versus* good	0.77	0.87 (0.34 – 2.23)
Poor *versus* good	0.27	1.73 (0.66 – 4.60)
Estrogen-receptor status	0.79	0.91 (0.48 – 1.74)
HER2/NEU receptor status	0.90	1.04 (0.54 – 2.02)
Surgery (mastectomy *versus* BCT)	0.29	1.35 (0.77 – 2.38)
Chemotherapy	0.41	0.50 (0.10 – 2.63)
Endocrine therapy	0.01	0.42 (0.22 – 0.81)
Prognosis-signature (poor *versus* good)	0.01	5.50 (1.47 – 20.62)
Prognosis-signature * chemotherapy	0.95	0.95 (0.17 – 5.39)

Abbreviations: CI, confidence interval; BCT, breast-conserving therapy.

* Multivariate model included 320 patients due to missing values in 27 patients.

A

B
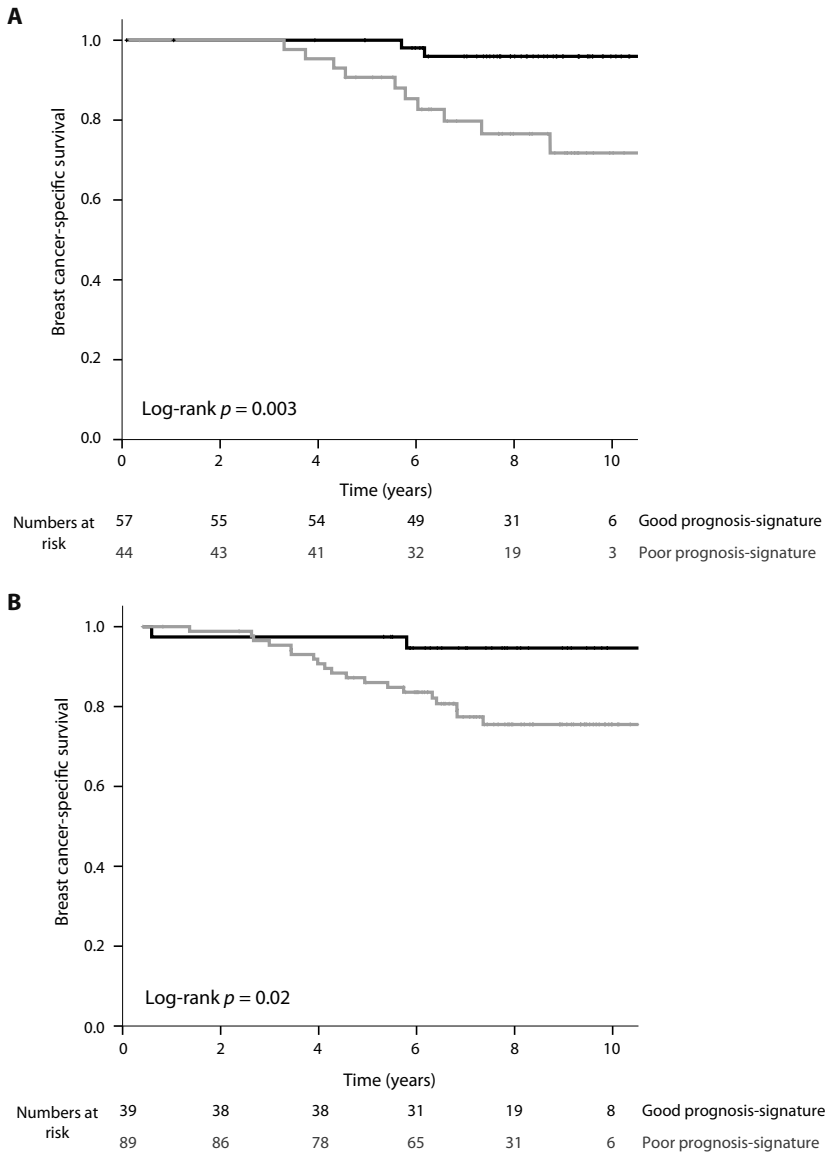
Supplementary figure 3. Kaplan-Meier curves by 70-gene prognosis-signature for breast cancer specific survival among the 241 patients.
A) Chemotherapy naïve patients (n=101)
B) Chemotherapy-treated patients (n=128).