EUROPEAN JOURNAL OF CANCER 45 (2009) 2528–2536

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Lack of TIMP-1 tumour cell immunoreactivity predicts effect of adjuvant anthracycline-based chemotherapy in patients (*n* = 647) with primary breast cancer. A Danish Breast Cancer Cooperative Group Study

Gro L. Willemoe[a,f], *Pernille B. Hertel*[b,c,f], *Annette Bartels*[b], *Maj-Britt Jensen*[d], *Eva Balslev*[e], *Birgitte B. Rasmussen*[e], *Henning Mouridsen*[c,d], *Bent Ejlertsen*[c,d], *Nils Brünner*[b,*]

[a]*Department of Pathology, Rigshospitalet, Copenhagen, Denmark*
[b]*Section of Biomedicine, Department of Disease Biology, Faculty of Life Sciences, University of Copenhagen, Ridebanevej 3, 1870 Frederiksberg C, Copenhagen, Denmark*
[c]*Department of Oncology, Rigshospitalet, Copenhagen, Denmark*
[d]*Danish Breast Cancer Cooperative Group, Copenhagen, Denmark*
[e]*Department of Pathology, Herlev University Hospital, Herlev, Denmark*

ARTICLE INFO

Article history:
Received 2 March 2009
Received in revised form 20 April 2009
Accepted 22 May 2009
Available online 15 June 2009

Keywords:
TIMP-1
Breast cancer
Resistance
Anthracyclines

ABSTRACT

Purpose: A number of prospective studies have shown that adjuvant CEF significantly improves disease-free and overall survival as compared to CMF in breast cancer patients. Our aim was to determine whether the benefit of epirubicin versus methotrexate differs according to TIMP-1 tumour cell immunoreactivity.

Experimental design: Tissue micro arrays from 647 patients randomly assigned to CMF or CEF in DBCG trial 89D were included. The primary end-point was invasive disease-free survival (IDFS). A central assessment of tissue inhibitor of metalloproteinases 1 (TIMP-1) status was performed using immunohistochemistry (IHC). Tumours were regarded as TIMP-1 positive if epithelial breast cancer cells were stained using the anti-TIMP-1 monoclonal antibody VT7.

Results: By central assessment 75% of tumours were classified as tumour cell TIMP-1 positive. Among CEF-treated patients, individuals with TIMP-1 negative tumours had a significant longer IDFS than patients with TIMP-1 positive tumours ($p = 0.047$). The multivariate Cox regression analysis of IDFS showed that CEF was superior to CMF among patients with TIMP-1 negative tumours (hazard ratio (HR) = 0.51; 95% confidence interval (CI): 0.31–0.84, $p = 0.0085$), while no significant difference could be demonstrated among patients with TIMP-1 positive tumours (HR = 0.88; 95% CI: 0.68–1.13, $p = 0.32$). A non-significant TIMP-1 status (positive or negative) versus treatment (CMF or CEF) interaction was detected for IDFS ($p = 0.06$) and OS ($p = 0.21$).

Conclusion: Lack of TIMP-1 tumour cell immunoreactivity seems to predict a favourable effect of epirubicin-containing adjuvant therapy in primary breast cancer. However, an independent study is awaited to validate the potential predictive value of TIMP-1 immunoreactivity.

* *Corresponding author:* Tel.: +45 26144708; fax: +45 35353514.
E-mail address: nbr@life.ku.dk (N. Brünner).
[f] Contributed equally to the study.

0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.05.029

1. Introduction

Chemotherapy regimens including an anthracycline were introduced into clinical practice primarily for metastatic breast cancer from the late 1970s and then in the adjuvant setting from the early 1990s.[1] In a series of meta-analyses the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) have established the importance of anthracyclines in the adjuvant setting. Compared with cyclophosphamide, methotrexate and fluorouracil (CMF) regimens, anthracycline-based adjuvant regimens significantly reduce the risk of recurrence with additional 11% and mortality with additional 16%.[2]

However, the benefit from anthracyclines is most probably restricted to a subgroup of the patients. If this subgroup could be identified, the remaining patients could avoid the additional toxicity induced by anthracyclines. Topoisomerase 2 alpha (*TOP2A*) and Human Epidermal Growth Factor 2 Receptor (*HER2*) gene aberrations have been suggested as predictive markers for anthracycline sensitivity in breast cancer.[3–7] However, neither *TOP2A nor HER2 have* as yet been taken into daily clinical decision making in general.[8,9] We observed in a previous study that high tumour tissue levels of TIMP-1 protein were associated with significantly less likelihood of obtaining an objective response to chemotherapy in patients with metastatic breast cancer.[10] Moreover, we have reported that experimental cancer cells made deficient for the TIMP-1 gene were significantly more sensitive to etoposide than their wild-type counterparts.[11] In addition to its antiproteolytic function, TIMP-1 can stimulate cell proliferation and inhibit apoptosis.[12] TIMP-1 has been shown to bind to a cell surface protein complex consisting of CD63 and beta 1 integrin.[13] By binding to this complex, TIMP-1 induces intracellular signalling through phosphorylation of PI-3 kinase, and thereby activating the Akt survival pathway, which in turn might lead to the inhibition of apoptosis including chemotherapy-induced apoptosis.[14] We therefore raised the hypothesis that breast cancer patients whose tumours contained TIMP-1 protein would be less sensitive to adjuvant chemotherapy than the patients in whom the breast cancer cells were devoid of TIMP-1 protein.

The present study was undertaken to address this hypothesis. A total of 647 paraffin embedded tumour tissue sections obtained from the patients enrolled in the DBCG 89D randomised trial were immunostained for TIMP-1 protein in order to explore whether the benefit from adjuvant chemotherapy might differentiate according to TIMP-1.

2. Materials and methods

2.1. Patients and methods

We followed the REMARK recommendations[15] wherever applicable.

The design of the original DBCG 89D clinical trial and the biological sub-study has previously been described.[5,16] Briefly, DBCG trial 89D was an open-labelled randomised, phase III trial comparing CEF (Cyclophosphamide, Epirubicin and Fluorouracil) against CMF (Cyclophosphamide, Methotrexate and Fluorouracil). Eligible for the 89D trial were patients with node positive (or tumour size $\geqslant$5 cm) and hormone receptor negative breast cancer, and premenopausal patients with node negative and malignancy grade II or III tumours. All patients gave an informed consent to the trial. The DBCG 89D trial did not include patients with node positive, hormone receptor positive tumours. These patients were included in trials with endocrine treatment.[17] The DBCG prepared the original protocol as well as the biomarker supplements and The Danish National Committee on Biomedical Research Ethics approved the original protocol as well as the supplements before their activation (V.200.1616/89, KF 12 295 003).

2.1.1. Pathology assessments

The pathological procedure included classification of histological type according to WHO, examination of tumour margins, invasion into skin or deep fascia, measurement of gross tumour size, number of metastatic and total number of lymph nodes identified. All invasive ductal carcinomas were graded for malignancy.[18] All sections have subsequently been analysed centrally for ER by immunohistochemistry and these centrally obtained ER data were used in the present analyses. Tumours with $\geqslant$10% stained tumour cells were considered ER positive.[19]

2.1.2. Retrospective collection of archival tumour tissue and construction of TMA's

From June 1990 to January 1998, 1224 patients were randomised in the DBCG trial 89D and 980 of these were recruited in Denmark. Archival paraffin embedded tissue blocks from 806 Danish patients enrolled in the trial were collected between September 2001 and August 2002 from the study sites and were stored centrally (Fig. 1). Tissue Micro Arrays (TMAs) were successfully constructed from 707 of 797 blocks still assessable by means of a TMA-builder from Histopathology Ltd. (AH-diagnostics, Denmark). A target area was identified in the donor block on haematoxylin stained sections and two 2 mm tissue cores were transferred to the recipient TMA block. For orientation the upper corners were marked using cores of kidney tissue.[20] For the present study, a total number of 659 tumours were available for TIMP-1 analysis. The lack of tumours (659–707) was due to their prior use in other studies resulting in no left over tissue for the present study.

2.1.3. TIMP-1 immunostaining

The mouse monoclonal antibody (clone VT7) raised against recombinant human TIMP-1 was included. We have previously validated this antibody for immunostaining.[21] The VT7 antibody is of the IgG_1 subtype and was used in the concentration 0.25 μg/ml. In addition, an irrelevant IgG_1 monoclonal antibody (anti-TNP) raised against tri-nitro-phenol hapten was used as a control. For each immunohistochemical experiment, a positive control case (human mammary carcinoma known to contain TIMP-1) was included.

Reagents used for IHC staining were obtained from Dako A/S and were used according to the manufacturer's instructions.

In brief, paraffin sections (4 μm) were dewaxed in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was carried out by boiling the sections for 10 min

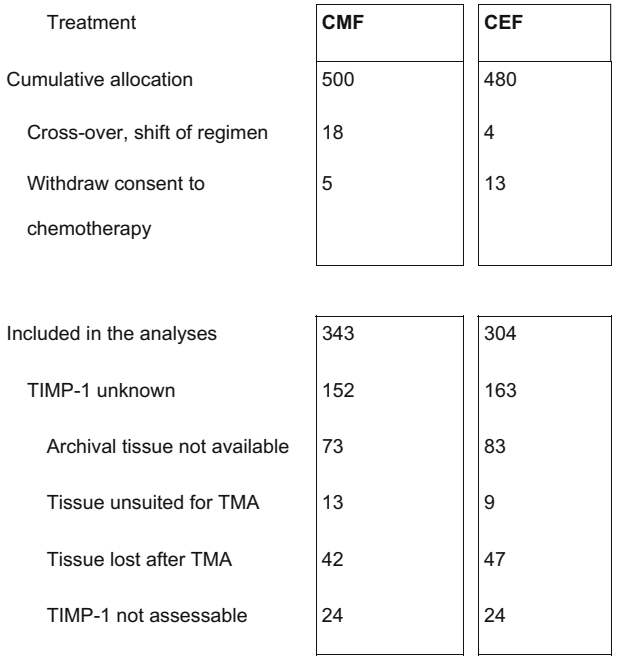

Fig. 1 – Diagram showing the patient flow.

in a conventional microwave oven in 10 mM citrate buffer pH 6.00 followed by 30 min in the hot buffer at room temperature. To block endogenous peroxidase activity, the sections were treated with 1% hydrogen peroxide for 10 min. Sections were incubated with primary antibody overnight at 4 °C. The monoclonal antibodies were detected with Advance HRP (Code No. K4068), and the reactions were visualised by incubating the sections with DAB+ (Code No. K5007) for 5 min. Washes between incubations were carried out with TBS containing 0.5% Triton X-100, pH 7.6. The sections were counterstained with Mayer's haematoxylin, and all staining procedures were performed manually.

Immunostaining of tissue sections was assessed semiquantitatively using + and – symbols as a measure of TIMP-1 immunoreactivity in the epithelial breast cancer cells. Scoring of the intensity of the signal was not included. The scoring of the tissue sections was performed blinded by two independent pathologists (GW and EB). In case of discrepancies, agreement was reached by looking at the slides together.

2.1.4. *Statistical methods*

The immunostaining results were transferred to the DBCG secretariat for statistical analyses.

Follow-up time was quantified in terms of a Kaplan–Meier estimate of potential follow-up.[22] IDFS (Invasive Disease-Free Survival) was the primary and OS (Overall Survival) the secondary end-point. IDFS was defined as the elapsed time from randomisation until invasive breast cancer recurrence irrespective of localisation, second primary invasive cancer or death attributable to any cause. OS was defined as the elapsed time from randomisation until death attributable to any cause.[23] IDFS and OS were analysed using Kaplan–Meier estimates and the log rank test. The effect of treatment regimen as well as centrally assessed TIMP-1 on IDFS and OS was quantified in terms of the hazard ratio, estimated unadjusted and adjusted using the Cox proportional hazards model. The multivariate Cox proportional hazards model was also applied to investigate the interaction of treatment and TIMP-1 using the Wald test. The multivariate model included TIMP-1, menopausal status, tumour size, positive lymph nodes, histological type and grade, central ER hormone receptor status, treatment regimen and interaction terms of TIMP-1 and treatment. The proportional hazard assumptions were not fulfilled for histological type and grade and ER receptor status, and these were included in the model as stratification variables. Differences between patients with and without information about biomarkers, between treatment regimens, and correlations between TIMP-1 status and clinico-pathological variables were tested by χ^2-test excluding unknowns. p-Values are two-tailed. Statistical analyses were done with the SAS 9.1 program package.

3. Results

The total number of tumour samples investigated was 659, among whom 12 did not receive CMF or CEF, resulting in a final number of 647 patients for subsequent analyses. Three hundred and fifty seven of these patients received CMF and 290 patients received CEF. Fig. 1 shows the flow of the original patients enrolled in the Danish part of DBCG 89D study and how we ended up with a total of 647 patients to be included in the final analyses. At the time of the present analyses (1st August 2007), 308 (48%) have died and 312 (48%) have had an event corresponding to IDFS. For the patients receiving CEF 123 (42%) had died and 129 (44%) had had an event corresponding to IDFS. Among CMF-treated patients, 185 (52%) had died and 183 (51%) had had an IDFS event. The median potential follow-up time with respect to IDFS was 9.8 years and 13.8 years with respect to OS.

Table 1 shows the base-line characteristics of the intention to treat population. As can be seen, patients included in the present study had significantly larger tumours ($p < 0.0001$) and significantly higher grade of malignancy ($p = 0.02$) than the remaining patients. No significant differences were found for the other classical base-line characteristics. When dividing the 647 patients into the two treatment groups (CMF versus CEF) no differences in base-line characteristics were observed, indicating that although approximately one third of the patients were lost for the present study, the included patients had retained a balanced distribution.

75% of the tumour samples showed positive TIMP-1 immunoreactivity. The pattern of immunoreactivity ranged from almost all epithelial cancer cells displaying TIMP-1 immunoreactivity (Fig. 2A) through scattered and focalised TIMP-1 immunoreactivity (Fig. 2B) (TIMP-1 positive) to total absence of TIMP-1 tumour cell immunoreactivity (not shown). In some tumours, distinct stromal cell TIMP-1 immunoreactivity was observed, but if these tumours were devoid of epithelial cancer cell TIMP-immunoreactivity, they were counted as TIMP-1 negatives (Fig. 2D). Fig. 2C is a negative control.

Table 2 shows the base-line characteristics between patients having TIMP-1 positive and patients having TIMP-1 negative tumour cells. Patients with TIMP-1 positive tumour cells had significantly more tumour positive axillary lymph

Table 1 – Base-line characteristics of the Danish intention to treat population (n = 980).

	Excluded n = 333 (34%)		Included n = 647 (66%)	
	No.	(%)	No.	(%)
Age at enrolment				
⩽39 Years	65	20	99	15
40–49 Years	165	50	316	49
50–59 Years	57	17	149	23
60–69 Years	46	14	83	13
Menopausal status				
Premenopausal	246	74	450	70
Postmenopausal	87	26	197	30
Nodal status				
Negative	121	36	233	36
1–3 positive	122	37	206	32
⩾4 positive	90	27	208	32
Tumour size[*]				
0–20 mm	179	55	253	39
21–50 mm	130	40	336	52
>50 mm	19	6	56	9
Unknown	5	2	2	0
Histologic type				
Infiltrating ductal carcinoma	313	94	602	93
Other carcinomas	17	5	44	7
Unknown	3	1	1	0
Malignancy grade (ductal carcinomas only)[**]				
Grade I	27	9	43	7
Grade II	177	57	298	50
Grade III	104	33	259	43
Unknown	5	2	2	0
Oestrogen-receptor status				
Positive	7	2	199	31
Negative	26	8	401	62
Unknown	300	90	47	7
Hormone-receptor status				
ER or PgR positive	88	26	167	26
ER and PgR negative	201	60	431	67
Unknown	44	13	49	8
Chemotherapy				
CMF	158	47	357	55
CEF	157	47	290	45
None	18	5	0	0

[*] $p < 0.0001$.
[**] $p = 0.02$.

nodes ($p = 0.02$) and significantly more ER positive tumours ($p = 0.04$). Among the TIMP-1 negative tumours ($n = 160$), the majority were ER-negative ($n = 107$). However, among the TIMP-1 positive tumours ($n = 487$) there was also a large proportion being ER-negative ($n = 294$). This suggests that even though TIMP-1 negativity primarily is found among ER-negative tumours, TIMP-1 is not a general surrogate for ER. No other differences in base-line characteristics between TIMP-1 negative/positive patients could be demonstrated.

The multivariate analysis (adjusted) included treatment arm, menopausal status, tumour size, number of positive axillary lymph nodes, histological type and malignancy grading, ER centrally measured and TIMP-1 tumour cell immunoreactivity. As stated above, the proportional hazard assumptions were not fulfilled for histological type and grade and ER receptor status, and these were therefore included in the multivariate model as stratification variables.

We first analysed the effect on IDFS and OS of CEF versus CMF in the 647 patients included in the present study. Thus, TIMP-1 immunoreactivity in the cancer cells was not taken into consideration. Patients who received CEF had a superior IDFS (adjusted hazard ratio (HR) = 0.78 (95% confidence interval (CI): 0.62–0.98; $p = 0.03$) and superior OS (adjusted HR = 0.77 (95% CI: 0.61–0.97; $p = 0.03$) when compared with patients receiving CMF (not shown). These figures are not different from those of the original study (IDFS: HR = 0.76 and OS: HR = 0.73),[16] suggesting that the studied subgroup is representative of the whole study group.

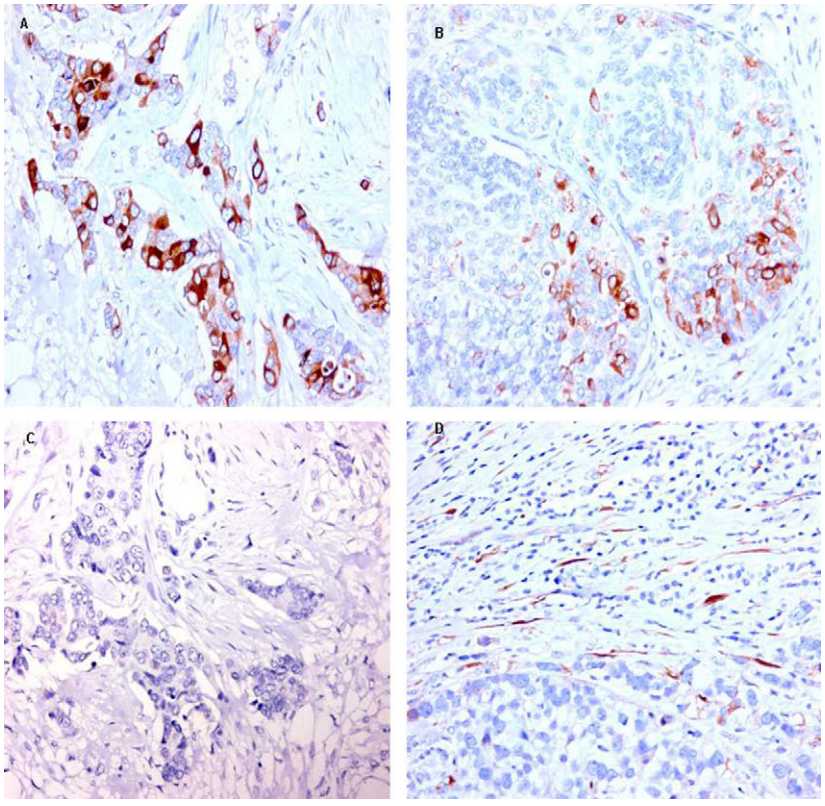

Fig. 2 – (A–D) TIMP-1 IHC. (A) A large proportion of epithelial cancer cells are TIMP-1 positive. (B) Scattered and focalised TIMP-1 immunoreactivity in epithelial cancer cells. (C) Negative control. (D) TIMP-1 immunoreactivity in fibroblasts but not in epithelial cancer cells.

We then analysed the association between TIMP-1 cancer cell immunoreactivity and IDFS and OS for the whole included patient cohort ($n = 647$). No significant differences were seen between TIMP-1 positive and TIMP-1 negative patients with regard to IDFS; unadjusted HR = 1.18 (95% CI: 0.91–1.54; $p = 0.22$) and adjusted HR = 0.95 (95% CI: 0.72–1.24; $p = 0.69$). For OS the figures were: unadjusted HR = 1.17 (95% CI: 0.89–1.53; $p = 0.25$) and adjusted HR = 0.97 (95% CI: 0.73–1.28; $p = 0.82$).

Subgroup analyses, taking the two different treatment arms and tumour cell TIMP-1 immunoreactivity into consideration, were then performed. In the CEF-treated patients ($n = 290$), individuals with TIMP-1 positive tumours had a significant shorter IDFS than patients with TIMP-1 negative tumours; unadjusted HR = 1.56 (95% CI: 1.01–2.41; $p = 0.047$) (Fig. 3A). In contrast, in the CMF-treated patients ($n = 347$), no differences in IDFS were seen between TIMP-1 positive and negative patients; unadjusted HR = 0.97 (95% CI: 0.69–1.35; $p = 0.84$) (Fig. 3A). The corresponding figures for OS were: CEF: unadjusted HR = 1.41 (95% CI: 0.91–2.18; $p = 0.13$) and CMF: unadjusted HR = 1.02 (95% CI: 0.72–1.43; $p = 0.93$) (Fig. 3B).

In the multivariate analyses, no significant differences were seen between TIMP-1 positive and TIMP-1 negative patients treated with CEF with regard to IDFS: adjusted HR = 1.30 (95% CI: 0.83–2.02; $p = 0.25$) and OS: adjusted HR = 1.21 (95% CI: 0.77–1.90; $p = 0.42$). Nor were significant differences observed in the patients treated with CMF; IDFS: adjusted HR = 0.76 (95% CI: 0.54–1.07; $p = 0.12$) or OS: adjusted HR = 0.84 (95% CI: 0.59–1.19; $p = 0.32$).

When comparing IDFS in CEF-treated patients versus CMF-treated patients in the group with TIMP-1 immunoreactive cancer cells the HR between the two treatment groups was: adjusted HR = 0.88 (95% CI: 0.68–1.13; $p = 0.32$) (Fig. 4A). The corresponding figures for OS were: adjusted HR = 0.83 (95% CI: 0.64–1.08; $p = 0.17$) (Fig. 4B). In contrast, comparing IDFS between CEF-treated patients and CMF-treated patients with lack of TIMP-1 cancer cell immunoreactivity showed an adjusted HR = 0.51 (95% CI: 0.31–0.84; $p = 0.0085$) (Fig. 4A) and OS adjusted HR = 0.58 (95% CI: 0.35–0.96; $p = 0.03$) (Fig. 4B) in favour of patients treated with CEF. A non-reduced Cox proportional hazards model was used to test for interactions between treatment effect and TIMP-1 with respect to IDFS and OS. A non-significant TIMP-1 profile (positive or negative immunoreactivity) versus treatment (CEF or CMF) interaction was detected for IDFS ($p = 0.06$) (Fig. 4A) and OS ($p = 0.21$) (Fig. 4B).

4. Discussion

This study shows for the first time that lack of TIMP-1 cancer cell immunoreactivity is associated with a favourable effect of adjuvant epirubicin-containing adjuvant therapy in primary breast cancer as compared with CMF, suggesting a predictive value of TIMP-1 immunoreactivity for anthracyclines. Compared with CMF, anthracycline-based adjuvant treatment of

Table 2 – Base-line characteristics in relation to TIMP-1.

	TIMP-1 negative (n = 160)		TIMP-1 positive (n = 487)	
	No.	(%)	No.	(%)
Age at enrolment				
⩽39 Years	26	(16)	73	(15)
40–49 Years	78	(49)	238	(49)
50–59 Years	36	(23)	113	(23)
60–69 Years	20	(13)	63	(13)
Menopausal status				
Premenopausal	118	(74)	332	(68)
Postmenopausal	42	(26)	155	(32)
Nodal status				
Negative	72	(45)	161	(33)
1–3 positive	44	(28)	162	(33)
⩾4 positive	44	(28)	164	(34)
Tumour size				
0–20 mm	62	(39)	191	(39)
21–50 mm	81	(51)	255	(52)
>50 mm	16	(10)	40	(8)
Unknown	1	(1)	1	(0)
Histologic type				
Infiltrating ductal carcinoma	146	(91)	456	(94)
Other carcinomas	14	(9)	31	(6)
Malignancy grade (ductal carcinomas only)				
Grade I	9	(6)	34	(7)
Grade II	66	(45)	232	(51)
Grade III	70	(48)	189	(41)
Unknown	1	(1)	1	(0)
Oestrogen-receptor status				
Positive	38	(24)	161	(33)
Negative	107	(67)	294	(60)
Unknown	15	(9)	32	(7)
Hormone-receptor status				
ER or PgR positive	36	(23)	131	(27)
ER and PgR negative	115	(72)	316	(65)
Unknown	9	(6)	40	(8)
Chemotherapy				
CMF	86	(54)	271	(56)
CEF	74	(46)	216	(44)

TIMP-1 negative patients significantly reduces the risk of recurrence with 49% and mortality with 42%.

The VT7 anti-TIMP-1 monoclonal antibody was previously selected among a panel of anti-TIMP-1 antibodies for its superiority regarding immunostaining.[21] VT7 recognises a linear TIMP-1 epitope located between amino acid 169–174.[24] The VT7 immunostaining was thoroughly validated with regard to sensitivity and specificity (VT7 does not bind TIMP-2, 3 or 4) and the staining conditions were optimised regarding antigen retrieval protocol, antibody concentration and time of incubation, etc.[21] In addition, the potential influence of fixation time (24–72 h) was tested.[21] On each TMA, a negative control antibody of the same IgG_1 subtype (anti-TNP) was used and a slide of a known TIMP-1 positive breast cancer was included in each assay run as a positive control.

Only minor differences were observed in the characteristics of the 647 patients included in present analyses compared to the 980 Danish patients included in the original 89D trial, which indicates that the present 647 patients are representative for the whole DBCG 89D Danish study cohort. The overall benefits reported in the original 89D trial were reproduced in the present subset, which further support that the 647 patients are representative for the entire cohort of Danish patients in the DBCG trial 89D.

We have previously published that murine fibrosarcoma cells derived from TIMP-1 gene-deficient mice are significantly more sensitive to etoposide (a topoisomerase II inhibitor) in vitro than wild-type murine fibrosarcoma cells expressing TIMP-1.[11] By applying an apoptosis assay, it was demonstrated that TIMP-1 protected the fibrosarcoma cells against apoptosis. That TIMP-1 can protect against chemotherapy-induced apoptosis has also been demonstrated by others.[25] It is at present not clear why TIMP-1 in the present study predicts sensitivity/resistance to CEF and not to CMF. Suggestions have been made regarding the signalling pathways possibly regulated by TIMP-1. In the MCF10A breast epithelial cell line

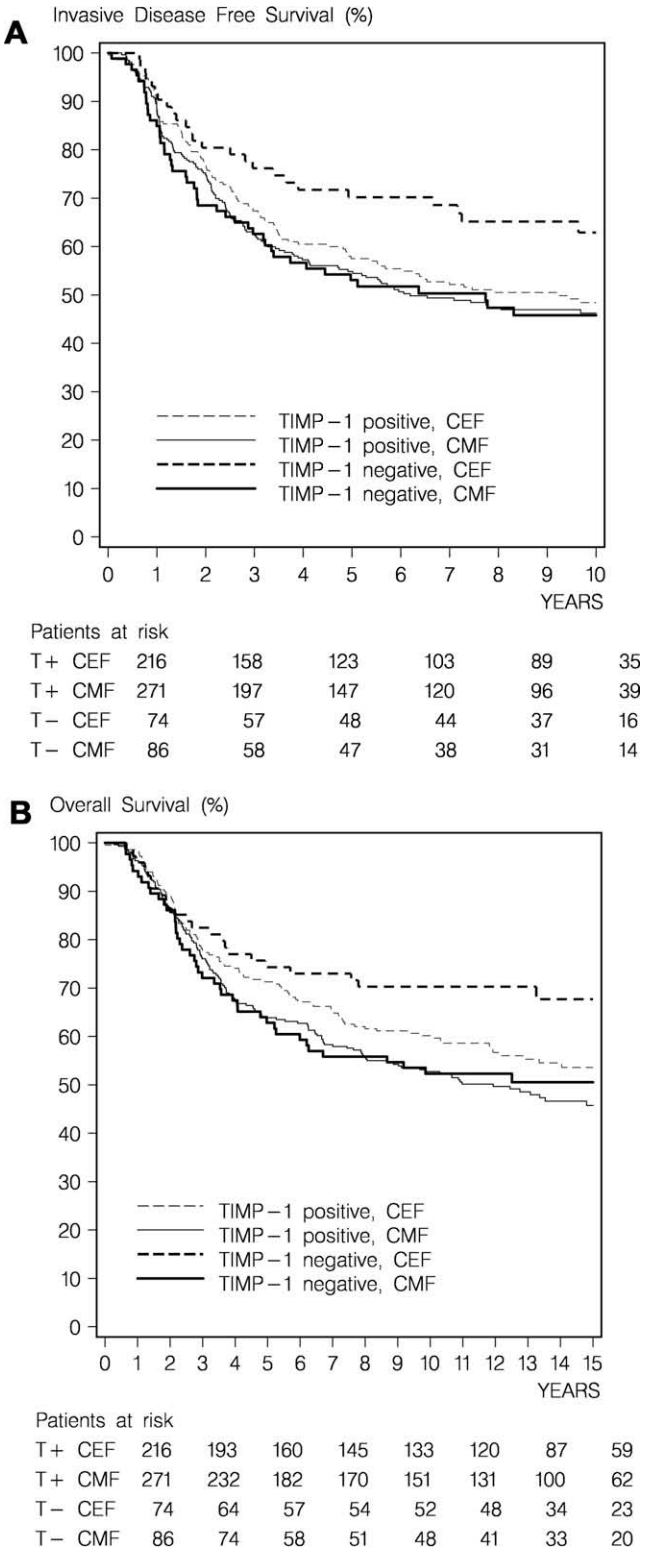

Fig. 3 – Invasive disease-free survival (IDFS) (A) and overall survival (OS) (B) probabilities for patients with known TIMP-1 status. T+ and T– means patients with and without TIMP-1 immunoreactivity in their breast cancer cells, respectively. CEF and CMF refer to received treatment.

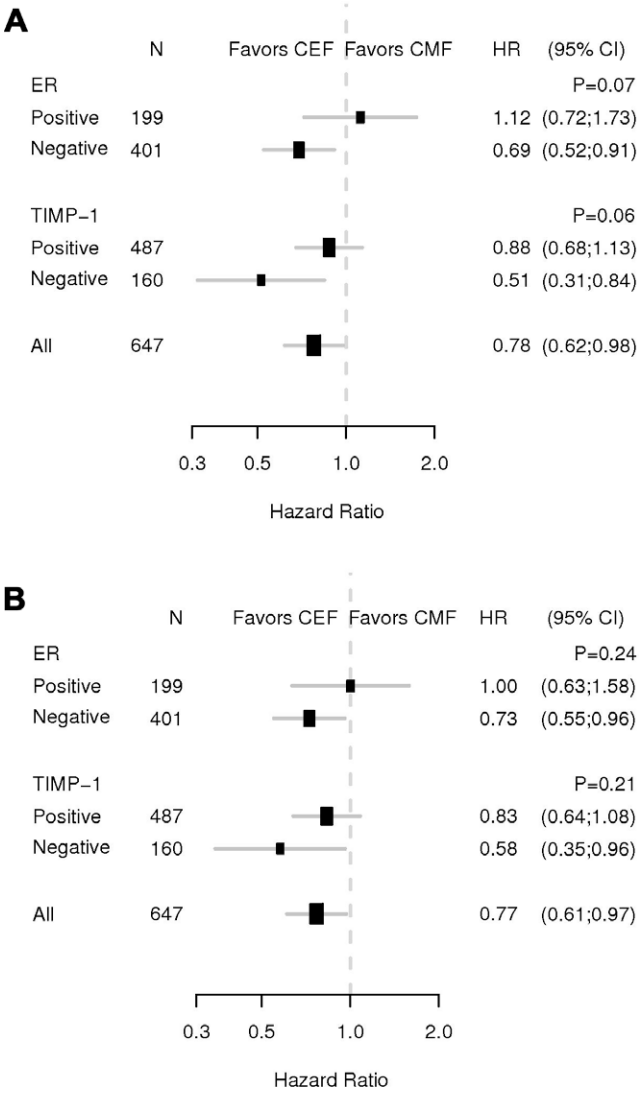

Fig. 4 – Forest plots illustrating hazard ratios from multivariate models for effect of CEF with CMF as baseline in TIMP-1 subgroups and ER subgroups of patients. (A) IDFS and (B) OS.

over-expression of TIMP-1 was shown to induce constitutive activation of focal adhesion kinase (FAK) through tyrosine phosphorylation.[25,26] FAK has previously been shown to be upstream regulator of the phosphatidylinositol-3 kinase (PI-3 kinase) leading to the regulation of the bcl-2 family members, a well-characterised signalling pathway leading to cell survival. Phosphorylated FAK associates with and thereby activates the PI-3 kinase, which in turn activates the Akt-kinase. Akt phosphorylates the protein Bad, which as a result is sequestered in the cytoplasm by the capture protein 14-3-3 and can therefore no longer interact with and inhibit bcl-2 and bcl-X_L. Bcl-2 and bcl-X_L are proteins situated in the mitochondrial membrane and when activated these anti-apoptotic proteins inhibit Bax thereby preventing the release of cytochrome c from the mitochondria. This in turn prevents the activation of the caspase cascade and accordingly prevents apoptosis. Thus, TIMP-1 may inhibit apoptosis by acting like a trophic factor initiating the survival pathway including FAK, PI-3 kinase, Akt and bcl-2 family members resulting in the inhibition of caspase activation and thereby inhibition of apoptosis.

By testing for TIMP-1 immunoreactivity in tumour tissue obtained from patients who were enrolled in the DBCG 89D trial, we have now shown that patients who lack TIMP-1 immunoreactivity in their breast cancer cells and who are treated with anthracycline-containing combination chemotherapy have a significantly better outcome than patients treated with CMF. In the multivariate analyses, patients with TIMP-1 negative tumours had a 49% reduced risk of recurrence and 42% reduced risk of death when treated with CEF rather than with CMF. These clinical results are thus yet another support for our hypothesis that the TIMP-1 protein is associated with sensitivity/resistance to anthracycline treatment. However, an independent study is awaited to confirm the significant association between TIMP-1 immunoreactivity and anthracycline sensitivity/resistance in the adjuvant setting. Moreover, we are currently comparing the TIMP-1 results with those of *HER2* and *TOP2A* gene aberration assays, both of which have been associated with sensitivity to anthracyclines.

We have previously published that the level of TIMP-1 protein in primary breast cancers carries prognostic information.[27,28] It can thus be speculated whether the observed effect of TIMP-1 immunoreactivity on IDFS is prognostic or predictive. As no effect of TIMP-1 immunoreactivity was observed among CMF patients but only among CEF-treated patients, the present results suggest that TIMP-1 immunoreactivity carries some predictive value and the present study is thus in line with our preclinical observations. In the prior prognostic studies, TIMP-1 protein was extracted from the whole tumour and the measured TIMP-1 protein could thus be derived from contaminating blood, from stromal cells, from extracellular matrix and from the cancer cells. In contrast, in the present study, only TIMP-1 protein localisation in the epithelial cancer cells was included in the final analyses, which may be another reason for the differences between the present and the previous studies.

In conclusion, the present study, demonstrates for the first time that tumours being devoid of TIMP-1 protein immunoreactivity in the epithelial cancer cells are more sensitive to anthracycline treatment than to CMF treatment. Future studies will be aimed at establishing the relationship between TIMP-1 immunoreactivity, *HER2, TOP2A* and effect of anthracyclines. Moreover, the present results will be validated in an independent patient cohort.

Conflict of interest statement

The authors do not have any personal commercial interest in TIMP-1 as a predictive biomarker in adjuvant treatment of breast cancer. University of Copenhagen is holding a patent application on this subject.

Disclaimer

These results have not been published elsewhere. The main clinical study (DBCG89D) was previously published (Ref. [16]).

Acknowledgements

This study was supported financially by the Foundation: A Race against Breast Cancer, The Danish Strategic Research Counsel, Breast Friends Foundation and Mogens Balslev Foundation.

REFERENCES

1. Mouridsen HT. New cytotoxic drugs in treatment of breast cancer. *Acta Oncol* 1990;**29**:343–7.
2. 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**:1687–717.
3. Gennari A, Sormani MP, Pronzato P, et al. HER2 status and efficacy of adjuvant anthracyclines in early breast cancer: a pooled analysis of randomized trials. *J Natl Cancer Inst* 2008;**100**:14–20.
4. Dhesy-Thind B, Pritchard KI, Messersmith H, et al. HER2/neu in systemic therapy for women with breast cancer: a systematic review. *Breast Cancer Res Treat* 2008;**109**:209–29.
5. Knoop AS, Knudsen H, Balslev E, et al. Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. *J Clin Oncol* 2005;**23**:7483–90.
6. Pritchard KI, Messersmith H, Elavathil L, et al. HER-2 and topoisomerase II as predictors of response to chemotherapy. *J Clin Oncol* 2008;**26**:736–44.
7. Di Leo A, Dowsett M, Horten B, Penault-Llorca F. Current status of HER2 testing. *Oncology* 2002;**63**(Suppl 1):25–32.
8. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol* 2007;**25**:5287–312.
9. Goldhirsch A, Wood WC, Gelber RD, et al. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol* 2007;**8**:1133–44.
10. Schrohl AS, Meijer-van Gelder ME, Holten-Andersen MN, et al. Primary tumor levels of tissue inhibitor of metalloproteinases-1 are predictive of resistance to chemotherapy in patients with metastatic breast cancer. *Clin Cancer Res* 2006;**12**:7054–8.
11. Davidsen ML, Wurtz SO, Romer MU, et al. TIMP-1 gene deficiency increases tumour cell sensitivity to chemotherapy-induced apoptosis. *Brit J Cancer* 2006;**95**:1114–20.
12. Wurtz SO, Schrohl AS, Sorensen NM, et al. Tissue inhibitor of metalloproteinases-1 in breast cancer. *Endocr Relat Cancer* 2005;**12**:215–27.
13. Jung KK, Liu XW, Chirco R, Fridman R, Kim HR. Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. *EMBO J* 2006;**25**:3934–42.
14. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. *Sci Signal* 2008;**1**:re6.
15. McShane LM, Altman DG, Sauerbrei W, et al. Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumor marker prognostic studies. *J Clin Oncol* 2005;**23**(36):9067–72.
16. Ejlertsen B, Mouridsen HT, Jensen MB, et al. Improved outcome from substituting methotrexate with epirubicin: results from a randomised comparison of CMF versus CEF in

patients with primary breast cancer. *Eur J Cancer* 2007;**43**:877–84.
17. Moller S, Jensen MB, Ejlertsen B, et al. The clinical database and the treatment guidelines of the Danish Breast Cancer Cooperative Group (DBCG): its 30-years experience and future promise. *Acta Oncol* 2008;**47**:506–24.
18. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991;**19**:403–10.
19. Andersen J, Thorpe SM, King WJ, et al. The prognostic value of immunohistochemical estrogen receptor analysis in paraffin-embedded and frozen sections versus that of steroid-binding assays. *Eur J Cancer* 1990;**26**:442–9.
20. Henriksen KL, Rasmussen BB, Lykkesfeldt AE, et al. Semi-quantitative scoring of potentially predictive markers for endocrine treatment of breast cancer: a comparison between whole sections and tissue microarrays. *J Clin Pathol* 2007;**60**:397–404.
21. Sorensen IV, Fenger C, Winther H, et al. Characterization of anti-TIMP-1 monoclonal antibodies for immunohistochemical localization in formalin-fixed, paraffin-embedded tissue. *J Histochem Cytochem* 2006;**54**:1075–86.
22. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials* 1996;**17**:343–6.
23. Hudis CA, Barlow WE, Costantino JP, et al. Proposal for standardized definitions for efficacy end points in adjuvant breast cancer trials: the STEEP system. *J Clin Oncol* 2007;**25**:2127–32.
24. Sorensen NM, Dowell BL, Stewart KD, et al. Establishment and characterization of 7 new monoclonal antibodies to tissue inhibitor of metalloproteinases -1. *Tumor Biol* 2005;**26**:71–80.
25. Li G, Fridman R, Kim HR. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer Res* 1999;**59**:6267–75.
26. Liu XW, Bernardo MM, Fridman R, Kim HR. Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells against intrinsic apoptotic cell death via the focal adhesion kinase/phosphatidylinositol 3-kinase and MAPK signaling pathway. *J Biol Chem* 2003;**278**:40364–72.
27. Schrohl AS, Pedersen AN, Jensen V, et al. Tumor tissue concentrations of the proteinase inhibitors TIMP-1 and PAI-1 are complementary in determining prognosis in primary breast cancer. *Mol Cell Proteom* 2003;**3**:164–72.
28. Schrohl AS, Holten-Andersen MN, Peters HA, et al. Tumor tissue levels of tissue inhibitor of metalloproteinase-1 as a prognostic marker in primary breast cancer. *Clin Cancer Res* 2004;**10**:2289–98.