Time Independence of the Prognostic Impact of Tumor Cell Detection in the Bone Marrow of Primary Breast Cancer Patients

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ABSTRACT

Purpose: **Tumor cell detection (TCD) in bone marrow is an outstanding prognostic factor in breast cancer. There is only one other study that has investigated more than 300 patients with a median follow-up of more than 5 years (J. L. Mansi *et al.*, Lancet, *354:* 197–202, 1999). We report data from 727 patients with a median follow-up period of 6.5 years.**

Experimental Design: **In a prospective study, intraoperatively aspirated bone marrow was screened for micrometastatic cancer cells. We used an immunocytological method (monoclonal mucin antibody 2E11; the avidin-biotin complex method).**

Results: **Forty-three percent of the patients were TCD positive. Sixty percent of the patients with distant metastases were tumor cell positive (155 of 258 patients). Forty-nine percent of the patients with positive TCD developed distant metastases (155 of 315 patients). TCD was an independent prognostic factor for clinical outcome after a median follow-up time of 6.5 years. The prognostic impact of TCD and tumor size remains constant with the time, whereas the impact of grading and progesterone receptor on risk seems to decrease with longer follow-up time.**

Conclusions: **TCD remains an independent prognostic factor The impact of TCD does not change with longer follow-up time. TCD is a reliable prognostic factor and provides important information about the process of metastasis.**

INTRODUCTION

The fate of patients with breast cancer is determined by the appearance of distant metastases. Tumor cell shedding is an important step in the process of metastasis, and the disseminated tumor cells can persist for up to 20–30 years (1). "Minimal residual disease" is diagnosed by detection of microscopic and submicroscopic tumor residues by means of molecular biological and immunological methods (2). Synonyms for minimal residual disease are TCD[2] and detection of single tumor cells, micrometastatic cells, and epithelial cells. Caution is needed with the term "micrometastases." Micrometastases are tumor cell groups smaller than 2 mm with stroma involvement that have found access to the capillary system (3, 4). A micrometastasis is diagnosed by histological analysis of a bone sample. Random perioperative bone biopsy cannot be recommended because of the low detection rate in patients with primary breast cancer (5). Individual tumor cells, as a correlate of minimal residual disease, are precursors of micrometastases. In minimal residual disease, in contrast to micrometastasis, stroma invasion has not occurred, and there was no previous metastasis. This stage is potentially curable. However, it is not possible to differentiate between cells that will die and those that carry the stigmata of metastatic potential.

Disseminated tumor cells can be detected in bone marrow smears using immunocytochemical methods (6–13). In most of studies, patients with positive TCD had a worse prognosis than patients without tumor cells in the bone marrow. It is remarkable that almost all studies found in medical literature involve small numbers of patients (<300) and short follow-up times (<5 years). There is only one long-term follow-up study that reports on the experiences of 350 patients with a median follow-up of 12.5 years (13). At the primary diagnosis, tumor cells were found in 25% of patients. TCD was associated with a shorter relapse-free survival (RR = 1.82) and with a shorter overall survival (RR = 1.72). TCD was not an independent prognostic factor in the multivariate analysis.

The largest study (n = 727) was published in 1996 by our research group. The TCD rate was 43% (n = 315). TCD was associated with shorter distant disease-free survival and shorter overall survival. The direct comparison between TCD and nodal status in patients with T_1 tumors showed TCD to be strikingly better at predicting metastases than nodal status. These results have raised the question of whether TCD can replace axillary dissection in some subgroups of breast cancer patients (*e.g.*, those with tumors smaller than 2 cm; Ref. 14). This article updates our investigation of the original 727 patients. The main

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[2] The abbreviations used are: TCD, tumor cell detection; RR, relative risk; CI, confidence interval; EMA, epithelial membrane antigen.

goal of this research was to evaluate the prognostic value of TCD after long-term median follow-up of 6.5 years.

PATIENTS AND METHODS

Patient Population. The characteristics of the patients have been described elsewhere (14). Data from 727 patients (surgery between May 1985 and July 1994) with primary breast cancer were analyzed. Exclusion criteria were visceral and/or bone metastases within 3 months after surgery, breast biopsy and/or lumpectomy before definitive surgery and bone marrow aspiration, anticancer treatment before surgery, a history of malignant disease or simultaneous second primary tumor, and incomplete follow-up data. Bone marrow samples from 21 patients without malignant disease were used as controls. The study design was examined and approved by the board of review for ethical practice. Informed consent was obtained from all participants.

Surgical and Systemic Adjuvant Treatment. Primary surgery consisted of either mastectomy and axillary lymph node dissection ($n = 263$) or breast-conserving therapy ($n = 464$; *i.e.*, lumpectomy with free margins or segmentectomy plus axillary dissection plus irradiation of the remaining breast). Five hundred and thirty-seven patients received adjuvant systemic therapy [tamoxifen, 30 mg daily ($n = 213$); goserelin, 3.6 mg monthly for 2 years ($n = 61$); chemotherapy ($n = 263$)]. One hundred and ninety patients received no further systemic treatment. Follow-up examinations were performed routinely at the outpatient clinic.

Immunocytology. The immunocytochemical staining method has been presented in detail in our previous study (14). Briefly, the aspirate was separated by density centrifugation (Ficoll), and the cell suspension ($4–5 \times 10^6$ cells) was smeared onto slides. Immunocytochemical staining was performed using murine monoclonal antibody 2E11 with the avidin-biotin complex technique. Our method is sensitive enough to detect 1 tumor cell (T47D) among 10^6 normal bone marrow cells. One negative and one positive smear were used as controls in all staining series. Four smears were analyzed per patient. The membrane and cytoplasm of the tumor cells stained bright red. Positive smears were defined as those containing one or more than one tumor cell. All slides included in our study were assessed by two independent investigators, with an interobserver agreement of 99%. Discordant findings occurred in only five cases, and the corresponding patients were eventually considered tumor cell negative. The analysis was performed without knowledge of the surgical procedure, tumor stage, and prognostic factors.

Statistical Methods. The association of TCD with established prognostic markers was analyzed by χ^2 test. Overall survival and distant relapse-free survival were analyzed. Distant disease-free survival was defined as survival without the development of distant metastases. Survival curves were calculated by the Kaplan-Meier method, and the comparison of two survival curves was based on the log-rank test according to Peto and Peto. A stepwise multivariate Cox regression analysis was performed to assess the independent prognostic value of TCD adjusted for other prognostic factors. The impact of each variable in the Cox regression model was tested by the Wald χ^2 test and described by the risk ratio (*i.e.*, the hazard ratio). All reported *P*s are two-sided.

The Cox model implies that the ratio of two hazards is independent of time, *i.e.*, the impact of each predictor included in the model does not change during the observation period, and therefore the RR regarding two levels x_i and x_j of an explanatory variable is $e^{\beta(x_i - x_j)}$.

However, it could be that this assumption does not hold for some variable included in the model. In that case, the coefficient β_i and therefore the RR are functions of time [$\beta_i = \beta_i(t)$, RR = $e^{b_{i(t)x}}$]. Two methods for revealing the time-varying effect of the predictors are applied in the present study: (*a*) a test proposal of Grambsch and Therneau (15); and (*b*) the time-varying coefficient model (Hastie and Tibshirani; Ref. 16).

The test of Grambsch and Therneau is a weighed Schoenfeld residuals score test. It assumes that the *i*th coefficient has the time-dependent form $\beta_i(t) = \beta_{0i} + \beta_{1i} f(t)$ and tests for $\beta_{1i} = 0$. The time-varying coefficient model is simply an extension of the Cox model in which the time consistency assumption on β_i is relaxed and allowed to be a function of time. A cubic spline with knots at each failure time point is fitted to assess $\beta_i(t)$. $\beta_i(t)$ is then compared to the constant coefficient assessed from the proportional harzard model. The statistical analysis was done with S-plus 4.5 (Systat, Evanstone, IL) and SPSS software.

RESULTS

TCD in Bone Marrow and Established Prognostic Factors. The median patient age was 53 years (range, 22–83 years). The characteristics of the patients are given in Table 1. Immunocytochemical TCD in bone marrow was positive in 315 patients (43%). The rate of TCD did not differ significantly among patients receiving different systemic adjuvant treatments. In the untreated low-risk group ($n = 190$), the TCD rate was only 32%. Table 1 shows the relationship between conventional prognostic markers and TCD. The prevalence of positive tumor cells is significantly higher with tumor size ($P < 0.001$), with positive nodal status ($P = 0.001$), and with tumor grade III ($P = 0.002$). Positive cell detection was more frequent in postmenopausal patients ($P = 0.010$).

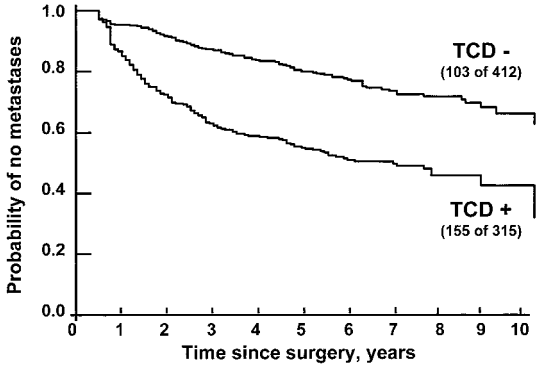
TCD in Bone Marrow and Survival Data. The median follow-up time was 77 months (range, 7–144 months). Distant metastases were diagnosed in 258 patients. One hundred and fifty-five (60%) of these patients were tumor cell positive. Interestingly, there was no difference in tumor cell positivity between patients whose primary site of metastasis was bone ($n = 91$; 57%) and those with visceral or multiple metastases ($n = 167$; 62%). Among the 119 patients with local and/or regional recurrences, 65 (55%) were tumor cell positive. Of the 169 women who died of breast cancer, 119 (70%) had micrometastatic cells in bone marrow. Distant metastases were observed in 49% of TCD-positive patients (155 of 315 patients). The other 160 TCD-positive patients (51%) showed no evidence of disease after a median follow-up of 77 months.

Patients with tumor cells in bone marrow had a shorter distant disease-free and overall survival than those who were TCD negative (Figs. 1 and 2). The RRs and their 95% CIs taken from a univariate Cox regression are RR = 2.5 (95% CI, 1.97–3.26) and RR = 3 (95% CI, 2.76–5.35), respectively.

Table 1 Clinical and pathological features of 727 patients with breast cancer in relation to tumor cell detection in bone marrow at primary surgery[a]

Prognostic marker	No. of patients	TCD				P^b
		Positive		Negative		
		No.	%	No.	%	
Tumor size						
T_1	258	77	30	181	70	<0.001
T_2	323	137	42	186	58	
T_3	69	43	62	26	38	
T_4	77	58	75	19	25	
Nodal status						
N_0	360	112	31	248	69	0.001
N_+	367	203	55	164	45	
Estrogen receptor (n = 617)						
Positive[c]	410	186	45	224	55	0.74
Negative	207	91	44	116	56	
Progesterone receptor (n = 588)						
Positive[c]	341	145	42	196	58	0.17
Negative	247	119	48	128	52	
Menopausal status						
Premenopausal	296	112	38	184	62	0.01
Postmenopausal	431	203	47	228	53	
Grade (n = 682)						
I + II	403	159	39	244	61	0.002
III	279	144	52	135	48	
S-phase fraction (n = 646)						
<5%	271	113	42	158	58	0.21
≥5%	375	175	47	200	53	
Tumor cells in bone marrow	727	315	43	412	57	

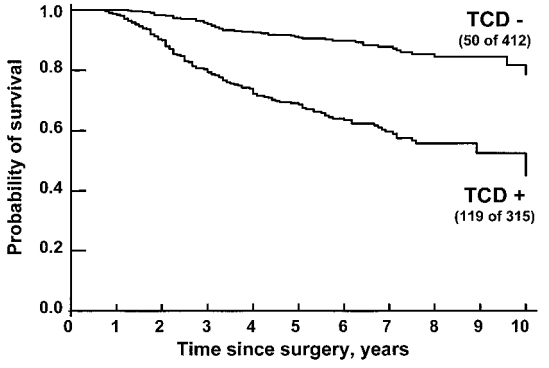
[a] Tumor staging according to UICC criteria.
[b] χ^2 test for contingency tables.
[c] Positive ≥20 fmol/mg protein.



Patients at risk

TCD -	412	391	374	348	326	265	186	132	87	41	21
TCD +	315	272	223	189	173	146	95	69	31	10	4

Fig. 1 Distant disease-free survival of patients with primary breast cancer according to the presence or absence of micrometastatic tumor cells in the bone marrow.



Patients at risk

TCD -	412	409	400	381	360	298	236	152	102	51	29
TCD +	315	308	276	239	216	182	134	86	40	14	7

Fig. 2 Overall survival of patients with primary breast cancer according to the presence or absence of micrometastatic tumor cells in the bone marrow.

Interestingly, cell detection also predicted locoregional relapse (P = 0.001; RR, 1.84; 95% CI, 1.26–2.69).

TCD was a significant independent factor for distant disease-free survival (RR, 1.41; 95% CI, 1.19–1.67; $P < 0.001$) and for overall survival (RR, 1.31; 95% CI, 1.08–1.55; P = 0.005) in Cox multivariate analysis (Table 2). The therapy was used as a stratification variable in Cox regression analysis. The stratified model was compared to a model with an interactive term from TCD and therapy as a predictor. The conclusion was that the stratified model fits the data better.

Our main interest was to investigate whether the prognostic value of TCD and other variables depends on the time after primary surgery. The results of the Grambsch and Therneau test are depicted in Table 3. Therapy is used as a covariate. No

Table 2 Results of multivariate analysis comparing TCD in bone marrow with other risk factors in patients with breast cancer[a]

Variable	*P*	RR	95% CI
A. Distant disease-free survival			
TCD (positive, negative)	<0.001	1.41	1.19–1.67
Grade (I + II, III)	<0.001	1.38	1.16–1.64
Nodal status (N_0, $N_{1–3}$, $N_{4–9}$, $N_{>9}$)	<0.001	1.25	1.15–1.37
Tumor size (T_1, T_2, T_3, T_4)	0.070	1.22	0.98–1.52
B. Overall survival			
Progesterone receptor (positive,[b] negative)	0.006	1.29	1.08–1.55
TCD (positive, negative)	0.005	1.31	1.08–1.58
Grade (I + II, III)	0.024	1.25	1.03–1.51
Nodal status (N_0, $N_{1–3}$, $N_{4–9}$, $N_{>9}$)	<0.001	1.23	1.12–1.35
Tumor size (T_1, T_2, T_3, T_4)	0.089	1.10	0.98–1.23

[a] Cox regression stratified by adjuvant therapy; nodal status and tumor size were each included in the model as one variable with values 1, 2, 3, and 4 given to the groups as indicated. RR therefore refers to the comparison of one category to the next. Tumor staging according to UICC criteria.

[b] Positive ≥20 fmol/mg protein.

Table 3 Time dependency of the prognostic factors

Results of the Grambsch and Therneau test with regard to metastasis-free survival and overall survival.

	Metastasis-free interval	Overall survival
Prognostic factors	*P*	*P*
TCD	0.063	0.858
Tumor size	0.811	0.210
Therapy	0.198	0.163
Nodal status	0.935	0.505
Progesterone receptor	0.210	0.157

variable was found to have a time-varying effect. Then, the dynamic Cox model is fitted, allowing variation to one variable at a time and adjusting for the other predictors. The estimated varying coefficient is plotted with the equivalent from the PH model in Fig. 3. With regard to the overall survival time, it seems that tumor size does not present any important departure from the PH assumption. However, the RR of progesterone receptor and grading decreases after 60 months, and the RR due to nodal status has a constant increase in the first 8 years. We also tested for time-varying effects by comparing the change in deviance between the Cox proportional hazards model and the time-varying model, allowing variation to one prognostic factor at a time. For tumor size, the test resulted in a *P* of 0.90. However, nodal status, grading, and progesterone receptor status have been found to have a time-dependent effect at a 10% level.

In contrast to those variables, TCD seems to have a constant effect on the overall survival time ($P = 0.98$; Fig. 5). Although this consistency is more doubtful with regard to the metastasis-free time (Fig. 4), the change in the deviance gives a *P* of 0.97. Therefore, TCD can be considered as a prognostic factor for which the impact remains unaffected by time.

DISCUSSION

Numerous research groups have demonstrated a poorer prognosis in patients with positive TCD. Our study is the only study involving more than 500 patients ($n = 727$) and a follow-up period of more than 5 years. There is only one other study with more than 300 patients and a follow-up period of more than 5 years, *i.e.*, that of Mansi *et al.* (1999) with the anti-EMA antibody (350 patients; median follow-up, 12.5 years; Ref. 13).

Immunocytology is currently the standard method for TCD. Newer methods such as enrichment of the tumor cells (*e.g.*, beads), PCR, and flow cytometry are still being evaluated. An important issue in immunocytology appears to be the choice of antibody. Mucin antibodies (*i.e.*, 2E11), epithelial antibodies (*i.e.*, EMA), or cytokeratins can be used to detect disseminated tumor cells. Our antibody, 2E11, is a mucin antibody directed against the tumor-associated antigen TAG12. The advantage of this antibody is its high sensitivity (it reacts with almost all breast cancer cells). With the 2E11 antibody, the detection rate in our study (43–45%) is higher than that with either cytokeratin (30–38%) or anti-EMA (25%).

Specificity is generally a problem for all antibodies, and this is also true for 2E11. We cannot rule out with absolute certainty that no cross-reaction occurred in TCD-positive patients. Muc-1 epitopes are expressed on 2–10% of normal bone marrow cells and particularly on cells of the erythroid lineage (17). With the correct concentration of the 2E11 antibody, only tumor cells and not normal bone marrow cells will be stained (18). If the staining protocol is not followed correctly, normal bone marrow cells may also be stained (19). However, no positive cells were found in our control group ($n = 21$). Likewise, in the other long-term follow-up study with the anti-EMA antibody, cross-reaction cannot be entirely ruled out. Cytokeratin antibodies also react with nonmalignant cells (20), and therefore it is very important to analyze the morphological criteria of the stained cells. Objective criteria for the evaluation of immunostained cells, which were recently published by the European ISHAGE Working Group for Standardization of Tumor Cell Detection, should be observed (21).

The study with the best monoclonal antibody at the moment (A45-B/B3 antibody against cytokeratin) was published by Braun *et al.* (12) They used the monoclonal antibody. The TCD-positive rate was 36% and correlated with tumor size but not with axillary lymph node status. TCD was also an independent prognostic factor for disease-free survival and for overall survival after a median follow-up time of 38 months (12).

TCD also provides independent, significant information in addition to that provided by nodal status, even after a median follow-up time of 6.5 years. The tumor cell dissemination reflects the ability of the primary tumor to metastasize and shows that the tumor cells are a marker for systemic disease.

Not all disseminated tumor cells cause metastasis. Fifty-one percent of patients with positive TCD at diagnosis (160 of 315 patients) remained relapse free after a median follow-up time of 6.5 years. The same percentage (46 of 89) of TCD-positive patients was found after a median follow-up of 6 years by Mansi *et al.* (10).

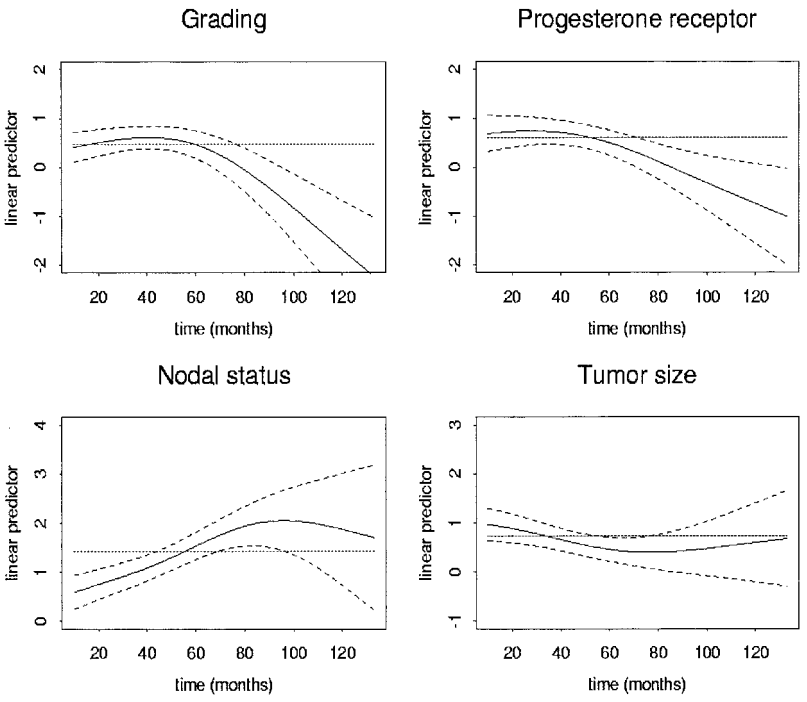


Fig. 3 Estimation of the time dependency of established prognostic factors. Comparison of the time-varying Cox model (*solid lines*) with the PH model (*dotted lines*) for grading, progesterone receptor status, nodal status, and tumor size.

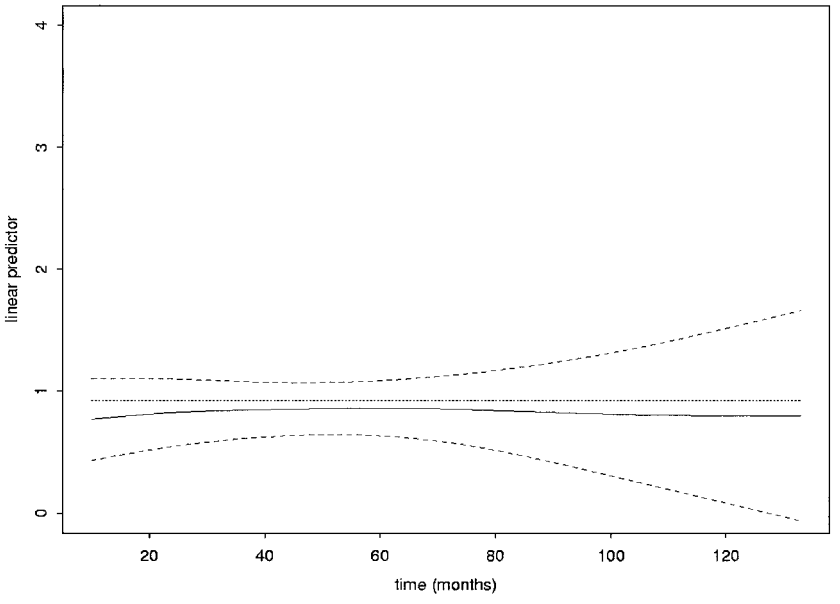


Fig. 4 Estimations of the time dependency of TCD with regard to overall survival time. Comparison of the time-varying Cox model (*solid lines*) with the PH model (*dotted lines*) for TCD.

After a median follow-up of 12 years, only 34% of patients were relapse free (30 of 89 patients; Ref. 13). This fact suggests that tumor cell shedding is an important step in the process of metastasis development, but that it is not sufficient in itself to cause metastasis (22). Only tumor cells that possess certain biological qualities can lead to the development of metastatic disease. These properties of micrometastatic tumor cells can include proteases (23–25), proliferation markers (Ki-67 and p120), and growth factor receptors (HER-2; Refs. 26 and 27).

Most prognostic factors are strongly related to outcome in early studies with short follow-up times but not in studies with long-term follow-up analysis. There are only a few long-term follow-up reports about the time dependency of prognostic factors. Our main interest was to analyze the time dependency of TCD. There is only one clinical study with a long median follow-up time (12.5 years; $n = 350$; Ref. 13). In 1999, Mansi *et al.* (13) found that TCD is a significant prognostic factor for relapse-free survival and overall survival; however, in contrast to the previous report, the prognostic impact of TCD was not more independent. However, analysis of the time dependency of TCD was missed. The reason

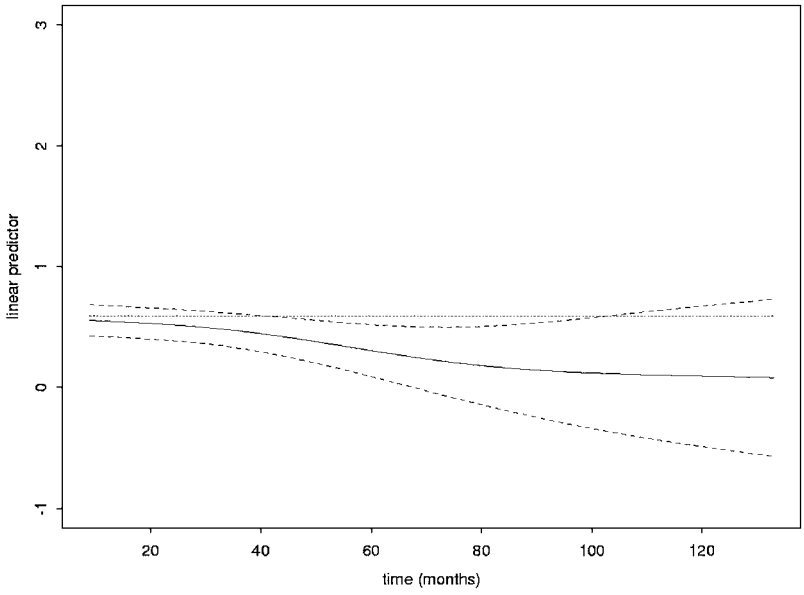


Fig. 5 Estimations of the time dependency of TCD with regard to distant disease-free survival time. Comparison of the time-varying Cox model (*solid lines*) with the PH model (*dotted lines*) for TCD.

for these results could be the low incidence of detected tumor cells in the bone marrow (25%; Ref. 13).

Our main purpose was to describe the prognostic behavior of TCD with regard to follow-up time and to test for the consistency of its impact on overall survival and metastasis-free time. The results from the metastasis sample were not able to be clearly interpreted, but they point to the same direction (*i.e.*, no time variation for TCD). In our study, the prognostic impact of TCD seems to decrease with regard to the decease-free time, but this variation was not statistically significant. With regard to overall survival time, there were no changes with longer follow-up time. TCD shows no time dependency and can be used as a marker not only for early dissemination but also for later dissemination. Therefore, we can exclude the possibility that the prognostic impact of TCD changes significantly after longer follow-up.

However, the time-varying hazard ratio function of grading and progesterone receptor is not horizontal, indicating that the impact of these factors decreases with time. Changes in the prognostic impact of estrogen receptor status is very well known (28, 29). The good prognostic effect of estrogen receptor-positive status on patients changes at about 3 years after diagnosis. Tumor size and nodal status are the best classical prognostic factors in breast cancer, and their prognostic impact remains independent of the follow-up time (28).

Today, the prognostic value of TCD is certain, and it is opening up new avenues in the treatment of breast cancer (replacement of axillary lymphadenectomy, bisphosphonates, immunotherapy, and better risk assessment) that must now be investigated in prospective randomized studies. However, studies with short-term follow-up can yield valid conclusions for early prognostic effect, and late effects cannot be determined without a longer follow-up time. The prognostic impact of minimal residual disease must be analyzed in long-term follow-up studies.

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