

Serum thymidine kinase activity compared with CA 15-3 in locally advanced and metastatic breast cancer within a randomized trial

J. Bjöhle · J. Bergqvist · J. S. Gronowitz · H. Johansson · L. Carlsson · Z. Einbeigi · B. Linderholm · N. Loman · M. Malmberg · M. Söderberg · M. Sundquist · T. M. Walz · M. Fernö · J. Bergh · T. Hatschek

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Abstract The primary objective was to estimate serum thymidine kinase 1 (TK1) activity, reflecting total body cell proliferation rate including cancer cell proliferation, in women with loco regional inoperable or metastatic breast cancer participating in a prospective and randomized study. Secondary objectives were to analyze TK1 in relation to progression-free survival (PFS), overall survival (OS), therapy response and other tumour characteristics, including CA 15-3, widely used as a standard serum marker for disease progression. TK1 and CA 15-3 were analysed in 198 serum samples collected prospectively from women included in the randomized TEX trial between December 2002 and June 2007. TK1 activity was determined by the ELISA based DiviTumTM assay, and CA 15-3 analyses was generated with

the electrochemiluminescence immunoassay Cobas Elecsys CA 15-3 II. High pre-treatment TK1 activity predicted shorter PFS (10 vs. 15 months $p = 0.02$) and OS (21 vs. 38 months, $p < 0.0001$), respectively. After adjustment for age, metastatic site and study treatment TK1 showed a trend as predictor of PFS ($p = 0.059$) and was an independent prognostic factor for OS, (HR 1.81, 95 % confidence interval (CI) 1.26–2.61, $p = 0.001$). There was a trend of shortened OS for women with high CA 15-3 ($p = 0.054$) in univariate analysis, but not after adjustment for the above mentioned covariates. Both TK1 ($p = 0.0011$) and CA 15-3 ($p = 0.0004$) predicted response to treatment. There were statistically different distributions of TK1 and CA 15-3 in relation to the site of metastases. TK1 activity measured by DiviTumTM predicted

J. Bjöhle · H. Johansson · T. M. Walz · J. Bergh · T. Hatschek
Department of Oncology, Karolinska Institutet and University Hospital, Radiumhemmet, 171 76 Stockholm, Sweden
e-mail: judith.bjohle@karolinska.se

J. Bergqvist (✉)
Karolinska Institutet and ASIH Långbro Park, Stockholm, Sweden
e-mail: jenny.bergqvist@yahoo.co.uk

J. S. Gronowitz
Biovica International AB, Uppsala, Sweden

J. S. Gronowitz
Department of Medical Sciences, Uppsala University, Uppsala, Sweden

L. Carlsson
Department of Oncology, Sundsvall General Hospital, Sundsvall, Sweden

Z. Einbeigi · B. Linderholm
Department of Oncology, Sahlgrenska University Hospital, Göteborg, Sweden

B. Linderholm
Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden

N. Loman · M. Söderberg · M. Fernö
Division of Oncology, Department of Clinical Sciences, Lund University, Lund, Sweden

M. Malmberg
Department of Oncology, Helsingborg General Hospital, Helsingborg, Sweden

M. Sundquist
Department of Surgery, Kalmar General Hospital, Kalmar, Sweden

T. M. Walz
Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden

therapy response, PFS and OS in loco regional inoperable or disseminated breast cancer. These results suggest that this factor is a useful serum marker. In the present material, a prognostic value of CA 15-3 could not be proven.

Keywords TK1 · CA 15-3 · Breast cancer · Prognostic factor · Predictive factor · DiviTum

Introduction

It has been claimed that the development of better prognostic and therapy predictive factors can improve metastatic breast cancer survival [1]. A retrospective analysis of 1,038 women, who presented with metastatic breast cancer between 1975 and 2000, reported age at primary diagnosis, hormone receptor status, metastasis free interval and site of metastasis to be the most important prognostic factors for prediction of post recurrence survival [2]. Furthermore, numbers of metastatic sites, prior adjuvant therapy, prior therapy for metastatic disease and performance status have been found to be important biological and clinical prognostic factors associated with survival of recurrent breast cancer [2–5]. However, relatively few tumour markers can be recommended for use in clinic practice for screening, surveillance and monitoring of breast cancer and treatment response [1]. No serum biomarker can be recommended for monitoring on its own, but according to the ASCO guidelines published in 2007 CA 15-3 can be used together with imaging and physical examination for treatment evaluation of advanced breast cancer. CA 15-3 (MUC1) is a soluble transmembrane glycoprotein overexpressed on tumour cells and associated with stage of disease and increased metastatic potential [6].

Thymidine kinase 1 (TK1) is another interesting serum marker discussed in previous literature and hypothetically a useful prognostic and/or predictive proliferation marker for individualised therapy decision making [7–9]. TK1 is a historically well-known proliferation marker first reported in foetal tissue in the 60s [10, 11]. It is a cell cycle dependent enzyme for dTTP (deoxy-thymidine-tri-phosphate) synthesis via the nucleotide DNA salvage pathway with rapidly increased activity after the G1-S transition checkpoint, which quickly declines in G2 when DNA replication is completed in normal cells [12, 13]. High levels of TK1 have been associated with other well-known negative prognostic factors (large tumour size and high histologic grade (III)) as well as with hormone receptor negativity, and with the ability to predict disease recurrence in various types of cancer [8, 14–19].

We have devoted this study to compare and combine two serum expressed markers, TK1 and the standard tumour marker CA 15-3. Thymidine kinase activity

reflecting total body cell division including tumour cell proliferation and CA 15-3 a differentiation related marker reflecting total tumour volume. The primary objective was to estimate serum TK1 in women with loco regional inoperable or metastatic breast cancer participating in the randomized TEX trial. Secondary objectives, with a long term goal to investigate whether TK1 can be used in the everyday clinic situation, were to analyze TK1 in relation to therapy response, progression-free survival (PFS), overall survival (OS) and other patient and tumour characteristics, including the standard tumour marker CA 15-3. Our study will be presented in line with the REMARK criteria for reporting tumor marker results [20].

Patients and tumour material

Patients and treatment

Between December 2002 and June 2007, 287 women with disseminated or loco regional inoperable breast cancer were randomized to treatment with both epirubicin and paclitaxel (ET, 143 patients), or the same combination together with capecitabine (TEX, 144 patients) [21]. Epirubicin and paclitaxel were given once every 3 weeks; capecitabine was taken BID on days 1–14. Dose adjustments were made according to toxicity, which was graded according to NCI CTC version 2.0. Treatment was discontinued due to progression ($n = 89$), unacceptable toxicity ($n = 54$), or on patients' request ($n = 23$). No statistically significant difference in terms of PFS and OS was observed between the two treatment arms. Patients received on average seven cycles (median) in both treatment arms. Approximately 40 % (83 women) had a disease free interval (DFI) of less than 2 years, varying between 6 months and 22 years, before inclusion in the TEX study. Forty-two patients had experienced a previous local relapse, which after radical surgery was treated by chemotherapy in five cases. None of the patients included in the present trial had received adjuvant treatment during the 12 months prior to randomization. However, 86 (30 %) women had previously received endocrine treatment for metastatic disease. Patient age at study entry ranged from 30 to 75 years. Thirty-four women presented with metastatic disease at the time of primary diagnosis. Tissue and blood samples were collected prospectively as part of the initial study protocol, but were not mandatory for inclusion in the TEX trial. Serum samples were taken shortly before start of the first treatment. All blood and tumour samples were banked and stored at the Cancer Centre Karolinska, Karolinska Institutet, for future studies. Pre-treatment serum samples were available from 198 women (Fig. 1). Patient and tumour characteristics of these 198 patients in our study

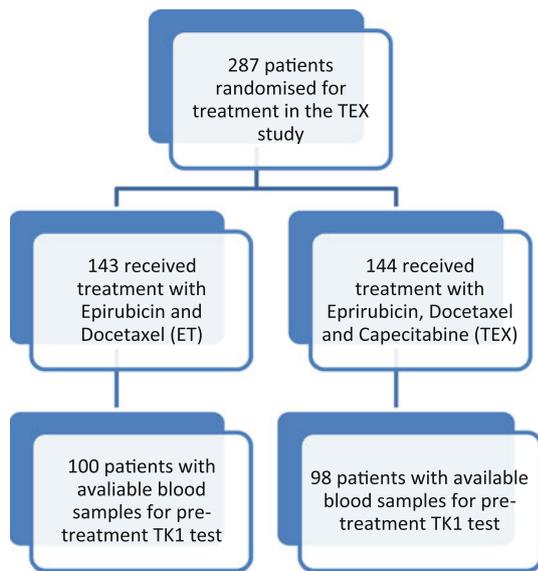


Fig. 1 Blood samples available for TK1 analyses within the TEX trial

are outlined in Table 1. According to a predefined sub-protocol for the present study TK1 analyses were performed in all 198 samples followed by CA 15-3 analyses in 194 cases (no serum left for 4 cases after TK1 analyses).

Response evaluation

Response evaluation using RECIST version 1.0 was performed after every third course in the TEX trial [22]. In patients with skeletal metastases, WHO classification [23] criteria for evaluation of skeletal metastases were used. In the present analysis, we used ‘first response’ defined as objective response of the earliest treatment evaluation after three treatment cycles, but not yet confirmed by a subsequent re-evaluation, and best response, confirmed according to RECIST criteria. We separated patients according to progressive disease (PD), stable disease (SD) and objective response (OR).

Laboratory analyses

Analyses of TK1 and CA 15-3 were performed on serum samples collected before start of chemotherapy.

TK1 activity

The TK1 activity of the samples was determined by a refined ELISA based method, the DiviTumTM assay (Biovica International, Uppsala, Sweden), according to the manufacturer’s instruction (www.biovica.com). The

analyses were performed at the Biovica laboratory in Uppsala, Sweden without access to, or any knowledge of, patient or tumour characteristics. A brief description of the assay: On the first day 10 μ L of serum sample was added to in total 500 μ L dilution buffer in a well on a microtiter-plate. Then 10 μ L diluted sample was transferred to a well with 100 μ L reaction mixture on the assay plate. Bromo-deoxyuridine, a thymidine analogue, was used as a substrate to the thymidine kinase in the serum sample. The assay plate including two control samples, was sealed and incubated at 32 $^{\circ}$ C for 18 h. The product of the reaction binds to the bottom of the well. On day 2, the plates were washed and 100 μ L antibody conjugate solution was added to each well. The conjugate consists of a monoclonal anti-BrdU antibody linked to alkaline phosphatase. The plate was incubated for 1 h at 32 $^{\circ}$ C. The wells were then washed to remove unbound antibody and 120 μ L of substrate solution [alkaline phosphatase (4-nitrophenyl phosphate)] was added to each well. The product of the alkaline phosphatase reaction is yellow, and hence the absorbance was measured at 405 nm with the reference wavelength of 630 nm. Absorbance was measured after 30 and 180 min of incubation at 32 $^{\circ}$ C. The measured signal is proportional to the thymidine kinase activity of the tested sample. The DiviTumEval software was used for data evaluation. The coefficient of variation of the DiviTum assay measuring at 100 Du/L is 8.6 %.

CA 15-3

The CA 15-3 analysis was performed with the Roche Diagnostic test (Cobas Elecsys CA 15-3 II) on the fully automated Roche Cobas8000, e602 analyser for the CA 15-3 analysis. The tests were done in the laboratory of the Department of Clinical Chemistry and Pharmacology, Uppsala University Hospital, Uppsala, Sweden. Analyses were performed according to manufacturer’s instructions and reported in kE/L. The samples were tested without any knowledge of patient or tumour characteristics.

Statistics

Time to event in analyses regarding PFS was calculated from date of randomization to date of either progression or death, whichever occurred first. Event-free patients were followed until last follow-up. Similar calculations were applied for OS. Comparisons between groups were made by use of the logrank test. Effects were estimated using Cox Proportional Hazards models for both univariate and multivariate analyses. Parameters included were TK1, CA 15-3, age at randomization, metastatic site and study treatment. Results are presented as hazard ratios together with 95 % confidence intervals (CI). Interactions between

Table 1 Characteristics of patients and metastases

	All patients		ET		TEX	
Age at diagnosis in median (range)	49 (27–69)		50 (27–68)		48(28–69)	
Age at study entry in median (range)	56 (30–75)		56 (56–75)		56 (30–73)	
Years of DFI in median (range)	3.5(0–22.5)		3.1 (0–22.5)		3.9 (0–20.5)	
<i>N</i>	198		100		98	
Adjuvant systemic therapy	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%
No adjuvant systemic therapy	60	29.8	33	33	27	27.6
Endocrine therapy	44	22.2	19	19	25	25.5
Chemotherapy	36	18.2	16	16	20	20.4
Endocrine- and chemotherapy	57	28.8	31	31	26	26.5
Unknown	1	0.5	1	1		
Metastases						
Only loco regional metastases	32	16.1	18	18.0	14	14.2
Only distant metastases	70	35.4	33	33.0	37	37.8
Loco regional and distant metastases	96	48.5	49	49.0	47	48.0
Bone metastases	103	52	51	51.0	52	53.1
Liver metastases	83	41.9	38	38.0	45	45.9
Hormone receptor status ^a						
ER– and PR–	25	12.6	15	15.0	10	10.2
ER+ and PR+	36	18.2	16	16.0	20	20.4
ER+ and PR–	33	16.7	16	16.0	17	17.3
ER– and PR+	3	1.5	2	2.0	1	1.0
Not done on metastasis	101	51.0	51	51.0	50	51.0
HER2 status						
Negative	194	98.0	98	98.0	96	98.0
Positive	4	2.0	2	2.0	2	2.0

DFI disease-free interval

^a Includes only hormone receptor status from FNAs on metastases. Cut-off point 10 %

TK1 and CA 15-3 were tested by inclusion of product terms into the model for both PFS and OS.

Chi square and Kruskal–Wallis procedures were used for comparison of groups with regard to TK1, CA 15-3, metastatic site, best response and first response. For the analyses of treatment response we tested both objective response after three cycles of treatment (‘first response’) and best response achieved and confirmed during the study treatment. TK1 and CA 15-3 levels were used as dichotomized variables and the definition high TK1 and CA 15-3 levels refer to levels above median compared with low levels below median.

At time of analysis of TK1 and CA 15-3 in relation to metastatic sites patients were categorized into four hazard groups based on the presence of metastatic ‘worst’ sites [2, 4, 5]; 1. Loco regional metastases included advanced disease in the breast and/or regional lymph nodes and/or local skin metastases and occurred in 29 women; 2. Skeletal metastases included 27 women with bone metastases with or without concurrent loco regional metastases; 3. Visceral

metastases included lung/pleura and abdominal sites other than liver regardless of loco regional or skeletal disease and applied to 59 patients; and 4. Liver metastases, which were present in 83 patients with or without other metastatic sites.

Results

TK1 activity and CA 15-3 in relation to metastatic site

Levels of TK1 activity were significantly different in relation to the ‘worst’ metastatic locations, with median values of 57 Du/L (range 5–16,976) for loco regional/skin, 63Du/L (range 3–2,811) for bone, 101 Du/L (range 5–45,674) for visceral other than liver and 579 Du/L (range 11–16,753) for liver metastases ($p < 0.0001$). The distribution of median levels for CA 15-3 was similar: loco regional/skin 27.5 kE/L (range 6–293), bone 42 kE/L (range 13–15,320), visceral other than liver 59.5 kE/L (range 8–1,308), and liver 235 kE/L (range 16–4,616;

$p < 0.0001$). PFS was significantly more favourable for patients with loco regional (15.4 months) and skeletal metastasis (15.9 months) compared with visceral (11.5 months) and liver metastases (10.0 months), $p = 0.004$. Corresponding periods for OS were 46.9 months (loco regional), 38.1 months (bone), 34.9 months (visceral) and 21.3 months (liver), respectively ($p = 0.0009$).

TK1 activity and CA 15-3 in relation to PFS and OS

Median pre-treatment level of TK1 was 235Du/L. Levels of TK1 above median predicted shorter PFS compared with low levels (10 vs. 15 months $p = 0.02$) and worse OS (21 vs. 38 months, $p < 0.0001$, Fig. 2a, b). The correlation between TK1 and CA 15-3 was $\rho = 0.38$ (Spearman). Multivariate Cox regression analysis of pre-treatment levels of TK1 showed TK1 to be an independent prognostic factor for OS (HR 1.81, 95 % CI 1.26–2.61, $p = 0.001$), adjusted for CA 15-3, age at randomization, metastatic sites and study treatment (Table 2). A similar, though not significant trend was shown for TK1 in relation to PFS (HR 1.37 95 % CI 0.99–1.91, $p = 0.059$, Table 2). The median pre-treatment level of CA 15-3 was 87kE/L. There was a trend for shorter OS for women with high pre-treatment levels of CA 15-3 ($p = 0.054$), but this marker did not predict PFS nor OS in univariate (Fig. 2c, d) or multivariate analysis (Table 2). Inclusion of product terms in the

multivariate models did not show significant interaction between TK1 and CA 15-3 with regard to PFS ($p = 0.74$) and OS ($p = 0.69$). Besides age, metastatic site and study treatment, we also considered adjustment for the hormone receptor status. However, receptor status from metastatic tissue was only available in 48 % of the cases. Inclusion of the receptor status from the primary tumor showed that this variable tended to reduce the risk of progression (HR 0.68, $p = 0.075$) and survival (HR 0.45, $p = 0.001$), and affected the impact of TK1 on PFS (HR 1.25, $p = 0.22$) and OS (HR 1.54, $p = 0.03$) in the model, while the role of the other variables was broadly unchanged. The fact that analysis techniques for hormone receptors changed over time, and that presence of receptors was closely correlated with the use of adjuvant tamoxifen, led to the decision to refrain from including hormone receptor status in the multivariate model, similar to the decision to forgo adjuvant chemotherapy

TK1 and CA 15-3 levels in relation to treatment response

Pre-treatment levels of TK1 and CA 15-3 significantly predicted both first (TK1 $p = 0.0001$; CA 15-3 $p = 0.0003$) and best response (TK1 $p = 0.0005$; CA 15-3 $p = 0.0002$) to treatment in all women. The highest levels of TK1 and CA 15-3 were reported in women who had PD by the first evaluation compared with those who had an OR

Fig. 2 TK1 and CA 15-3 in relation to progression-free and overall survival

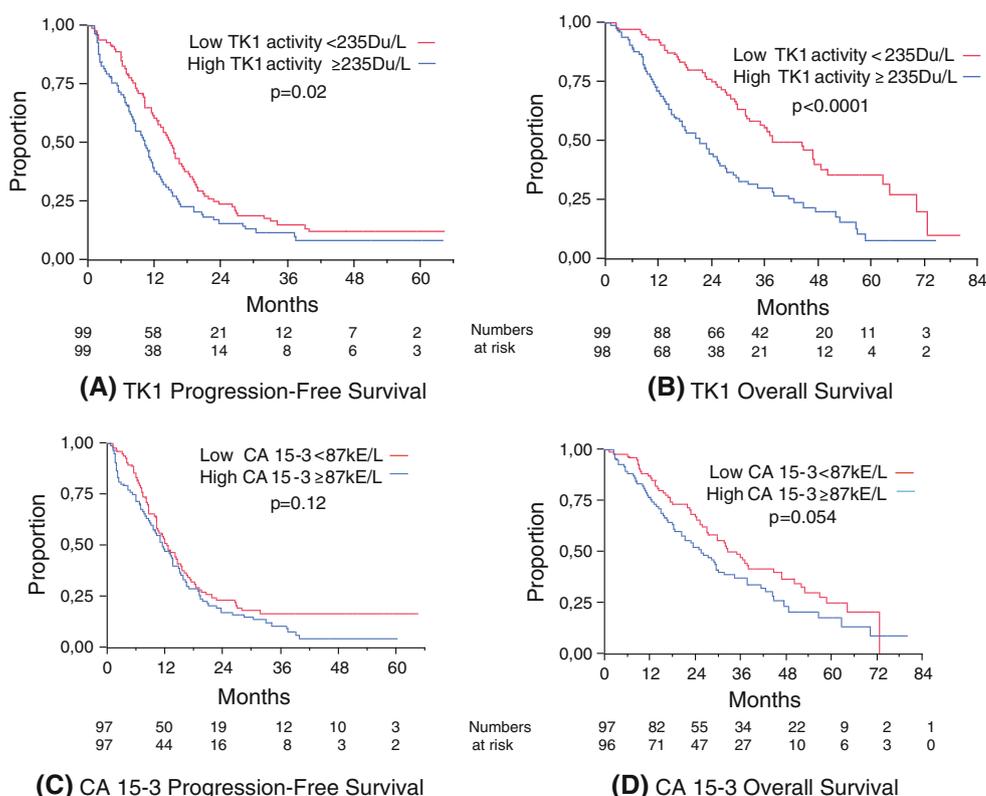


Table 2 Multivariate Cox models including TK1 and CA 15-3 serum levels in relation to progression-free and overall survival, adjusted for age, metastatic sites and study treatment

	Progression-free survival			Overall survival		
	Hazard ratio	95 % CI	<i>p</i> Value	Hazard ratio	95 % CI	<i>p</i> Value
Thymidine kinase 1	<i>n</i> = 194			<i>n</i> = 194		
High vs. low	1.37	0.99–1.91	0.059	1.81	1.26–2.61	0.001
CA 15-3						
High vs. low	1.07	0.76–1.51	0.70	1.11	0.76–1.63	0.58
Age at randomization						
>50 vs. ≤50 years	1.65	1.16–2.33	0.005	1.67	1.13–2.47	0.009
Metastatic sites vs. loco regional						
Bone	0.82	0.43–1.56		1.01	0.49–2.06	
Visceral not liver	1.72	1.01–2.92		1.09	0.58–2.05	
Liver	1.78	1.04–3.06	0.011	1.92	1.03–3.60	0.027
Study treatment						
TEX vs. ET	0.93	0.68–1.28	0.66	1.02	0.72–1.45	0.99

Table 3 Pre-treatment TK1 and CA 15-3 in relation to first and best response to treatment

Response	First response	Best response
Median TK1 levels (numbers)		
OR	193(77)	142(107)
SD	110(91)	124(60)
PD	1,492(16)	1,002(22)
Median CA 15-3 levels (numbers)		
OR	48(74)	60(104)
SD	88(91)	106(60)
PD	873(15)	618(21)

OR objective response, SD stable disease, PD progressive disease

to treatment (Table 3). CA 15-3 increased with lack of response. TK1 was lower in patients with SD compared with those with OR. The difference was more apparent at first response than at best response.

Discussion

This is the first study of total body cell proliferation, including tumour cell proliferation, using the DiviTumTM assay in serum samples from women with inoperable locally advanced or metastatic breast cancer within a prospective randomized study. TK1 activity and standard tumour marker CA 15-3 were analysed in serum from more than 190 women participating in the TEX trial. TK1 predicted PFS, OS and therapy response.

The median TK1 level in our study was 235 Du/L. In comparison, a previous study reported significantly lower serum TK1 activity in healthy blood donors compared to women with early breast cancer, 16 (7–35) versus 38 (21–97) [16]. In addition, a pre-operative level of

TK1 ≥ 134 Du/L was previously reported to be an independent predictor of recurrent disease in women with early breast cancer [16].

Both TK1 and CA 15-3 levels were significantly associated with different metastatic sites and increased with visceral metastases, particularly in the liver. Our data are in agreement with a recent report on TK1 in patients with metastatic malignant melanoma [24]. However, the range of TK1 and CA 15-3 varied a lot within each metastatic site.

We found high levels of TK1 to predict unfavourable PFS and OS. Women with low TK1 had approximately 4.5 months longer PFS compared with women who had high pre-treatment serum levels. In addition, OS was more than 16 months longer. Our results are in agreement with recently published data in early breast cancer, renal cell carcinoma and metastatic lung cancer, respectively, where high levels of TK1 activity was significantly associated with shorter DFI [16, 17, 25] and OS [26]. Furthermore, previously published results using different TK assays and different clinical specimens, i.e. serum and/or tumour biopsies/extracts show high levels of TK1 to be associated with other well-known negative prognostic and predictive factors [8, 14–18, 27, 28].

TK1 and CA 15-3 both predicted first and best response to treatment. CA 15-3 increased with lack of response. In contrast, TK1 was higher in women who had OR compared with those who had SD at first therapy evaluation, a similar trend was found with regard to best response. One explanation is that TK1 is, in contrast to CA 15-3, a proliferation marker with a half-life of less than 2 days [29], and not a tumour marker related to tumour volume. Furthermore, data indicate a favourable chemotherapy response for tumours with a moderate proliferation rate compared with more slowly proliferating tumours. However, even slowly proliferating tumours may respond to chemotherapy with

delay, since SD observed at first response turned into OR as best response in some cases. Those who never responded to treatment had the highest levels of both TK1 and CA 15-3. Previously published results of the association between TK1 levels and therapy response are conflicting. High levels of TK1 in tumour cytosols predict a better response to adjuvant chemotherapy according to some [14] but not according to others [30]. Foekens et al. [30] showed low levels of TK1 in tumour cytosols to be predictive of a favourable response to tamoxifen therapy. To some extent, conflicting results can be explained by the different methods and tumour templates used for TK1 evaluation and by different administered therapies. For example, the difference in clinical resolution capacity between DiviTum and Liason based assay for primary breast cancer was recently published [31].

CA 15-3 is a widely used marker for monitoring and surveillance of breast cancer with the advantage of being a well standardised method for evaluation in serum. A disadvantage is that approximately 20 % of metastatic breast cancer lack overexpression of CA 15-3, and can not be monitored by this tumour marker [32]. Furthermore, CA 15-3 is associated with tumour volume, and may change more slowly due to a change in tumour activity compared with tumour proliferation rate. Ki-67 assessed by IHC analysis on tumour tissue is the most commonly used proliferation marker in the adjuvant treatment algorithm. However, the lack of standardised methods in addition to tumour heterogeneity complicates the interpretation of this widely used proliferation marker. Several attempts have been made to design an assay for Ki-67 in serum but standardization of staining techniques, counting methods and cut points are needed [33]. Chemotherapy in general is directed against DNA or cell cycle processes, which affects proliferation rate. As a consequence, changes in proliferation rate during treatment would be expected to reflect response to chemotherapy. A serum-based proliferation marker analysed with standardised techniques, like TK1 might result in an earlier evaluation of therapy response compared with radiology.

The results from this study stimulate a prospective comparison of TK1 as a serum-borne marker with currently used IHC based proliferation markers.

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Conflict of interest J Bergqvist has a family member who works as a consultant for Biovica International AB. S Gronowitz is employed by Biovica International AB and has family members who own shares in Biovica International AB.

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