Plasma and Serum Levels of Tissue Inhibitor of Metalloproteinases-1 Are Associated with Prognosis in Node-negative Breast Cancer

A PROSPECTIVE STUDY*

Sidse Ørnbjerg Würtz‡§¶, Susanne Møller||, Henning Mouridsen**, Pernille Bræmer Hertel**, Esbern Friis‡‡, and Nils Brünner‡§

The tumor level of TIMP-1 has been suggested as a new prognostic marker in breast cancer. The purpose of this study was to investigate whether TIMP-1 also carries prognostic information when measured in blood as this is a much more preferable material compared with tumor extracts. Using ELISA, TIMP-1 was measured in prospectively collected preoperative plasma and serum samples from 519 patients with primary breast cancer, and the measurements were related to patient outcome. The median age of the patients was 58 years (range, 38–80 years), and the median follow-up time was 1043 days (range, 300–1630 days). Plasma and serum TIMP-1 measurements correlated significantly with each other with a Pearson correlation coefficient of 0.75 (p < 0.0001). For univariate survival analysis, patients were divided into quartiles according to increasing TIMP-1 levels (Q1–Q4). Analysis of all patients showed that high TIMP-1 plasma levels were significantly associated with a shorter disease-free survival. Subgroup analysis showed that plasma TIMP-1 significantly predicted the prognosis of node-negative patients but not of node-positive patients. Importantly plasma TIMP-1 was able to further stratify low risk node-negative patients. High serum TIMP-1 levels were associated with a shorter disease-free survival; however, the association was not statistically significant. In contrast, serum TIMP-1 significantly predicted the prognosis of node-negative and low risk patients. In multivariate survival analysis of node-negative patients including all the classical prognostic parameters, plasma TIMP-1 remained significantly associated with prognosis when comparing Q1 with Q2 and Q4. Serum TIMP-1 remained significant when comparing Q1 with Q4. Taken together, this study is to our knowledge the first large prospective study suggesting that TIMP-1 carries independent prognostic information when measured in blood, especially plasma. This was especially true in the node-negative group of patients and in patients already defined as low risk patients using the currently available prognostic parameters. Molecular & Cellular Proteomics 7: 424–430, 2008.

First line treatment of patients diagnosed with primary breast cancer is surgical removal of the tumor with or without adjuvant radiotherapy. Subsequently patients may be offered adjuvant systemic therapy depending on a prognostic evaluation. Today prognosis is estimated using both classical prognostic parameters (lymph node status, tumor size, grade of malignancy, and age) as well as HER2/neu gene expression (1). In addition, estrogen/progesterone receptor status is used by some as a predictive factor and by others as a prognostic and predictive factor. Axillary lymph node status is currently recognized as the best clinical discriminate between a good and poor prognosis. Based on these prognostic factors patients are allocated to different risk groups, i.e. low risk, intermediate risk, or high risk group. Patients in the low risk group are offered minimal or no adjuvant therapy following removal of the primary tumor. In contrast, intermediate risk and high risk patients are recommended adjuvant systemic therapy. Unfortunately it is becoming increasingly clear that the currently available prognostic parameters are relatively inadequate to precisely define the prognosis of individual patients (2). In this regard, a substantial proportion of breast cancer patients allocated to the intermediate risk or high risk group are given adjuvant therapy although they are not in need of this treatment. These patients are therefore overtreated. Also some patients who are allocated to the low risk group and therefore do not receive adjuvant therapy do nevertheless experience recurrence of the disease, and these patients are therefore undertreated. Thus, additional prognostic markers, which can be used either alone or in combination with the traditional markers, need to be identified to ensure a more precise stratification and thereby a more effective management of breast cancer patients.
TIMP-1 Carries Prognostic Information When Measured in Blood

Tissue inhibitor of metalloproteinases-1 (TIMP-1) \(^1\) is a naturally occurring inhibitor of the matrix metalloproteinases (MMPs), and several studies have demonstrated that the tumor tissue level of TIMP-1 is increased in a number of malignancies including breast cancer. In addition, the increased level of TIMP-1 has been shown to be associated with a poor prognosis of breast cancer patients, and accordingly TIMP-1 has been suggested as a new potential prognostic marker in breast cancer (3–7). So far, most of the studies investigating the prognostic value of TIMP-1 have been performed on tumor tissue extracts. However, due to the fact that effective diagnostic methods and thus earlier detection of the disease have made it increasingly difficult to acquire frozen breast tumor samples, other more accessible materials for TIMP-1 measurements need to be identified. In this regard, results from a recent study including 71 patients have indicated that TIMP-1 measured in serum also carries prognostic information in breast cancer (8). This is a very important finding because blood is a much more preferable material compared with tumor tissue extracts. Sample collection is easy and non-invasive, and samples can be taken continuously throughout the treatment. Thus, the aim of the present study was to validate and investigate further the previous preliminary finding concerning the prognostic value of TIMP-1 in blood. In addition, we wished to analyze whether both serum and plasma samples were suitable for TIMP-1 tumor marker studies. We therefore measured TIMP-1 in prospectively collected preoperative plasma samples as well as in the corresponding serum samples from 519 patients with primary breast cancer. The results showed that the measurements were associated with each other and with patient outcome.

**EXPERIMENTAL PROCEDURES**

**Patients and Sample Collection**—Blood samples were collected from a total of 685 women from a prospective, standard operative procedure-driven study. All individuals had undergone surgery at Rigshospitalet, Copenhagen, Denmark and were consecutively included in the period of 2002–2006. A total of 166 women were excluded from the study; 67 women had benign tumors, 22 women had recurrence of disease, and 77 women could not follow guidelines for treatment, and therefore no follow-up was registered. This resulted in 519 patients with primary breast cancer that were included in the present study. For 10 of these 519 patients, plasma samples were not available, and for 10 other patients, serum samples were not available. Thus, 509 patients were available for analysis of plasma and serum, respectively. Primary surgical procedures included mastectomy or lumpectomy alone or in combination with radiotherapy. The median age of the patients was 58 years (range, 38–80 years). The median follow-up time was 1043 days (range, 300–1630 days). The end point used in the statistical survival analyses was disease-free survival (DFS). During the follow-up a total of 82 patients had an event; 40 patients experienced recurrence of disease, 32 patients died, and 10 patients were diagnosed with another malignancy. Clinicopathological data registered for the patients were provided by the Danish Breast Cancer Cooperative Group. The lymph node status was known for all patients of which 242 (48%) had lymph node-positive tumors. A total of 364 (70%) patients were postmenopausal.

The hormone receptor status was known for 503 of the patients of which 418 were hormone receptor-positive. Patients allocated to the low risk group (145 patients, 28% did not receive any adjuvant therapy, whereas intermediate risk and high risk patients (374 patients) were given chemotherapy (cyclophosphamide, epirubicin, and 5-fluorouracil or cyclophosphamide, methotrexate, and 5-fluorouracil), endocrine treatment (tamoxifen or an aromatase inhibitor), or a combination of both. Some patients received trastuzumab. Low risk patients were in this patient material defined as patients over the age of 35 years with lymph node-negative tumors smaller than 2 cm with malignancy grade 1 and that were hormone receptor-positive. Intermediate risk and high risk patients (in the following termed high risk patients) were defined as patients with lymph node-positive tumors or lymph node-negative tumors with one of the following characteristics: age below 35 years, tumor size above 2 cm, tumors with malignancy grade 2 or 3, or hormone receptor-negative.

Blood samples were collected from the patients preoperatively (on average 4 days from operation, 90% CI = 1–19 days) following a standardized protocol. Plasma samples were prepared by collecting blood in EDTA tubes, which were left on ice immediately following sampling and until centrifugation. Serum samples were prepared by collecting blood in empty tubes and left at room temperature (for a maximum of 30 min) until centrifugation. Samples were centrifuged for 10 min at 4000 rpm after which plasma and serum were transferred to new tubes and immediately stored at −80 °C until TIMP-1 measurements. The samples had only undergone one freeze/thaw cycle before the measurements were conducted.

The study was approved by the ethics committee. All patients entered in the study signed an informed consent.

**TIMP-1 Measurements**—The level of total TIMP-1 was measured in each serum and plasma sample using an established and highly validated in-house ELISA (9). In brief, wells were coated with a polyclonal sheep anti-TIMP-1 antibody overnight at 4 °C. Following coating, wells were incubated with the sample (diluted 1:101 in sample dilution buffer). All samples were run in duplicate. TIMP-1 was detected by incubation with a monoclonal anti-TIMP-1 antibody (Mac15). Finally wells were treated with a secondary alkaline phosphatase-conjugated rabbit anti-mouse antibody, and p-nitrophenyl phosphate was added as a substrate. Color development was measured spectrophotometrically at 405 nm. KinetiCalc II software (Bio-Tek Instruments, Winooski, VT) was used for calculation of TIMP-1 concentrations. On every plate, serial dilutions of recombinant TIMP-1 were included to allow for determination of TIMP-1 concentrations in individual samples. As internal control, duplicates of a control plasma pool were included on every plate. All incubations were performed in volumes of 100 µl for 1 h at 30°C. Details regarding buffers and other analytical conditions have been published previously (9).

**Statistical Analysis**—The SAS software package was used to analyze the data. For analysis of association between the level of TIMP-1 and traditional clinicopathological parameters and DFS, patients were divided into four groups (Q1–Q4) of equal size according to increasing TIMP-1 levels as specified in the figure and table legends. Associations were tested by using \( \chi^2 \) tests. The Kaplan-Meier method was used to estimate survival probabilities in the univariate survival analysis, and the groups were compared by the log rank test. The Cox proportional hazard model was used for multivariate analysis. Patients with missing values were excluded from the calculations. When a parameter had more levels, all levels were included in the test for significant effect. \( p \) values less than 5% were considered significant.

---

\(^1\) The abbreviations used are: TIMP-1, tissue inhibitor of metalloproteinases-1; DFS, disease-free survival; MMP, matrix metalloproteinase; CI, confidence interval.
TIMP-1 Carries Prognostic Information When Measured in Blood

**Table I**

| Parameter               | n   | Plasma<sup>a</sup> | Serum<sup>b</sup> | \(\chi^2\) test <br> (\(p\) value) | \(\chi^2\) test <br> (\(p\) value) |
|-------------------------|-----|--------------------|-------------------|-----------------------------------|-----------------------------------|
| **Plasma**              |     | Q1  | Q2  | Q3  | Q4  | Q1  | Q2  | Q3  | Q4  | Q1  | Q2  | Q3  | Q4  |
| Tumor size (mm)         | 509 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0–20                    | 329 | 25.8| 22.5| 26.8| 24.9| 0.213| 24.0| 26.1| 25.5| 24.3| 0.944|
| 20+                     | 180 | 23.9| 28.9| 20.0| 27.2| 0.0159| 25.0| 24.4| 24.4| 26.1|        |
| Age (yr)                | 509 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 20–29                   | 4   | 60.0| 20.0| 20.0| 0.0001| 0.0001| 50.0| 25.0| 25.0| 25.0| 0.0001| 0.0001| 26.7| 33.3| 23.3| 16.7|
| 30–39                   | 30  | 44.9| 27.6| 24.1| 3.45  | 0.0001| 37.2| 26.9| 16.7| 19.2| 0.001  | 0.001 | 25.6| 23.2| 26.8| 24.4|
| 40–49                   | 78  | 39.7| 24.4| 21.8| 14.1  | 0.0001| 22.7| 31.1| 29.4| 16.8|        |        | 13.6| 20.0| 24.6| 41.8|
| 50–59                   | 168 | 22.0| 31.7| 28.7| 17.7  |        | 22.7| 31.1| 29.4| 16.8|        |        | 13.6| 20.0| 24.6| 41.8|
| 60–69                   | 119 | 27.1| 24.6| 21.3| 27.1  |        | 22.7| 31.1| 29.4| 16.8|        |        | 13.6| 20.0| 24.6| 41.8|
| ≥70                     | 110 | 10.8| 14.4| 23.4| 51.4  |        | 22.7| 31.1| 29.4| 16.8|        |        | 13.6| 20.0| 24.6| 41.8|
| Nodal status            | 509 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Neg                     | 267 | 24.2| 24.2| 26.0| 25.7  | 0.814 | 22.9| 24.7| 26.6| 25.8| 0.724  |        | 26.0| 26.5| 23.8| 24.0|
| Pos                     | 242 | 26.3| 25.4| 22.5| 25.8  |        | 26.0| 26.5| 23.8| 24.0|        |        | 26.0| 26.5| 23.8| 24.0|
| Menopausal status       | 509 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Pre                     | 151 | 37.8| 27.8| 23.2| 11.3  | 0.0001| 30.5| 26.5| 25.2| 17.9| 0.054  |        | 21.8| 25.1| 25.1| 27.9|
| Post                    | 358 | 19.9| 23.5| 24.9| 31.8  |        | 30.5| 26.5| 25.2| 17.9| 0.054  |        | 21.8| 25.1| 25.1| 27.9|
| Receptor status         | 503 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Neg                     | 85  | 25.3| 29.9| 16.1| 28.7  | 0.542 | 24.7| 30.6| 22.4| 22.4| 0.539  |        | 24.2| 24.6| 26.1| 25.1|
| Pos                     | 418 | 25.0| 23.8| 26.0| 25.2  |        | 24.2| 24.6| 26.1| 25.1|        |        | 24.2| 24.6| 26.1| 25.1|

<sup>a</sup> Plasma TIMP-1 groups: Q1, 1–75 ng of TIMP-1/ml of plasma; Q2, 76–88 ng of TIMP-1/ml of plasma; Q3, 89–109 ng of TIMP-1/ml of plasma; and Q4, 110–600 ng of TIMP-1/ml of plasma.

<sup>b</sup> Serum TIMP-1 groups: Q1, 1–127 ng of TIMP-1/ml of serum; Q2, 128–145 ng of TIMP-1/ml of serum; Q3, 146–166 ng of TIMP-1/ml of serum; and Q4, 167–600 ng of TIMP-1/ml of serum.

plasma concentration was 96.7 ng/ml (range, 45.5–498.1 ng/ml), and the mean TIMP-1 serum concentration was 152.1 ng/ml (range, 49.6–515.6 ng/ml). There was a significant correlation between measurements in the two types of specimens (Pearson correlation coefficient = 0.75, \(p < 0.0001\)). The association between TIMP-1 measurements and the traditional clinicopathological parameters used in breast cancer today is summarized in Table I for both plasma and serum. For the analysis, patients were divided into quartiles according to increasing TIMP-1 levels (Q1–Q4, specified in the figure and table legends) where Q1 is the group with the lowest TIMP-1 levels. As is evident from Table I, TIMP-1 in plasma was significantly associated with age and menopausal status (\(p = 0.0001\) and \(p = 0.0001\), respectively) and in serum with age (\(p = 0.001\)). More importantly, neither plasma nor serum TIMP-1 levels were correlated with lymph node status.

**Univariate Survival Analysis**—Plasma samples were available from 509 patients. For survival analysis, patients were divided into the four groups (Q1–Q4) as mentioned above. As can be seen in Fig. 1A, patients with high plasma TIMP-1 levels had a significantly shorter DFS compared with patients with lower TIMP-1 plasma levels (\(p = 0.0159\)). Analysis of patients with node-negative (plasma samples were available from 269 of the 271 node-negative patients) and node-positive tumors (\(n = 248\)) separately showed that high plasma TIMP-1 levels significantly predicted a shorter DFS in the node-negative group (Fig. 1B, \(p = 0.0181\)) but not in the node-positive group (\(p = 0.2897\), data not shown).

Serum samples were available from 509 patients. As is evident from Fig. 2A, increasing serum levels of TIMP-1 were associated with a shorter DFS; however, this association was not statistically significant (\(p = 0.1565\)). However, the subgroup analysis revealed that high serum TIMP-1 levels were significantly associated with a shorter DFS in the node-negative group of patients (Fig. 2B, \(p = 0.0347\), serum samples were available from 267 of the 271 node-negative patients). In the node-positive group, no significant association was found (\(p = 0.8782\), data not shown).

Importantly when analyzing separately the subgroup of patients with node-negative tumors defined as low risk patients (\(n = 145\)) based on the traditional prognostic parameters, TIMP-1 significantly predicted DFS when measured both in plasma (Fig. 1C, \(p = 0.0019\), plasma samples were available from 143 patients) and in serum (Fig. 2C, \(p = 0.0204\), serum samples were available from 143 patients). No significant association was found in patients with high risk node-negative tumors for either plasma or serum (\(p = 0.2725\) and \(p = 0.6731\), respectively, data not shown). Taken together, TIMP-1 was found to be associated with prognosis in primary node-negative breast cancer, especially when measured in plasma, even within patients in the low risk group already defined using the currently available prognostic parameters. Other prognostic factors that were significantly associated with DFS in univariate survival analysis were age (\(p = 0.001\)), tumor size (\(p = 0.022\)), tumor grade (\(p = 0.002\)), and hormone receptor status (\(p < 0.0001\)).

**Multivariate Survival Analysis**—To investigate whether TIMP-1 was an independent predictor of DFS, we performed multivariate analysis with respect to all the clinicopathological parameters for the node-negative group of patients (Table II). Concerning plasma TIMP-1 and using Q1 as the base line it was found...
that Q2 (\(p = 0.026\), hazard ratio = 5.671, 95% CI = 1.23–26.24) and Q4 (\(p = 0.012\), hazard ratio = 7.054, 95% CI = 1.54–32.3) remained, as the only prognostic parameters, significantly associated with DFS in a multivariate model of node-negative patients including all the traditional prognostic parameters, suggesting that plasma TIMP-1 carries independent prognostic information in node-negative breast cancer. When comparing Q1 with Q3, plasma TIMP-1 was not significant in the multivariate model (\(p = 0.123\), hazard ratio = 3.480, 95% CI = 0.71–16.99). The overall \(p\) value for all four groups reached borderline significance (\(p = 0.058\)). For serum TIMP-1, borderline significance was retained when comparing Q1 with Q3 (\(p = 0.061\), hazard ratio = 3.652, 95% CI = 0.94–14.17). When comparing Q1 with Q4 serum TIMP-1 remained significantly associated with DFS (\(p = 0.020\), hazard ratio = 4.757, 95% CI = 1.29–17.62). The overall \(p\) value for all four groups was 0.098.

**DISCUSSION**

In breast cancer, a significant proportion of node-negative breast cancer patients are offered adjuvant therapy, although many of these patients are cured by the primary surgery alone and therefore do not need the treatment. Instead they may suffer from the substantial side effects often caused by the drugs. A minor proportion of the node-negative patients are allocated to the low risk group, and accordingly they are spared from adjuvant therapy; however, some of these patients nevertheless experience recurrence of disease. Therefore, new and more effective prognostic markers are urgently needed in breast cancer to correctly identify those patients who need adjuvant therapy and those who do not.

TIMP-1 is one of four naturally occurring inhibitors of the MMPs, which are proteolytic enzymes capable of degrading almost every component of the extracellular matrix (10). The MMPs are believed to play a central role in cancer cell dissemination by paving the way for metastasizing cancer cells. In this regard, one would expect high tumor tissue levels of TIMP-1 to be associated with a favorable prognosis because of the TIMP-1-mediated inhibition of cancer metastasis. Surprisingly several studies have shown that high tumor tissue levels of TIMP-1 are associated with a poor prognosis in breast cancer, and therefore tumor TIMP-1 has been suggested to be a new potential prognostic marker in this disease (3–7). The unexpected association between high tumor tissue levels of TIMP-1 and a poor prognosis in breast cancer has been hypothesized to be the result of cancer-promoting func-

![Fig. 1. Univariate survival analysis of DFS in plasma for all patients (A), node-negative (neg) patients (B), and low risk patients (C). Patients are divided into four groups of equal size (Q1–Q4) according to increasing plasma TIMP-1 levels: Q1, 1–75 ng of TIMP-1/ml of plasma; Q2, 76–88 ng of TIMP-1/ml of plasma; Q3, 89–109 ng of TIMP-1/ml of plasma; and Q4, 110–600 ng of TIMP-1/ml of plasma. The number of events were: for all patients: Q1, 14; Q2, 22; Q3, 15; Q4, 31; for node-negative patients: Q1, 3; Q2, 11; Q3, 9; Q4, 15; and for low risk patients: Q1, 2; Q2, 6; Q3, 7; Q4, 10.](image-url)
tions recently demonstrated for TIMP-1, such as stimulation of proliferation and inhibition of apoptosis (11–16).

The use of tumor tissue in tumor marker studies, however, has been a matter of debate partly because of the heterogeneity of this material. Furthermore because of more effective diagnostic methods, tumors of still smaller size are obtained from the patients making it increasingly difficult to acquire frozen tumor tissue samples for analyses. Therefore, recent studies have been aimed at identifying new and more assessable specimens for TIMP-1 measurements. In ovarian and colon cancer, TIMP-1 has been shown to carry prognostic information when measured in blood (17, 18). In this regard, results from a recent study including 71 patients have indicated that this could also be the case concerning breast cancer (8). That study showed that TIMP-1 measured in serum significantly predicted the outcome of patients with primary breast cancer, especially in the node-negative group of patients.

The purpose of the present study was, in a prospective manner, to validate and investigate further the prognostic value of TIMP-1 measurements in blood. We therefore measured the total level of TIMP-1 in 519 prospectively collected plasma and corresponding serum samples obtained preoperatively from patients with primary breast cancer, and the measurements were related to the outcome of the patients. TIMP-1 was measured using an ELISA highly validated for plasma and serum measurements. In univariate survival analysis we showed that high plasma levels of TIMP-1 were significantly associated with a shorter DFS. The same was shown to be true for serum; however, the association was not statistically significant. We also performed univariate survival analysis of patients with node-negative and node-positive tumors separately. In this case, both high plasma and serum levels of TIMP-1 significantly predicted a shorter DFS in the node-negative group of patients but not in the node-positive group. Importantly TIMP-1 significantly predicted prognosis in the subgroup of patients with node-negative tumors defined as low risk patients when measured in both plasma and serum. In this material, low risk patients are women with node-negative tumors who are over the age of 35 years, have tumors below 2 cm, have tumors with malignancy grade 1, and have tumors that are hormone receptor-positive. These patients are considered at very low risk of experiencing relapse, and therefore they are offered only minimal or no adjuvant therapy following surgery. However, based on the present study it seems that by using plasma or serum TIMP-1, the

**Fig. 2. Univariate survival analysis of DFS in serum for all patients (A), node-negative (neg) patients (B), and low risk patients (C).** Patients are divided into four groups of equal size (Q1–Q4) according to increasing serum TIMP-1 levels: Q1, 1–127 ng of TIMP-1/ml of serum; Q2, 128–145 ng of TIMP-1/ml of serum; Q3, 146–166 ng of TIMP-1/ml of serum; and Q4, 167–600 ng of TIMP-1/ml of serum. The number of events were: for all patients: Q1, 13; Q2, 20; Q3, 14; Q4, 24; for node-negative patients: Q1, 4; Q2, 7; Q3, 12; Q4, 14; and for low risk patients: Q1, 2; Q2, 4; Q3, 10; Q4, 9.
low risk group of patients can be further stratified because patients with high levels of TIMP-1 have a significantly shorter DFS as compared with patients with low TIMP-1 levels. Thus, plasma or serum TIMP-1 may aid in selecting a group of patients within the low risk group that may need adjuvant treatment despite their expected favorable prognosis. Indeed this is illustrated by the present data indicating an inferior prognosis in the node-negative low risk subgroup compared with the total node-negative group. This may be explained by the fact that the low risk subgroup was left untreated, whereas the high risk node-negative subgroup received adjuvant systemic therapy.

In multivariate survival analysis of node-negative patients including all the classical prognostic parameters normally used in breast cancer, plasma TIMP-1 remained as the only prognostic factor significantly associated with prognosis when comparing patients with the lowest TIMP-1 levels with patients with low-intermediate and the highest TIMP-1 levels, ensuring that plasma TIMP-1 significantly and independently predicted prognosis in this patient material. Concerning serum, TIMP-1 remained significant in multivariate analysis when comparing patients with the lowest levels with patients with the highest levels. The fact that TIMP-1 measured in blood carries prognostic information in subgroups of node-negative breast cancer patients is a very important finding because new and more precise prognostic markers are urgently needed in breast cancer as mentioned earlier. Especially blood-based markers would be very useful because of the accessibility of this specimen compared with tumor tissue extracts. In addition, sample collection is much easier, and the problem with tissue heterogeneity is circumvented as blood is a more homogenous material. Furthermore the possibility of taking blood samples during follow-up of patients after primary surgery allows for sequential measurements of TIMP-1 during treatment. It should also be mentioned that an advantage of using TIMP-1 measurements in prognostic evaluation is that the measurements are completely objective as opposed to parameters such as tumor grade and nodal status, which rely on the subjective determination by a pathologist.

Recently a number of preanalytical issues concerning the use of blood, especially serum, in tumor marker studies have been stressed (19–23). In general, levels of TIMP-1 measured in serum are higher compared with the levels in the corresponding plasma samples. This is believed to be caused by the fact that serum contains platelets, which store TIMP-1 in their α-granules. Upon blood coagulation these platelets are activated and disintegrated and may thus release TIMP into the sample resulting in an increase in the level of the inhibitor. Based on this, plasma has been suggested to be a better choice for TIMP-1 measurements. To investigate the usability of plasma versus serum in TIMP-1 tumor marker studies we analyzed the association between TIMP-1 measurements in the two types of specimens. We found that the TIMP-1 levels in serum were generally higher than the TIMP-1 levels determined in plasma as was expected from the above mentioned discussion. However, plasma and serum measurements correlated significantly with each other with a Pearson correlation coefficient of 0.75. Thus, based on the present study it appears that the two types of specimens carry similar informa-

---

**Table II**

*Multivariate analysis using Cox proportional hazard model on DFS in node-negative patients (plasma: n = 269, serum: n = 267)*

Neg, negative; Pos, positive.

| Parameter                  | Plasma p value | Plasma Hazard ratio | 95% CI  | Serum p value | Serum Hazard ratio | 95% CI  |
|----------------------------|---------------|---------------------|---------|---------------|---------------------|---------|
| Hormone receptor status    | 0.541         | 1.000               | 0.45–4.62 | 0.790         | 1.000               | 0.36–3.87 |
| Pos                        |               | 1.438               |          |               | 1.175               |          |
| Tumor size (mm)            | 0.996         | 1.000               | 0.41–2.43 | 0.630         | 1.000               | 0.32–2.01 |
| 0–20                      |               | 1.000               |          |               | 0.797               |          |
| 20+                       |               | 1.003               | 0.41–2.43 | 0.942         | 1.000               |          |
| Tumor grade               | 0.916         | 1.000               | 0.48–2.28 | 0.347         | 1.000               | 0.47–2.28 |
| 1                         |               | 1.004               |          |               | 1.030               |          |
| 2–3                       |               | 1.043               |          |               | 1.063               |          |
| Menopausal status          | 0.341         | 1.000               | 0.39–15.05 | 1.000         | 2.352               | 0.40–14.00 |
| Pre                       |               | 2.245               |          |               | 2.352               |          |
| Age (yr)                   |               | 1.000               |          |               | 1.000               |          |
| 20–49                     |               | 1.000               |          |               | 1.000               |          |
| 50–59                     | 0.544         | 1.896               | 0.24–14.93 | 0.443         | 2.222               | 0.29–17.12 |
| 70+                       | 0.641         | 1.222               | 0.53–2.83 | 0.530         | 1.309               | 0.57–3.03 |
| TIMP-1*                   | 0.058*        | 0.098*              |          |               | 0.098*              |          |
| Group Q2 vs. Q1            | 0.026         | 5.671               | 1.23–26.24 | 0.230         | 2.327               | 0.59–9.25 |
| Group Q3 vs. Q1            | 0.123         | 3.480               | 0.71–16.99 | 0.061         | 3.652               | 0.94–14.17 |
| Group Q4 vs. Q1            | 0.012         | 7.054               | 1.54–32.30 | 0.020         | 4.757               | 1.29–17.62 |

* Plasma TIMP-1 groups: Q1, 1–75 ng of TIMP-1/ml of plasma; Q2, 76–88 ng of TIMP-1/ml of plasma; Q3, 89–109 ng of TIMP-1/ml of plasma; and Q4, 110–600 ng of TIMP-1/ml of plasma. Serum TIMP-1 groups: Q1, 1–127 ng of TIMP-1/ml of serum; Q2, 128–145 ng of TIMP-1/ml of serum; Q3, 146–166 ng of TIMP-1/ml of serum; and Q4, 167–600 ng of TIMP-1/ml of serum.

* Overall p value for all four TIMP-1 groups.
tion although at different overall levels, and accordingly both plasma and serum seem suitable for tumor marker studies. However, as described above, plasma TIMP-1 performed better in the survival analyses suggesting that plasma TIMP-1 is more informative as compared with serum TIMP-1.

In conclusion, tumor tissue levels of TIMP-1 have previously been recognized to carry prognostic information in primary breast cancer. The present study is to our knowledge the first large prospective study showing that this also applies to TIMP-1 measured in blood. This was especially true in the node-negative group of patients when TIMP-1 was measured in plasma even within prognostics groups (i.e. low risk) already defined by the traditional prognostic markers. The obvious advantage of the present study is the short storage time of the samples as well as few freeze/thaw cycles ensuring that no or minimal proteolytic degradation has occurred. Furthermore the highly standardized collection/protocol guaranties samples of high quality and makes comparison between samples highly reasonable. However, it should be emphasized that the results should be validated when the patients have been followed throughout more years. In addition, the results should be tested in an independent patient material. Finally it is important to note that if blood is going to be used in the clinical setting important preanalytical conditions, such as for example the impact of blood sampling, handling, and storage; daily variations; and menstrual cycle on the concentration of circulating TIMPs, need to be further elucidated. Thus, future studies should be aimed at addressing these issues.

Acknowledgement—This work was performed in collaboration with the Danish Centre for Translational Breast Cancer Research, Copenhagen, Denmark.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby advertised in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: University of Copenhagen, The Faculty of Life Sciences, Dept. of Veterinary Pathology, Ridebanevej 9, 1870 Frederiksberg C, Denmark. Fax: 45-3535-3514; E-mail: suw@life.ku.dk.

REFERENCES

1. Goldhirsh, A., Glick, J. H., Gelber, R. D., Coates, A. S., Thrulimann, B., and Senn, H. J. (2005) Meeting highlights: international expert consensus on the primary therapy of early breast cancer. Ann. Oncol. 16, 1569–1583

2. Thomassen, C., and Jäncke, F. (2003) Do we need better prognostic factors in node-negative breast cancer? Eur. J. Cancer 36, 293–306

3. Ree, A. H., Flabens, V. A., Berg, J. P., Malslandmo, G. M., Nesland, J. M., and Fodstad, Ø. (1997) High levels of messenger RNAs for tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of distant metastases. Clin. Cancer Res. 3, 1623–1628

4. Nakopoulou, L., Giannopoulou, I., Sefanaki, Κ., Panayotopoulos, E., Tsimpfa, I., Alexandrou, P., Mavromatis, J., Katsarou, S., and Davaris, P. (2003) Enhancement of tissue inhibitor of metalloproteinase-1 (TIMP-1) in breast carcinomas is correlated with adverse prognosis. J. Pathol. 207, 307–313

5. McCarthy, K., Maguire, T., McGreal, G., McDermott, E., O’Higgins, N., and Duffy, M. J. (1999) High levels of tissue inhibitor of metalloproteinase-1 predict poor outcome in patients with primary breast cancer. Int. J. Cancer 84, 44–48

6. Schröhl, A. S., Christensen, I. J., Pedersen, A. N., Jensen, V., Mouridsen, H., Murphy, G., Foekens, J. A., Brünnér, N., and Holten-Andersen, M. N. (2003) Tumor tissue concentrations of the proteinase inhibitors tissue inhibitor of metalloproteinases-1 (TIMP-1) and plasminogen activator inhibitor type-1 (PAI-1) are complementary in determining prognosis in primary breast cancer. Mol. Cell. Proteomics 2, 164–172

7. Schröhl, A. S., Holten-Andersen, M. N., Peters, H. A., Look, M. P., Meijer-van Gelder, M. E., Klijn, J. G. M., Brünnér, N., and Foekens, J. A. (2004) Tumor tissue levels of tissue inhibitor of metalloproteinases-1 as a prognostic marker in primary breast cancer. Clin. Cancer Res. 10, 2289–2298

8. Talvensaari-Mattila, A., and Turpeenniemi-Hujanen, T. (2005) High preoperative serum TIMP-1 is a prognostic indicator for survival in breast carcinoma. Breast Cancer Res. Treat. 89, 29–34

9. Holten-Andersen, M. N., Murphy, G., Nielsen, H. J., Pedersen, A. N., Christensen, I. J., Heyer-Hansen, G., Brünnér, N., and Stephens, R. W. (1999) Quantification of TIMP-1 in plasma from healthy blood donors and patients with advanced cancer. Br. J. Cancer 80, 495–503

10. Egelblad, M., and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. Nature 416, 161–174

11. Hayakawa, T., Yamashita, K., Tazawa, K., Uchiyama, E., and Iwata, K. (1992) Growth-promoting activity of tissue inhibitor of metalloproteinases-1 for a wide range of cells. FEBS Lett. 298, 29–32

12. Luparello, C., Avanzato, G., Carella, C., and Pucci-Minafra, I. (1999) Tissue inhibitor of metalloproteinase (TIMP-1) and proliferative behaviour of clonal breast cancer cells. Breast Cancer Res. Treat. 54, 235–244

13. Murphy, F. R., Issa, R., Zhou, X., Ratnajah, S., Nagase, H., Arthur, M. J. P., Benyon, C., and Iredale, J. P. (2002) Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition. J. Biol. Chem. 277, 11069–11076

14. Guedez, L., Stetler-Stevenson, W. G., and Wolff, L. (1998) In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. J. Clin. Investig. 102, 2002–2010

15. Li, G., Fridman, R., and Kim, H. R. C. (1999) Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. Cancer Res. 59, 6267–6275

16. Liu, X. W., Bernardo, M. M., Fridman, R., and Kim, H. R. C. (2003) 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. 278, 40364–40372

17. Holten-Andersen, M., Stephens, R., Nielsen, H. J., Murphy, G., Christensen, I. J., Stetler-Stevenson, W., and Brünner, N. (2000) High preoperative plasma tissue inhibitor of metalloproteinase-1 levels are associated with short survival of patients with colorectal cancer. Clin. Cancer Res. 6, 4292–4299

18. Rauvala, M., Puiiola, U., and Turpeenniemi-Hujanen, T. (2005) Gelatinases and their tissue inhibitors in ovarian tumors; TIMP-1 is a predictive as well as a prognostic factor. Gynecol. Oncol. 99, 656–663

19. Holten-Andersen, M. N., Brünnér, N., Christensen, I. J., Jensen, V., and Nielsen, H. J. (2002) Levels of tissue inhibitor of metalloproteinases-1 in blood transfusion components. Scand. J. Clin. Lab. Investig. 62, 223–230

20. Holten-Andersen, M. N., Schröhl, A. S., Brünnér, N., Nielsen, H. J., Hougdaill, C. K., and Hougdaill, E. V. (2003) Evaluation of sample handling in relation to levels of tissue inhibitor of metalloproteinases-1 measured in blood by immunoassay. Int. J. Biol. Markers 18, 170–176

21. Jung, K., Laube, C., Lien, M., Lichtinghagen, R., Tschesche, H., Schnorr, D., and Looming, S. A. (1998) Kind of sample as preanalytical determinant of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase-2 in blood. Int. J. Cancer 84, 223–230

22. Jung, K., Meisser, A., and Bischof, P. (2005) Blood sampling as critical preanalytical determinant to use circulating MMP and TIMP as surrogate markers for pathological processes. Int. J. Cancer. 116, 1000–1001

23. Jung, K. (2005) Serum or plasma: what kind of blood sample should be used to measure circulating matrix metalloproteinases and their inhibitors? J. Neuroimmun. 162, 1–2