

Total Levels of Tissue Inhibitor of Metalloproteinases 1 in Plasma Yield High Diagnostic Sensitivity and Specificity in Patients with Colon Cancer¹

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ABSTRACT

Purpose: The purpose of this study was to measure total levels of tissue inhibitor of metalloproteinases (TIMP-1) by ELISA in plasma from blood donors, patients with inflammatory bowel disease (IBD), and patients with cancer and to correlate the results to patient diagnosis.

Experimental Design: Total TIMP-1 plasma levels were measured by ELISA in blood samples from two different blood donor populations from IBD patients, and preoperative samples from patients with primary colon cancer (CC), rectal cancer (RC), or breast cancer.

Results: There were no significant differences in plasma TIMP-1 levels between healthy donors and IBD or breast cancer patients, whereas patients with CC or RC had significantly elevated TIMP-1 levels. Total TIMP-1 levels identified patients with CC with a sensitivity of 63% at 98% specificity, patients with early CC (Dukes' A+B) with a

sensitivity of 56% at 98% specificity, and patients with right-sided CC with a sensitivity of 72% at 98% specificity. Combining carcinoembryonic antigen and TIMP-1 measurements increased the sensitivities obtained from TIMP-1 measurements alone.

Conclusions: TIMP-1 was significantly elevated in plasma from CC and RC patients, including those with early-stage disease. Sensitivity and specificity were both sufficiently high to consider TIMP-1 as a marker for the early identification of CC patients, in particular, those with right-sided CC.

INTRODUCTION

The risk of recurrence and subsequent death of CRC³ is closely related to the stage of disease at the time of primary diagnosis. A reasonable assumption would therefore be that the survival rate of CRC could be improved if more patients could be diagnosed when their cancer is at an early stage. Indeed, recent studies have demonstrated that annual or biannual screening for CRC by FOBT shifts the detection to an earlier stage of cancer (1) and that intervention at this stage can reduce the risk of dying from CRC (2, 3).

CEA is one of the most extensively studied serological tumor markers. Despite the low sensitivity of serum CEA for early-stage CRC (4–6) and local recurrences (7, 8), serum CEA is still considered the standard serum tumor marker in the diagnosis and follow-up of CRC patients. Other potential tumor markers such as CA 242, CA 19-9, and CA 50 have also been studied extensively. However, none of these markers has demonstrated sensitivity for detection of cancer at an early stage (9, 10). Thus, there are no serological markers that currently demonstrate both high sensitivity and high specificity in routine use for the detection of patients with early-stage CRC.

In vitro studies have shown that during the process of cell transformation toward a malignant phenotype, the transformed cells may turn on the expression of proteases and/or their inhibitors (11, 12). In accordance, several reports have shown that proteases, their receptors, and/or their naturally occurring inhibitors can be overexpressed in cancerous tissue (13–16). Recently published data have indicated that some of these proteins, free or bound in complexes, are released from the tumor tissue and can be measured in the circulation (17, 18).

The naturally occurring inhibitors of MMPs, referred to as

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³ The abbreviations used are: CRC, colorectal cancer; CEA, carcinoembryonic antigen; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; IBD, inflammatory bowel disease; CC, colon cancer; RC, rectal cancer; BC, breast cancer; CD, Crohn's disease; UC, ulcerative colitis; ROC, receiver operating characteristic; FOBT, fecal occult blood test; CI, confidence interval; AUC, area under the curve.

TIMPs, form tight 1:1 stoichiometric complexes with the activated forms of MMPs (19, 20) and with their secreted proenzymes (21, 22), thereby regulating enzyme activity (23, 24). Of the four known TIMPs, TIMP-1 and TIMP-2 are the best characterized (19, 25–28). Beyond its well-known enzyme-inhibitory properties, TIMP-1, an M_r 28,000 glycoprotein present in various nonmalignant and malignant human tissues and bodily fluids (29, 30), likely serves additional functions apart from its protease-inhibitory action. Several investigators have demonstrated that TIMP-1 has growth-promoting properties (25, 31, 32), and recent experimental findings support the hypothesis that TIMP-1 might also stimulate tumor growth by inhibiting apoptosis (33, 34). In support of this hypothesis is the finding by Pellegrini *et al.* (35), who reported a positive correlation between serum TIMP-1 and serum levels of bcl-2 in CRC, suggesting an association between TIMP-1 and inhibition of apoptosis. However, this study was based on a limited number of patients.

TIMP-1 is readily measurable in plasma, and we have reported previously that plasma TIMP-1 levels are distributed over a narrow range in healthy individuals (36). In contrast, we and others have reported that patients with various types of metastatic cancer (CRC, BC, bladder cancer, and prostate cancer) have elevated levels of TIMP-1 (36–38). Of particular interest are the results of another study demonstrating that patients with localized prostate cancer have plasma TIMP-1 levels comparable with those of healthy individuals (38), suggesting that high plasma TIMP-1 levels are associated with advanced cancer disease. However, in a recent study by Simpson *et al.* (39), which included a limited number of patients, plasma TIMP-1 levels were found to be significantly elevated in both patients with early-stage CRC and patients with advanced CRC, with patients with metastatic disease having the highest values. Öberg *et al.* (40) have recently reported a significant difference in serum TIMP-1 levels as well between healthy individuals and CRC patients. Here again, Dukes' D patients had the highest TIMP-1 levels. Serum levels of TIMP-1 were also evaluated in a recent study in which it was confirmed that Dukes' D patients have significantly increased TIMP-1 levels when compared with patients with lower Dukes' stages (35). Furthermore, this same study also supported our recent findings that circulating TIMP-1 is a strong and independent prognostic factor for patient survival (41). The observation that plasma TIMP-1 levels are higher in Dukes' D patients compared with Dukes' A, B, or C patients is also in agreement with our previous findings (41).

In the present study, we have measured and compared plasma TIMP-1 levels among healthy blood donors, patients with IBD, and patients with a diagnosis of primary CC, RC, or BC.

PATIENTS AND METHODS

Blood Donors. Two sets of apparently healthy blood donors were included in the study. One set comprised 46 males and 62 females with a median age of 60 years (age range, 35–79 years). These age-matched donors were used as controls in a comparison with IBD and CRC patients. A second set of 69 apparently healthy, female blood donors with a median age of

41 years (age range, 18–58 years) was used as a control for the BC patients.

Patients. A total of 22 males and 28 females (median age, 36 years; age range, 18–85 years) with IBD were included in this study. This group consisted of 26 patients fulfilling the diagnostic criteria for CD (42) and 24 patients fulfilling the diagnostic criteria for UC (43). The clinical disease activity of these patients at the time of their blood draw was scored using a semiquantitative scale (0 = quiescent disease, 1 = mild disease activity, 2 = moderate disease activity, and 3 = severe disease activity) as described previously (44). None of the IBD patients had received systemic glucocorticoid treatment for 1 month prior to the study. However, some patients had received 5-aminosalicylic acid (mesalazine; 1.6–4.8 g daily).

All 588 CRC patients included in the study were referred for elective surgery for CRC based on biopsy-verified or barium enema-suspected primary colorectal adenocarcinoma. All patients were without clinical signs or symptoms of infectious disease, and none had been treated with systemic steroids, antibiotics, or antiviral drugs within 2 weeks prior to study entry. Patients with severe concurrent illness, such as HIV infection or prior cancer, were excluded from the study. All patients had histologically verified adenocarcinoma of the colon or rectum. The diagnosis and stage of CRC was established pathologically from the resected primary tissue and from biopsies of involved lymph nodes or distant metastases, when present. Patients were classified according to Dukes' stage: 58 (10%) Dukes' A patients; 218 (37%) Dukes' B patients; 175 (30%) Dukes' C patients; and 137 (23%) Dukes' D patients (45). A total of 338 patients had CC, and 250 patients had RC. The median age of the patients was 69 years (age range, 33–90 years), with 236 females and 352 males represented. Patients with Dukes' A, B, or C disease underwent complete resection of their tumors, whereas patients presenting with Dukes' D disease had resection of their primary tumor and distant metastases whenever possible. None of the patients received adjuvant chemo- or radiotherapy.

Also included in this study were 322 women with primary BC (stage I + II). The median age of these women was 57 years (age range, 27–90 years). Their patient characteristics appear in Tables 1 and 2.

Blood Samples. Blood samples were obtained with informed consent from all individuals in accordance with the Helsinki Declaration, with permission granted by the Central National Ethical Committees. All blood samples were collected according to a previously described protocol with minimal stasis to prevent platelet activation (36). EDTA plasma and sera were collected preoperatively from the 588 CRC patients and from the 177 apparently healthy blood donors. Nearly all donor sera (105 of 108) and CRC patient sera (577 of 588) were available for CEA analysis. In addition, EDTA plasma samples were obtained from 50 patients with IBD and obtained preoperatively from 322 patients with primary BC. All EDTA plasma and serum samples from CRC patients or healthy volunteers were collected at one sitting.

TIMP-1 ELISA. A rigorously validated kinetic rate ELISA demonstrating low intra- and interassay coefficients of variation (36) was used to measure total TIMP-1 (free and complexed forms). In brief, affinity-purified sheep polyclonal

Table 1 Plasma TIMP-1 levels in healthy blood donors and patients with IBD

Disease/healthy	Clinical activity	<i>n</i>	Gender (males/females)	Median age (yr) (range)	Median TIMP-1 (µg/liter) (range)
Healthy blood donors		108	46/62	60 (35–79)	88.9 (51.0–156) ^a
Female blood donors		69	0/69	41 (18–58)	86.5 (51.2–190) ^b
CD		26	10/16	32 (18–85)	96.9 (39.7–335) ^c
	Remission	7	3/4	30 (22–70)	63.9 (39.7–92.3)
	Low	6	1/5	33.5 (25–76)	82.2 (65.1–112)
	Medium	11	6/5	31 (18–62)	114 (74.2–155)
	High	2	0/2	69 (53–85)	275 (215–335)
UC		24	12/12	41 (19–82)	83.3 (54.9–226) ^d
	Remission	5	1/4	33 (31–63)	75.4 (54.9–92.4)
	Low	8	3/5	44.5 (25–82)	77.6 (61.1–117)
	Medium	9	7/2	33 (19–75)	88.8 (61.6–109)
	High	2	1/1	52.5 (52–53)	165 (104–226)

The 5–95% percentile (µg/liter): ^a 63.3–134; ^b 59.6–151; ^c 45.7–185; ^d 58.0–113.

Table 2 Plasma TIMP-1 levels in patients with CRC

Disease Localization	Dukes' stage	<i>n</i>	Gender (males/females)	Median age (yr) (range)	Median TIMP-1 (µg/liter) (range)
CRC		588	352/236	69 (33–90)	141 (53.7–789) ^a
	A	58	35/23	67 (36–85)	121 (62.6–420)
	B	218	128/90	70 (35–88)	137 (53.7–550)
	C	175	108/67	68 (33–90)	132 (58.0–410)
	D	137	81/56	68 (35–88)	201 (81.0–789)
Colon		338	193/145	70 (37–90)	159 (53.7–789) ^b
	A	19	7/12	67 (49–85)	131 (62.6–283)
	B	140	81/59	72 (47–88)	148 (65.5–550)
	C	94	60/34	70 (37–90)	142 (58.0–410)
	D	85	45/40	68 (39–88)	222 (87.3–789)
Right sided colon		116	55/61	72 (43–88)	169 (53.7–789)
Left sided colon		222	138/84	70 (37–90)	148 (58.0–747)
Rectum		250	159/91	67 (33–74)	126 (64.1–640) ^c
	A	39	28/11	65 (36–84)	118 (64.9–420)
	B	78	47/31	67 (35–85)	117 (74.9–510)
	C	81	48/33	67 (33–83)	124 (64.1–330)
	D	52	36/16	69 (35–83)	160 (81.0–640)
BC		322	0/322	57 (27–90)	95.6 (45.5–289) ^d

The 5–95% percentile (µg/L): ^a 82.7–376; ^b 83.7–411; ^c 81.0–310; ^d 61.1–159

anti-TIMP-1 antiserum (46, 47) was used as a capture antibody in a 96-well microtiter plate. A murine monoclonal anti-TIMP-1 IgG1 (MAC-15; Ref. 48) was used for detection, and a rabbit antimouse immunoglobulin/alkaline phosphatase conjugate (Dako, Glostrup, Denmark) was the secondary antibody that enabled a kinetic rate assay. Rate measurements were collected automatically over a 1-h incubation period in a Ceres 900 plate reader (Bio-Tek Instruments, Winooski, VT). A four-parameter fitted standard curve was generated using Kineticalc II software (Bio-Tek Instruments, Winooski, VT), from which the total TIMP-1 concentration of each sample was calculated. Because the monoclonal detection antibody MAC-15 binds both free TIMP-1 and TIMP-1 in complex with MMPs (48),⁴ total TIMP-1 levels of the sample captured by the sheep polyclonal anti-TIMP-1 antibody were determined by the ELISA. In the present study, the intra-assay coefficient of variation for the

TIMP-1 ELISA was 5.3% ($n = 16$), and the interassay coefficient of variation was 7.4%, determined using an internal, pooled plasma control included on each plate (plasma pool median, 60.7 µg/liter; SD, 4.4 µg/liter; $n = 50$).

CEA EIA. A commercially available solid-phase, chemiluminescent EIA kit (Immulite CEA; Diagnostic Products Corporation, Los Angeles, CA) was used for CEA determination. This assay has a detection limit of 0.2 µg/liter, recovery of approximately 100%, and intra- and interassay variations of 5% ($n = 20$) and 6% ($n = 10$), respectively (49).

Statistical Methods. The SAS software package (version 6.12; SAS Institute, Cary, NC) was used to manage patient data and to perform statistical analyses. Descriptive statistics for TIMP-1 and CEA levels included the median, range, percentiles, and 95% CIs of the median. Rank statistics were used to calculate correlation coefficients and to test hypotheses on location. Sensitivity and specificity for varying cutoffs were calculated. For the combination of TIMP-1 and CEA, sensitivity and specificity were calculated by logistic regression on the log-transformed values. Comparisons of sensitivity at a given

⁴ M. N. Holten-Andersen, unpublished observations.

specificity for TIMP-1 versus the combination of TIMP-1 and CEA were performed using the McNemars test. Sensitivity and specificity were summarized in ROC curves with the AUC and its SE calculated as described by Hanley and McNeil (50). The significance level was set at 0.05.

RESULTS

Plasma TIMP-1 Levels. TIMP-1 was measurable in all samples analyzed. For the 108 healthy donors, the median TIMP-1 level was 88.9 $\mu\text{g/liter}$ (range, 51.0–156 $\mu\text{g/liter}$); a narrow range of TIMP-1 levels was thus found in healthy donors. There was a nonsignificant trend toward a correlation between age and TIMP-1 levels in these healthy donors (Spearman-Rank test, $\rho = 0.17$; $P = 0.08$). No significant difference in plasma TIMP-1 levels could be seen between male and female blood donors (Mann-Whitney test, $P = 0.76$).

The median TIMP-1 level in plasma from patients with IBD was 89.0 $\mu\text{g/liter}$. Four patients with severe disease activity presented with the highest TIMP-1 levels in their disease group. There was no statistically significant difference between TIMP-1 levels from healthy donors and those from patients with IBD (Mann-Whitney test, $P = 0.45$). Separating this group into patients with CD and patients with UC, no statistically significant differences in TIMP-1 levels were found (Mann-Whitney test, $P = 0.32$). When patients with CD were stratified into subgroups according to clinically assessed disease activity, there was a statistically significant difference in TIMP-1 levels (Kruskal-Wallis rank, $P = 0.0005$) among the groups, with patients with severe disease activity having the highest TIMP-1 levels. This suggests a potential association between TIMP-1 and CD activity. In contrast, no statistically significant differences in TIMP-1 levels were found among the patients with UC (Kruskal-Wallis rank, $P = 0.1$).

The median TIMP-1 level for all CRC patients was 141.1 $\mu\text{g/liter}$ (range, 53.7–789 $\mu\text{g/liter}$). When comparing TIMP-1 levels in healthy donors with either CRC, CC, or RC patients, highly significant differences were found (Mann-Whitney test, $P < 0.0001$). Similarly, highly significant differences in TIMP-1 levels were found when comparing IBD patients with either CRC, CC, or RC patients (Mann-Whitney test, $P < 0.0001$). Comparing CC and RC patients revealed that the former had significantly higher TIMP-1 levels (Mann-Whitney test, $P < 0.0001$), with a median TIMP-1 value of 159 $\mu\text{g/liter}$ (95% CI, 148–167 $\mu\text{g/liter}$) for the CC patients and 126 $\mu\text{g/liter}$ (95% CI, 119–132 $\mu\text{g/liter}$) for the RC patients. Fig. 1 summarizes the TIMP-1 levels of healthy donors and patients. Median TIMP-1 levels and ranges for healthy donors and IBD patients are given in Table 1, and those for all cancer patients are given in Table 2.

CEA. The median CEA level in serum of healthy donors was 1.8 $\mu\text{g/liter}$ (range, 0.4–8.2 $\mu\text{g/liter}$; 5–95% percentile, 0.6–5.9 $\mu\text{g/liter}$) and 3.8 $\mu\text{g/liter}$ (range, 0.3–9820 $\mu\text{g/liter}$; 5–95% percentile, 0.8–206.0 $\mu\text{g/liter}$) in CRC patients. Dividing the CRC patients into CC patients and CR patients, the median CEA levels were 4.0 $\mu\text{g/liter}$ (range, 0.5–9820 $\mu\text{g/liter}$; 5–95% percentile, 0.8–286.0 $\mu\text{g/liter}$) and 3.5 $\mu\text{g/liter}$ (range, 0.3–7940 $\mu\text{g/liter}$; 5–95% percentile, 0.9–61.7 $\mu\text{g/liter}$), respectively. The differences in serum CEA levels in healthy

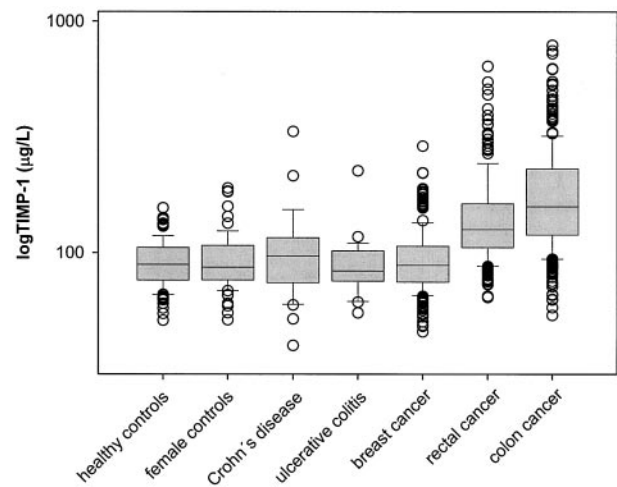


Fig. 1 Box and whiskers plot of plasma TIMP-1 levels (log scale) in healthy controls, healthy female controls, and patients with CD, UC, BC, RC, and CC. Boxes indicate 25th, 50th, and 75th centiles. Whiskers indicate 5th and 95th centiles.

donors and in CRC, CC, or RC patients were highly statistically significant (Mann-Whitney test, $P < 0.0001$). When comparing serum CEA levels in CC and RC patients, it was found that CC patients had higher serum CEA levels; however, this difference was not significant (Mann-Whitney test, $P > 0.05$).

Specificity and Sensitivity of TIMP-1. Fig. 2 illustrates the relationship between specificity and sensitivity of TIMP-1 measurements (ROC curves) for the detection of CC or RC. The curves are based on TIMP-1 levels in 108 healthy donors, 338 CC patients, and 250 RC patients. Also shown are ROC curves for patients with early-stage cancer: 159 Dukes' A and B CC patients and 117 Dukes' A and B RC patients. At 95% specificity, a sensitivity of 65% is achieved for TIMP-1 for CC patients, and a sensitivity of 42% is achieved for TIMP-1 for RC patients. Setting the specificity to 98% only slightly reduces the sensitivity for CC patients to 63% but significantly reduces the sensitivity for RC patients to 36%. For early-stage CC patients (Dukes' A and B), sensitivity was 58% and 56% at 95% or 98% specificity, respectively. Similarly, for RC patients, sensitivity for early-stage disease was 35% and 30% at the respective 95% and 98% specificity cutoffs. The AUC for all CC and RC patients was 0.89 (SE = 0.01) and 0.83 (SE = 0.02), respectively. For early-stage disease alone, the AUC was 0.87 (SE = 0.02) for CC and 0.81 (SE = 0.03) for RC.

When patients with right-sided CC (primary tumor localization in the cecum, ascending colon, or hepatic flexure) were analyzed separately, TIMP-1 sensitivity increased to 75% at 95% specificity and to 72% at 98% specificity (Fig. 3). For early-stage patients with right-sided CC, sensitivity of 69% and 65% was obtained at 95% and 98% specificity (Fig. 3). The corresponding AUC for all patients with right-sided CC was 0.93 (SE = 0.02). The AUC did not decrease significantly (0.91; SE = 0.03) for right-sided CC patients with early-stage disease.

Sensitivity and Specificity of TIMP-1 and CEA. As seen in Table 3, for CC patients, CEA alone had a sensitivity of 40% and 37% at 95% and 98% specificity, respectively, with a

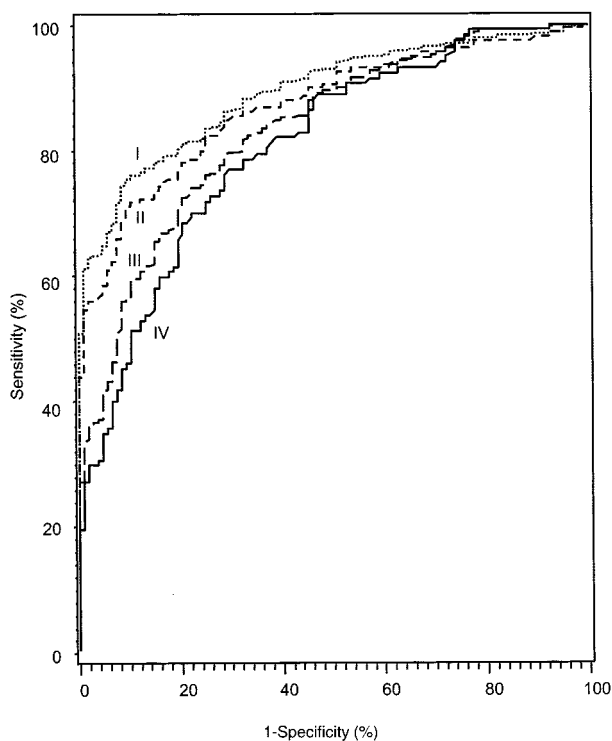


Fig. 2 ROC curves for total plasma TIMP-1 levels in CC (I), RC (III), Dukes' stage A + B CC (II), and Dukes' stage A + B RC patients (IV). AUC: I, 0.89 (SE = 0.02); II, 0.87 (SE = 0.02); III, 0.83 (SE = 0.02); and IV, 0.81 (SE = 0.03).

corresponding AUC of 0.75 (SE = 0.02). When combining TIMP-1 and CEA measurements in these patients, an improvement in sensitivity was observed from 65% to 75% and 63% to 67% at 95% ($P < 0.0001$) and 98% specificity ($P = 0.1$), respectively. The AUC for the combination of TIMP-1 and CEA also increased significantly to 0.91 (SE = 0.01). Of note, the addition of CEA also improved sensitivity for patients with early-stage CC and for patients with right-sided CC, as illustrated in Fig. 4 by ROC curves of TIMP-1, CEA, and the combination of the two markers. For CEA, a sensitivity of 50% and 46% was obtained at 95% and 98% specificity in patients with right-sided CC. The combination of CEA and TIMP-1 increased sensitivity to 82% and 74% at 95% and 98% specificity, respectively, demonstrating that the addition of CEA increased the sensitivity of TIMP-1 alone from 75% to 82% at 95% specificity ($P = 0.02$) and from 72% to 74% at 98% specificity ($P = 0.44$) in patients with right-sided CC. The corresponding AUC increased from 0.93 (SE = 0.02) for TIMP-1 alone to 0.95 (SE = 0.01) for the combination of TIMP-1 and CEA in this same patient cohort. For RC patients, CEA alone showed a sensitivity of 37% and 33% at 95% and 98% specificity (AUC, 0.71). However, the combination of markers increased sensitivity to 54% and 39% at 95% ($P < 0.0001$) and 98% specificity ($P = 0.39$), respectively, with an AUC of 0.86. The sensitivity and specificity data are summarized in Table 3.

Patients with Primary BC. To assess its expression in other cancers, we measured TIMP-1 concentration in plasma

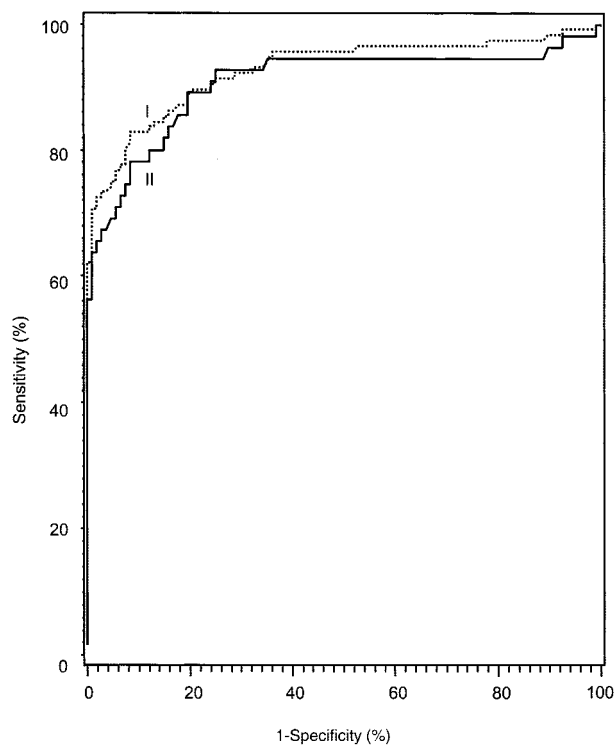


Fig. 3 ROC curves for total plasma TIMP-1 levels in right-sided CC patients (I) and Dukes' A + B right-sided CC patients (II). AUC for each ROC curve: I, 0.93 (SE = 0.02); and II, 0.91 (SE = 0.03).

samples from 322 women with primary BC. The median level was 95.6 $\mu\text{g/liter}$ (range, 45.5–289 $\mu\text{g/liter}$; 5–95% percentile, 61.1–159 $\mu\text{g/liter}$). As a control, we measured TIMP-1 levels in the second set of 69 age-matched, healthy female donors. The median TIMP-1 level in this control group was 86.5 $\mu\text{g/liter}$ (range, 51.2–190 $\mu\text{g/liter}$; 5–95% percentile, 59.6–151 $\mu\text{g/liter}$), which was not significantly different from the level found in the larger, gender-mixed donor population (Mann-Whitney test, $P = 0.8$). No correlation was evident between TIMP-1 levels and age for the healthy female donors (Spearman rank: $\rho = 0.012$; $P = 0.93$). Moreover, there was no statistically significant difference in TIMP-1 levels between the primary BC patients and either set of healthy controls (Mann-Whitney test, $P = 0.8$). TIMP-1 levels for the female donors and the BC patients are illustrated in Fig. 1.

DISCUSSION

The present study is the first to suggest that total plasma TIMP-1 could be a new, sensitive, and specific diagnostic marker for the identification of patients with early-stage CC. We have shown that patients with CC have significantly elevated plasma TIMP-1 levels compared with both healthy individuals and patients with IBD, a benign condition frequently present concomitantly with CC. Of particular interest was the observation that TIMP-1 measurements were very sensitive in the identification of both early-stage (Dukes' A + B) and right-sided CC. Furthermore, the addition of CEA significantly increased the sensitivity of TIMP-1 in identifying CC, albeit only

Table 3 Calculated sensitivities of TIMP-1 and CEA for screening for CRC at 95% and 98% specificity, respectively

Analysis	Cancer	Dukes' stage	n	Sensitivity (%) at 95% specificity	Sensitivity (%) at 98% specificity	AUC	SE
TIMP-1	CRC	All	588	55	52	0.87	0.014
CEA	CRC	All	577	39	35	0.74	0.022
TIMP-1 + CEA	CRC	All	577	65	52	0.89	0.013
TIMP-1	CC	All	338	65	63	0.89	0.015
CEA	CC	All	333	40	37	0.75	0.024
TIMP-1 + CEA	CC	All	333	75	67	0.91	0.015
TIMP-1	CC	A + B	159	58	56	0.87	0.021
TIMP-1 + CEA	CC	A + B	159	66	52	0.89	0.020
TIMP-1	CC right	All	116	75	72	0.93	0.018
CEA	CC right	All	115	50	46	0.80	0.030
TIMP-1 + CEA	CC right	All	115	82	74	0.95	0.015
TIMP-1	CC right	A + B	55	69	65	0.91	0.028
TIMP-1 + CEA	CC right	A + B	55	76	73	0.94	0.023
TIMP-1	RC	All	250	42	36	0.83	0.021
CEA	RC	All	244	37	33	0.71	0.027
TIMP-1 + CEA	RC	All	244	54	39	0.86	0.019
TIMP-1	RC	A + B	117	35	30	0.81	0.029

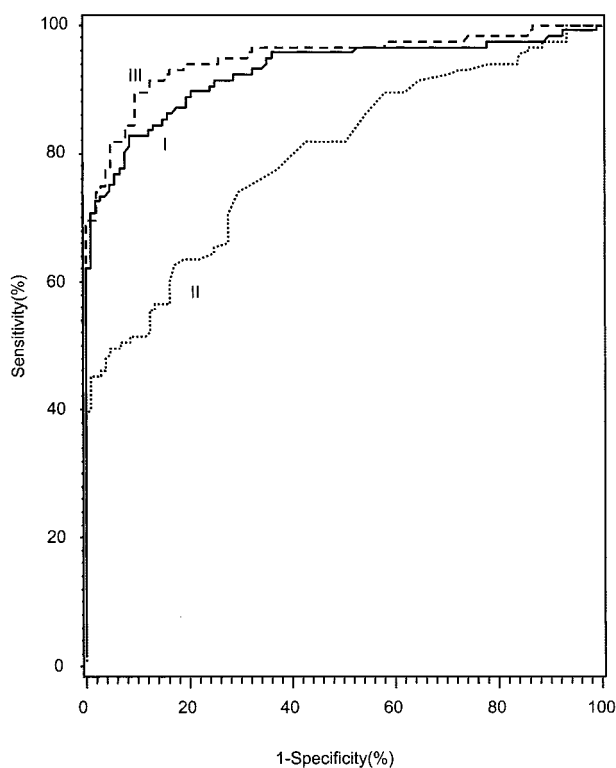


Fig. 4 ROC curves for total plasma TIMP-1 (I), serum CEA (II), and TIMP-1/CEA combined (III) in right-sided CC patients. AUC for each ROC curve: I, 0.93 (SE = 0.02); II, 0.80 (SE = 0.03); and III, 0.95 (SE = 0.02).

at a 95% specificity. This suggests that the addition of other markers, in a panel with TIMP-1, may improve the overall performance of TIMP-1 in this setting.

In this study, we also evaluated patients with RC. Whereas TIMP-1 levels were significantly elevated above the normal or benign controls in the plasma of these patients as well, it was

observed that the RC patients had significantly lower TIMP-1 levels than did CC patients. Consequently, the sensitivity of TIMP-1 was significantly lower for RC compared with CC patients, suggesting that TIMP-1 may have too low a sensitivity and specificity to be useful for the detection of RC.

The assay used detects all forms of TIMP-1 and measures the total amount of TIMP-1 in plasma. It could be speculated that measuring specific fractions of TIMP-1 might provide additional value. However, Zucker *et al.* (51) described an assay for the detection of MMP-9-TIMP-1 and reported on levels found in plasma from patients with gastrointestinal cancer, gynecological cancer, or BC. In that study, a significant difference in MMP-9-TIMP-1 complex levels was observed between control individuals and patients with stage IV gastrointestinal cancer. Complex levels in stage I, II, and III gastrointestinal cancer, as well as in the two other cancer types, were not significantly different from those in controls. Zucker *et al.* (51) concluded that the complex between MMP-9 and TIMP-1 may carry prognostic potential in the identification of metastatic disease but does not offer diagnostic potential in the detection of early-stage cancer of these specific types.

The specificity of a marker for the detection of early-stage CC in an average-risk population should be high to avoid a large number of false-positive results. We have shown that the normal range for TIMP-1 in healthy donors is relatively narrow, thus facilitating identification of individuals with marginally increased levels due to disease. Moreover, TIMP-1 levels in the two healthy donor populations in the present study were similar to those reported in a cohort of 100 healthy individuals from a previous study (median TIMP-1 level, 71.2 $\mu\text{g/liter}$; range, 49.0–121.4 $\mu\text{g/liter}$; Ref. 36), further supporting the low expression and tight distribution of TIMP-1 levels found in the normal population. This finding has been reproduced by two recent independent studies (39, 40).

To have value as a marker for detecting early-stage CC, it is also important that TIMP-1 is present at normal levels in patients with nonmalignant conditions, such as IBD. We found that TIMP-1 was not elevated in patients with quiescent, mild,

or moderate IBD. Only those with severe IBD had elevated TIMP-1 levels. These severe IBD patients typically present with acute symptoms and require immediate medical attention, distinguishing them clinically from other patients. As their condition improves and they return to a more quiescent stage of disease, TIMP-1 levels would likely return to normal. TIMP-1 elevations in this patient subset should not, therefore, confound a differential diagnosis of CC. Furthermore, in a recent report, normal levels of plasma TIMP-1 were found in patients with rheumatoid arthritis or osteoarthritis (52). However, most probably because of the wide distribution of TIMP-1 in serum caused by release of TIMP-1 from platelets during coagulation (53, 54), some reports have described increased serum TIMP-1 levels in patients with rheumatoid arthritis (55, 56).

Recent studies have reported normal TIMP-1 levels in patients with primary prostate cancer and renal cell cancer (38, 57). We also measured TIMP-1 in patients with other malignant disease. Our data from 322 patients with primary BC support this finding that plasma TIMP-1 is not elevated in patients with nonmetastatic cancer. However, we (36) and others (38) have shown that patients with metastatic disease, *e.g.*, breast and prostate cancer, often present with elevated levels of TIMP-1 and that absolute values have prognostic significance. This finding could imply that increasing TIMP-1 levels in these patients may signify progression from early-stage to metastatic disease.

To maximally identify cancer patients using a particular detection assay, its clinical sensitivity must be high. The sensitivity seen in the present study indicates that our plasma TIMP-1 assay detects the large majority of CC patients, including those with early-stage disease. Furthermore, the test is capable of detecting both right- and left-sided CC, the former of which is often otherwise difficult to detect due to a location too proximal to be visualized with a flexible sigmoidoscope.

A commonly accepted method for detection of early CC is testing for blood in the stool (FOBT). The sensitivity of FOBT, as reported in recent literature, ranges from 50–83 at 98% specificity (58) in the general, average-risk population. Our data indicate that TIMP-1 measurements provide comparable sensitivity. It should be stressed, however, that our data were obtained by analyzing preoperative samples from symptomatic patients with CRC. Evaluating TIMP-1 in a prospective screening population might result in different sensitivity or specificity.

The addition of CEA to TIMP-1 resulted in a significant increase in overall diagnostic sensitivity without compromising specificity. In addition to TIMP-1, we have evaluated members of several other families of proteases, their receptors, and inhibitors, as well as molecules involved in tumor angiogenesis, both in plasma and serum, as potential markers for early-stage CC (18, 59–62). Because CC is a heterogeneous disease, it is likely that a combination of markers in a panel will be necessary to optimize the clinical performance of any detection assay. Potential panel members may include various forms of TIMP-1 itself. In an attempt to improve assay performance, we are conducting additional studies evaluating the clinical significance of subfractions of total TIMP-1. Determining ratios of the various forms of TIMP-1 may improve clinical performance. Furthermore, to validate TIMP-1 as a marker for CC, a large prospective screening study should be performed.

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