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Plasma TIMP-1 and CEA as Markers for Detection of Primary Colorectal Cancer: A Prospective Validation Study Including Symptomatic and Non-symptomatic Individuals

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Abstract. *Background/Aim: The combination of plasma tissue inhibitor of metalloproteinases-1 (1) and CEA has been shown to have utility in early detection of colorectal cancer (2). A prospective study was performed to validate previous findings. Patients and Methods: Individuals undergoing large bowel endoscopy were prospectively included (N=1965). Baseline data and co-morbidity were recorded. The primary end-point was the detection of CRC. Plasma was obtained before endoscopy and TIMP-1 and CEA levels were determined using an automated analysis platform when all samples were collected. Results: CRC was detected in 32 individuals, 24 with colonic cancer (CC) and 8 with rectal cancer (RC). Other findings were 265 with adenomas and 889 with non-neoplastic pathology. The biomarker levels were elevated in plasma from patients with CRC, but also from patients with various co-morbidities compared to individuals without any findings at endoscopy. Univariate analysis demonstrated that both markers were significant predictors of CRC. The odds ratios (OR) for an elevated TIMP-1 level for the detection of CRC was 6.2 [95% confidence interval (CI)=3.1-13.0, p<0.0001] and for an elevated CEA level was 2.4 (95% CI=1.9-2.9, p<0.0001). A subset analysis with CC as the end-point showed an OR for TIMP-1 of 7.0 (95% CI=3.2-15.3, p<0.0001). Multivariable analysis including TIMP-1, CEA and age resulted in an OR for TIMP-1 of 2.0 (95% CI=0.7-5.2, p=0.078) and for CEA the OR was 2.2 (95% CI=1.8-2.8, p<0.0001). Conclusion: This prospective study validates a previous study testing the detection of CRC based on TIMP-1 and CEA levels (3).*

Population screening for CRC using faecal occult blood test (FOBT) results in more individuals being diagnosed with an early-stage disease (4, 5), which translates into improved survival compared to CRC cases not detected by screening (6, 7). However, due to a relatively low compliance with and thereby a low clinical sensitivity of FOBT screening (8-10), improved methods for screening and early detection of CRC are required (11-13).

Carcinoembryonic antigen (CEA) in serum was the first soluble biomarker for use in CRC and is still the only soluble biomarker recommended for used for monitoring purposes in CRC (14). However, the level of CEA is strongly dependent on the stage of disease, with a low positive rate being found in early-stage disease, and therefore the sensitivity of CEA in screening rays between 8% and 89% at specificities of 70% to 95% (15). The latest ASCO guidelines, accordingly, state that CEA is not recommended for use as a screening test for CRC (15).

Plasma tissue inhibitor of metalloproteinases-1 (TIMP-1) has been suggested for the early detection of CRC (1), as

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high plasma TIMP-1 levels were shown to identify patients with colonic cancer (CC) with a sensitivity of 63% at 98% specificity, patients with early CC (stage I and II) with a sensitivity of 56% at 98% specificity, and patients with right-sided CC with a sensitivity of 72% at 98% specificity (1). A subsequent study by independent investigators confirmed that the level of plasma TIMP-1 protein may be an important marker in early detection of CRC, showing 42% sensitivity at 95% specificity (16). Of specific interest was that by combining TIMP-1 with CEA measurements, the discrimination was significantly improved (1). Most previous studies used retrospectively collected plasma samples from patients with known CRC and healthy blood donors as control individuals. This approach may introduce bias since blood donors may not be representative of a CRC-related background population. Another potential confounder in such studies is that samples from patient cohorts and healthy volunteers may not be collected simultaneously and therefore not according to similar standard operating procedures.

The Tumor Marker Utility Grading System (TMUGS) guidelines (17) suggest that retrospectively obtained results must be prospectively validated in order for a biomarker to reach clinical acceptance and subsequent implementation. Such prospective studies should take all possible pre-, per- and postanalytical aspects into consideration, including the use of strict and identical sampling, handling and storage procedures for specimens from all recruited individuals (18). Furthermore, external validation is necessary for the final evaluation of a test for screening of CRC.

The present prospective study was designed to evaluate the utility of CEA and TIMP-1 as screening markers for CRC in order to validate our earlier prospective study (3). The present study was based on an independent population consisting of both symptomatic and non-symptomatic individuals from another continent (Australia) and therefore with a different distribution of baseline characteristics.

The design of the entire protocol and subsequent study was similar to the previous study, including blood collection, handling and storage (19). The St. George Hospital and Community Health Service Kogarah, NSW, Australia, approved the protocol (no. 04/122) including the Case Record Form), and the study was performed according to the Helsinki II declaration. In the informed consent, requests to analyse other relevant already established or new biomarkers pending *ad hoc* permission from the Ethics Committee were included and approved with the signature of the recruited individuals. We followed the REMARK guidelines whenever applicable for reporting the study (20).

Baseline data were collected on each recruited individual and included data on co-morbidity, medication and lifestyle. The latter two were self-reported. All findings and co-morbidities were registered using ICD-10 codes (HTTP://WWW.WHO.INT/CLASSIFICATIONS/ICL/EN/).

Table I. *Patients' characteristics*.

Finding	N	Male, (%)	Median age, years	Comorbidity, n (%)
Colon cancer	24*	75.0	65	17 (70.8)
Rectal cancer	8	75.0	71	4 (50.0)
Adenoma	271	60.2	65	224 (82.7)
Non-malignant	898	47.4	65	700 (78.0)
No finding	789	45.8	56	553 (70.1)

*Carcinoembryonic antigen (CEA) and Tissue inhibitor of metalloproteinases-1 (TIMP-1) levels were available for 23.

Plasma levels of TIMP-1 and CEA were determined using the Abbott ARCHITECT® i2000 automated immunoassay system utilizing a two-step dual monoclonal immunoassay. The ARCHITECT® i2000 is a high-throughput immunoassay analyser that utilizes paramagnetic microparticle capture and chemiluminescent detection technology. Assays were run at the Abbott Centrer of Excellence Research Laboratories in Munich, Germany. Abbott in-house research prototype TIMP-1 reagents (21), and on-market ARCHITECT CEA reagents were used for these analyses.

Patients and Methods

Statistics. The database was managed and calculations performed using the SAS system (SAS v9.2; SAS Institute, Cary, NC, USA). Descriptive statistics are presented by the median, minimum and maximum for continuous variables. Tests for location were performed using the Mann–Whitney test and the proportion of all possible pairs with the first biomarker level exceeding the other are presented. The estimation of differences between levels of the two biomarkers for individuals with co-morbidity or not, and lifestyle variables was performed using a linear model with the biomarkers log-transformed and adjusted for gender and age. The probability of CRC was estimated using logistic regression analysis modelling the probability for CRC. Goodness of fit was assessed using the Hosmer–Lemeshow test. Biomarkers were initially considered as continuous variables on the log scale and assessment of linearity was carried out. Continuous biomarker levels were scored using the log-transformed values (log base 2). The odds ratios (ORs) of CRC versus not having CRC are presented with 95% confidence intervals (CIs). Receiver operating characteristic curves (ROC) were estimated and the areas under the ROC curve (AUC) were calculated. Multivariable analyses were carried out on the entire set, as well as subsets, using logistic regression analysis. Age is categorized and entered as a class variable. The multivariable analyses also include covariates: gender, co-morbidity and lifestyle variables. Model selection was made including tests for possible interactions and the final multivariable model only included covariates which were significant. *p*-Values less than 5% were considered significant.

Table II. *Distribution of TIMP1* and CEA*.*

	Biomarker	N	Median	Minimum	Maximum	Lower quartile	Upper quartile
Adenoma	TIMP1	265	91.5	38.5	297.7	81.8	104.4
	CEA	265	1.3	0.0	55.8	0.7	2.1
Colonic cancer	TIMP1	23	108.1	71.1	343.9	95.7	138.7
	CEA	23	2.7	0.2	933.5	0.8	51.4
Non-malignant findings	TIMP1	889	85.9	47.7	325.3	75.2	101.3
	CEA	889	1.1	0.0	40.2	0.6	1.9
No findings	TIMP1	780	83.9	26.3	337.3	74.9	97.4
	CEA	780	1.1	0.0	36.2	0.6	1.8
Rectal cancer	TIMP1	8	98.1	61.4	297.3	71.2	142.6
	CEA	8	8.7	1.6	231.7	2.0	76.2

*Levels of TIMP-1 and CEA are in ng/ml.

Results

Two thousand and five individuals were included in the study and data for 1990 were evaluable. The median age was 59 years (20-104 years). The study included 1,016 males and 974 females. One thousand eight hundred and ninety were of European ethnicity and 98 were not (two had missing data regarding ethnicity).

The results of the endoscopy examination are shown in Table I, classified as colorectal cancer, adenoma, non-malignant findings and no findings, and include demographic data as well as co-morbidity (at least one co-morbidity registered). The TNM stages of the 32 patients with CRC detected were T1: 2, T2: 2, T3: 17, T4: 6, Tx: 1 and 4 were classified as *in situ*. For nodal status, 13 were N0, 11 N1 and 6 N2 (2 were Nx). Seven patients had M1 disease, 12 M0 and 13 Mx.

Complete blood collection sets were available for 1,965 individuals and EDTA plasma was used for biomarker analyses. Plasma TIMP-1 was a median level of 85.9 ng/ml (range 26.3-343.9 ng/ml , lower quartile 75.6, upper quartile 100.8 ng/ml) and plasma CEA had median level of 1.1 ng/ml (range 0-933.5, lower quartile 0.6, upper quartile 1.9 ng/ml). The rank correlation (Spearman) between the two biomarkers was 0.20 ($p<0.0001$). Similarly the correlations between TIMP-1, CEA and age were 0.49 ($p<0.0001$) and 0.25 ($p<0.0001$) respectively – both biomarker levels increase with age.

The distributions of TIMP-1 and CEA stratified by the results of the endoscopic examination are shown in Table II. There were 32 patients detected as having CRC, however one did not have CEA or TIMP-1 levels available.

The presence of co-morbidity was associated with the levels of the two biomarkers. Analysis of the levels of the biomarkers and the selected co-morbidities using a linear model and adjusted for age and gender are shown in Table III. The Table shows the relative difference between those with the denoted co-morbidity and those individuals not having any co-morbidity and include the 95% CI for the difference. Plasma TIMP-1 was significantly elevated in those with cardiovascular disease, chronic lung disease and diabetes, whereas CEA was only significantly elevated for those with cardiovascular disease.

The primary purpose of the study was to assess the discrimination between those with CRC and the remaining study subjects based on the biomarker levels. Univariate analyses between the results of the endoscopic examination and TIMP-1 and CEA are shown in Table IV. The Table gives the AUC under the ROC curve as a measure of discrimination for each pairwise comparison for TIMP-1 (upper) and CEA (lower). It can be seen that both TIMP-1 and CEA are significantly elevated for those with CC.

A univariate analysis of patients with CRC versus the remaining individuals using a logistic regression analysis demonstrated that TIMP-1, as well as CEA, were associated with the diagnosis of CRC: TIMP-1: OR=6.2, 95% CI=3.1-13.0, $p<0.0001$ (AUC=0.695); CEA: OR=2.4, 95% CI=1.9-2.9, $p<0.0001$ (AUC=0.731). A multivariate analysis including TIMP-1, CEA and age was then performed and resulted in only CEA being significantly indicative of CRC. The OR for TIMP-1 was 2.0 (95% CI=0.7-5.2) and CEA was 2.2 (95% CI=1.8-2.8). The AUC of this model was 0.753. The ROC curves for each of these analyses are shown in Figure 1.

A secondary univariate analysis evaluated the relationships between the two biomarkers and CC only. The results showed that TIMP-1 and CEA were significantly associated with CC [TIMP-1: OR=7.0, 95% CI=3.2-15.3,

Table III. *Association of TIMP-1 and CEA with co-morbidity. Examples of biomarker levels for patients with co-morbidities compared to individuals without co-morbidity adjusted for age and gender.*

Disease group	TIMP1		CEA	
	Elevated by	95% CI	Elevated by	95% CI
Cardiovascular disease	19%	12-26%	24%	0-53%
Chronic lung disease	9%	2-17%	24%	−6-23%
Diabetes	20%	10-30%	−5%	−29-27%
Metabolic disease	5%	−2-11%	−8%	−26-15%

CI: Confidence interval.

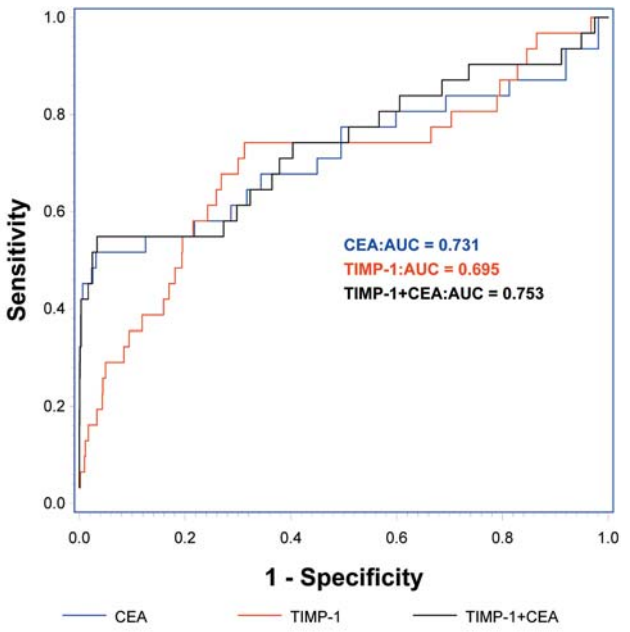

Figure 1. *ROC curves for CEA (blue line), TIMP-1 (red line) and the combined model for the discrimination of CRC (black line) versus all other patients included in the study.*

$p<0.0001$ (AUC=0.742); CEA: OR=2.3, 95% CI-1.80-2.8, $p<0.0001$ (AUC=0.667)]. A multivariable analysis was carried-out including only CEA, TIMP-1 and age due to the small number of CRC cases detected. The multivariable analysis showed that both biomarkers were significantly associated with the diagnosis of CRC (TIMP-1: OR=2.9, 95% CI=1.0-7.8, $p=0.041$; CEA: OR=2.1, 95% CI=1.6-2.7, $p<0.0001$).

Another secondary end-point was the comparison of adenoma *versus* all without significant findings or with non-malignant findings. TIMP-1 and CEA were both significantly associated with the diagnosis of adenoma [OR=2.3, 95% CI=1.6-3.3, $p<0.0001$ (AUC=0.597)] as well as CEA [OR=1.2, 95% CI=1.1-1.3, $p=0.004$, (AUC=0.554)]. However, adjustment for age led to non-significant results (TIMP-1: $p=0.28$; CEA: $p=0.26$).

Discussion

The present study was designed to validate the hypothesis that the levels of soluble TIMP-1 and CEA are associated with presence of CRC. The study was designed as a prospective study consecutively including individuals referred for endoscopy for various reasons, including symptoms which could be attributable to the presence of CRC, but also individuals with no symptoms. The primary end-point was CRC. Due to the design of the study, which is different from that of our previous study (3) in which we predominantly included symptomatic individuals, the present study only had 1.6% (95% CI: 1.1-2.3%) patients with CRC while our previous study had 6.5%. The present study thus more closely resembles a screening population where the incidence of CRC is estimated to be approximately 0.7% (22). The TIMP-1 and CEA measurements were analysed as in the former study and sampling done according to the same Standard Operating Procedure (19). The biomarker levels detected are also remarkably similar to those reported earlier (3).

Our previous prospective study (3) demonstrated a significant association between plasma levels of CEA and TIMP-1 and the presence of CRC. The AUCs for each biomarker and the multivariable analysis were 0.695 for TIMP-1, 0.731 for CEA and 0.752 for both combined, and thus comparable to the results of our previous study (0.71, 0.73 and 0.76, respectively). Of particular interest is that our very first study which was based on a retrospective analysis testing the discriminatory potential of TIMP-1 suggested that the association between plasma TIMP-1 levels and CRC was strongest for CC (1), and this finding was confirmed in the first study (3) as the results demonstrated a higher AUC when restricting the analysis to CC.

The number of patients with CRC was low due to the design of the study and accordingly the statistical power and also the validity of multivariable modelling must be interpreted with caution. The multivariable modelling for the primary end-point was therefore only performed for CEA and TIMP-1 when adjusted for age.

The results obtained on other the end-point, adenoma, were also comparable with our previous results. Both TIMP-1 and CEA levels were able to statistically discriminate those with adenomas from individuals with other non-malignant findings and healthy individuals, but

Table IV. *Discrimination using TIMP1 and CEA between the different endoscopy findings. Pairwise comparisons of the findings. The area under the receiver operating characteristic curve (AUC) for each parameter are shown with the p-value for the Mann–Whitney U-test in parentheses.*

		AUC (*p*-value)		
Endoscopy findings	Parameter	Colonic cancer	Adenoma	Non-malignant
Rectal cancer	TIMP1	0.64 (0.28)		
	CEA	0.64 (0.28)		
Adenoma	TIMP1	0.68 (0.004)		
	CEA	0.64 (0.02)		
Non-malignant	TIMP1	0.73 (0.0001)	0.58 (<0.0001)	
	CEA	0.67 (0.006)	0.55 (0.025)	
No findings	TIMP1	0.76 (<0.0001)	0.62 (<0.0001)	0.53 (0.06)
	CEA	0.68 (0.004)	0.56 (0.003)	0.51 (0.31)

when age was included in the model, the significance disappeared. Therefore a clinical use of plasma TIMP-1 or CEA in identifying individuals with adenoma is doubtful.

The occurrence of co-morbidities were registered and TIMP-1 as well as CEA levels were found elevated for several of these. For example, for cardiovascular disease, the plasma TIMP-1 level was 19% higher than in those not having this co-morbidity. This implies that co-morbidity should be included when using plasma TIMP-1 or CEA levels in the assessment of CRC.

Other blood-based markers have been suggested for the detection of CRC. Detection of methylated Septin 9 DNA has been shown to have high sensitivity and specificity (23). This study was retrospective and the healthy populations does represent a screening population. Testing for methylation of Septin 9 DNA in sera from a screening population (22) would most probably reduce the sensitivity and specificity of the test. Another potential serological marker for CRC is the receptor for the urokinase plasminogen activator (uPAR). Serum levels of uPAR and its cleaved forms was tested in a case–control study demonstrating a significant difference in serum levels of domain I between patients with CRC and patients without malignant findings or no significamt findings (24). However, additional studies are required before the potential value of cleaved forms of uPAR in early detection of CRC can be finally evaluated.

Another non-invasive method for CRC screening is the testing of stool for DNA mutations. This test has been used extensively and has been shown to result in reduced mortality from CRC in screened individuals (25). It has been recently been suggested that the stool DNA test is more accurate than the Septin 9 plasma test in the detection of CRC (2). There is, the poor compliance rate effectively reduces the clinical sensitivity of the test.

A final evaluation of the clinical relevance of the serological or stool test for CRC detection must be based on a stringently designed study which includes covariates that may influence outcome, as well as biomarker levels. Many of the published studies are based on retrospective populations, which may not be representative of screening populations and others are case–control designs. Although these are useful for initially testing potential biomarker usefulness, the hypothesis must be tested in prospective studies. A framework for the development of biomarkers for clinical use has been outlined (26, 27). The question of clinical relevance should also be addressed; Does the screening method provide benefit to the screened individuals (28).

The strength of our studies are its prospective design and inclusion of relevant covariates, the latter could be confounders or mediators. The first study was representative of the population of interest (19); individuals were referred to public hospitals for examination. All of these individuals had symptoms related to CRC. Individuals in the present cohort were examined at two private clinics and not necessarily referred due to symptoms. This may explain the difference in the rate of CRC detected.

In conclusion, our prior reported findings of an association between plasma TIMP-1 and CEA levels and CRC has now been validated in a prospective study including both symptomatic and non-symptomatic individuals. The estimated sensitivities and specificities in the present study, which approximated to our previous findings, are not considered sufficiently high to warrant the use of plasma TIMP-1 and CEA as a screening test for CRC. However, plasma TIMP-1 and CEA are easy to measure and may provide supplemental information and thus be combined with other biomarkers (3).

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