The tumor marker and prognostic impact of cathepsin B, cathepsin L, urokinase-type plasminogen activator and its inhibitor type-1 in colorectal cancer

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ABSTRACT

Background: Cathepsin B and L (CATB, CATL), urokinase-type plasminogen activator (UPA) and its inhibitor PAI-1 play an important part in colorectal cancer invasion. The tumor marker and prognostic impact of these proteases has not been evaluated in the same experimental setting, and compared with that of CEA and CA-19-9.

Methods: Protease, CEA, CA 19-9 serum or plasma levels were determined in 56 patients with colorectal cancer, 25 patients with ulcerative colitis, 26 patients with colorectal adenomas and 35 tumor-free control patients. Protease, CEA, CA 19-9 levels have been determined by ELISA and electrochemiluminescence immunoassay, respectively; their sensitivity, specificity, diagnostic accuracy have been calculated and correlated with clinicopathological staging.

Results: The proteases antigen levels were significantly higher in colorectal cancer than in other groups. Sensitivity of PAI-1 (94%), CATB (82%), UPA (69%), CATL (41%) were higher than those of CEA or CA 19-9 (30% and 18%, respectively). PAI-1, CATB and UPA demonstrated a better diagnostic accuracy than CEA or CA 19-9. A combination of PAI-1 with CATB or UPA exhibited the highest sensitivity value (98%). High CATB, PAI-1, CEA, CA 19-9 levels correlated with advanced Dukes stages. CATB (P=0.0004), CATL (P=0.02) and PAI-1 (P=0.01) had a significant prognostic impact. CATB proved the only Dukes-independent predictor variable for survival (P=0.02).

Conclusions: At the time of clinical detection proteases are more sensitive indicators for colorectal cancer than the commonly used tumor markers. Our results may indicate the benefit of multiparametric tumor marker analyses for the diagnosis of colorectal cancer. Determinations of CATB, CATL and PAI-1 have a major prognostic impact in patients with colorectal cancer.
**Background**

Colorectal cancer (CRC) is the most common gastrointestinal cancer in the Western world and it is an important cause of cancer-related death, tumor stage being generally considered the strongest prognostic factor in CRC [1, 2]. Great effort has been dedicated to the search of sensitive and specific markers of the disease and up to now, carcinoembryonic antigen (CEA) and the gastrointestinal cancer-associated carbohydrate antigen (CA 19-9) are the most widely applied markers in gastrointestinal malignancies, e.g., CRC or pancreatic cancer [3-7].

Prediction of survival is another capacity requested to tumor markers and elevated levels of both CEA and CA 19-9 have also been reported to be associated with poor prognosis in CRC [8-12]. However, because of their low sensitivity, CEA and CA 19-9 seem to be unacceptable both for screening for CRC [13, 14]. Therefore, there is a need for a search for additional tumor-related antigens, eligible as tumor markers, in gastrointestinal malignancies.

Tumor cells have been shown to produce and release several proteolytic enzymes, which are thought to be involved in tumor invasion and metastasis [15]. For instance, it has been found that cathepsin B (CATB) and cathepsin L (CATL), which are cysteine proteases, the serine protease urokinase-type plasminogen activator (UPA) and its inhibitor type-1 (PAI-1) play a crucial role in this process through the destruction of various elements of the cell-surrounding extracellular matrix [16-21].

Several human solid tumors have been reported to have increased levels of proteolytic enzymes in cancer tissue, strongly suggesting that proteases may be important in tumor invasion and metastasis. With respect to the gastrointestinal tract, we have previously demonstrated that proteolytic enzymes are widely distributed in gastrointestinal tissues, being implicated in processes of gastrointestinal tissue remodelling and angiogenesis [22], may have
a role not only in the process of esophageal [23], gastric [24, 25] or colorectal cancer invasion [26], but also in the progression of gastrointestinal precancerous changes into cancer [27].

Cathepsins and the plasminogen activator-inhibitor system could be demonstrated in various malignant tissues, e.g., breast cancer [28-30], lung cancer [31, 32], head and neck cancer [33], ovarian cancer [34], gastric cancer [35-38] or CRC [39-45] and might therefore be useful as a diagnostic tool.

With respect to the gastrointestinal tract, some studies, along with our own, have pointed to the prognostic value of proteases for survival, for instance, in gastric cancer [24, 25, 38, 46, 47] and CRC [26, 40, 43, 48-50].

Some studies have demonstrated an elevation of serum or plasma protease levels in patients suffering from CRC [51-56], however, to our knowledge, the tumor marker impact of CATB, CATL, UPA and PAI-1 has not been evaluated in the same experimental setting, and compared with that of the most commonly used gastrointestinal tumor markers, such as CEA and CA-19-9.

Therefore, the objective of the present study was to assess the possible clinical relevance of serum CATB, CATL and plasma UPA, PAI-1 antigen levels in the same CRC patients, compare it with the established serum markers CEA and CA 19-9, and to evaluate any correlation between these parameters and clinicopathological and prognostic staging of CRC.
Methods

The study involved 56 patients with CRC, who underwent colorectal resection, 29 males and 27 females, mean age 65.4 ±12.8 years (range 39-86 years) and 35 tumor-free control patients (controls) with negative gastroscopy and colonoscopy, 12 males and 23 females, mean age 46.3±13.4 years (range 24-85 years). For further comparison we also investigated 25 patients with ulcerative colitis (UC) without dysplasia as confirmed by colonoscopy and biopsy, 11 males and 14 females, mean age: 31.7±6.6 years (range 22-48 years), and 26 patients with colorectal adenoma confirmed by histology after endoscopic polypectomy (samples consisted of 16 tubular adenomas with low grade dysplasia and 10 tubulovillous adenomas with high grade dysplasia), 17 males and 9 females, mean age 57.8±6.9 years (range 47-72 years).

Informed consent was obtained from all patients.

In all instances, this was a first diagnosis of CRC, and no recurrences were taken into consideration. None of the patients received pre- or postoperative adjuvant chemotherapy.

Clinical data for the patients and histologic data for tumors were registered accurately. Pathologic staging was obtained for the presence (n= 37) or absence (n=19) of lymph node and/or distant metastases; for differentiation (well differentiated, G1 (n=17); moderately differentiated, G2 (n=30); or poorly differentiated, G3 (n=9). Finally, the tumors were subgrouped according to their tumor location (colon cancer, n=38; rectal cancer, n=18). The tumors were histologically classified according to Dukes classification [57], as modified by Turnbull et al. [58]. Dukes stage A tumors are confined to the bowel wall (n=7); Dukes stage B tumors have spread beyond the wall without involving lymph nodes (n=12); Dukes stage C are associated with regional lymph node metastases (n=23); and finally, Dukes stage D tumors are associated with distant metastases (n=14).

**Blood collection.** Serum and plasma samples were collected from patients with CRC at the time of clinical tumor detection. Serum and EDTA plasma samples were collected from
resting patients after a 12h fast between 8:00 and 10:00 a.m. to avoid possible influences of circadian variations on the fibrinolytic system [59]. The samples were stored at -70°C until analysis.

**Determination of established tumor markers and proteases**

Serum CEA (carcinoembryonic antigen electrochemiluminescence immunoassay “ECLIA”, Cobas®, Roche, Diagnostics, Mannheim, Germany; cut-off limit, 4.0 ng/ml) and serum CA 19-9 (carbohydrate electrochemiluminescence immunoassay “ECLIA”, Cobas®, Roche, Diagnostics, Mannheim, Germany; cut-off limit, 37.0 ng/ml) were determined by the use of commercially available test kits and monoclonal antibodies. Cut-off limits were taken as recommended by the manufacturers.

The assays for CATB, CATL, UPA and PAI-1 have been published elsewhere [22, 26]. Antigen levels were measured by using the enzyme-linked immunoadsorbent assay (ELISA) method as follows: briefly, cathepsin immunoassay is a solid-phase ELISA based on the sandwich principle (BiAss, Diesen, Germany). Absolute quantities of CATB and CATL antigens on the serum samples were calculated from a 7-point standard curve of CATB and CATL (0-250 ng/ml). The lowest detectable concentrations are estimated at ≅ 1 ng/ml.

The UPA antigen was determined by using the TintElize UPA-ELISA (Biopool, Umea, Sweden). The amount of UPA antigen in the plasma samples was calculated from a 6-point standard curve of UPA (0-4 ng/ml). The detection limit is ≅ 0.1 ng/ml for UPA.

PAI-1 antigen quantification was performed by using the Asserachrom PAI-1 ELISA (Diagnostica Stago, Asnières-sur-Seine, France). Absolute quantities of PAI-1 antigen in the plasma samples were calculated form a 7-point standard curve of PAI-1 (0-250 ng/ml). The detection limit is ≅ 0.5 ng/ml for PAI-1.
Statistics

Due to the high standard deviations of some of the series, results were expressed as median levels and, after evaluation by using the Kolmogorov-Smirnov test, differences between groups were tested statistically by using the Mann-Whitney U test and the Kruskall-Wallis analysis of variance, where applicable. Standard linear regression analysis was performed to evaluate the correlation between CATB, CATL, UPA, PAI-1, CEA and CA 19-9. Differences were considered significant with $P<0.05$. The receiver operating characteristics (ROC) curves were used to determine the optimal cut-off values (with the Youden J test for overall accuracy).

The association of proteases and CEA, CEA-19 and survival was tested using their median values in the group of CRC. The Kaplan-Meier method was used to estimate survival probabilities, and the log-rank test was used to test equality of strata. Group-oriented curves for survival were calculated according to the Kaplan-Meier method for CATB, CATL, UPA and PAI-1 antigen levels; CEA and CA 19-9; presence of metastases; Dukes classification; grade; tumor location; age and gender. The Cox proportional hazards model was applied for multivariate analysis. The SAS software package (SAS Institute, Cary, North Carolina) was used to perform statistical analyses.
Results

Serum and plasma concentrations for CATB, CATL, UPA, PAI-1, CEA and CA 19-9 in patients with CRC, UC, colorectal adenoma and controls, expressed in ng/ml, are shown in Table 1. Significantly higher CATB, CATL, UPA and PAI-1 antigen concentrations were observed in CRC patients compared with controls, patients with UC or colorectal adenomas. No statistically significant differences were seen with respect to CEA and CA 19-9 levels. Antigen levels of CATB, CATL and PAI-1 were significantly higher in blood samples from patients with colorectal adenomas than from controls. CATB, CATL, UPA and PAI-1 showed a trend toward higher levels in patients with UC than in controls, but the differences were not statistically significant (Table 1).

Antigen levels of CATB, PAI-1 and CA 19-9, as reported in Table 2, were significantly higher in blood samples from patients with lymph node and/or distant metastases than from patients without metastases. CEA levels also showed a trend toward higher, although this was not significant ($P=0.058$).

With respect to Dukes classification, CATB, PAI-1, CEA and CA 19-9 showed the highest antigen concentrations in patients with Dukes stage D tumors (Table 3).

Antigen levels of CATB, CATL, UPA, PAI-1 and CEA, CA 19-9 levels showed a trend toward higher levels in patients with colon cancer than in patients with rectal cancer, but the differences were not statistically significant (data not shown). No statistically significant changes were observed in association with tumor differentiation (grade), age or gender (data not shown).

Sensitivity was calculated as the percentage of individuals in the tumor groups, who showed concentrations of tumor markers above the respective cut-off limits. Specificity was calculated as the percentage of individuals who had concentrations of the tumor markers within the normal range. The receiver operating characteristics (ROC) curves were used to
determine the optimal cut-off values (with the Youden J test for overall accuracy). The optimal cut-off value for CATB was 4.6 ng/ml (Youden J=0.68), this discriminated 86 patients (61%) below and 56 patients (39%) above the cut-off value. The cut-off for CATL was placed at 1.12 ng/ml (Youden J=0.25), with 102 patients (72%) below and 40 patients (28%) above said level. For UPA, the selected value of 0.21 ng/ml (Youden J=0.53) distinguished between 88 patients (62%) below and 54 patients (38%) above the cut-off value.

The cut-off value for PAI-1 was 18.90 ng/ml (Youden J=0.75), with 75 patients (53%) below and 67 patients (47%) above the cut-off value. Cut-off limits for CEA (4.0 ng/ml) and CA 19-9 (37.0 ng/ml) were taken as recommended by the manufacturers. The cut-off for CEA distinguished between 116 patients (82%) below and 26 patients (18%) above the cut-off value. Finally, the cut-off value for CA 19-9 discriminated 126 patients (89%) below, and 16 patients (11%) above the cut-off limit.

When proteases, CEA and CA 19-9 were used as single markers, sensitivity of PAI-1 (94%), CATB (82%), UPA (69%) and CATL (41%) were more indicative for CRC than CEA or CA 19-9 (30% and 18%, respectively). Specificity of CATB (88%) and PAI-1 (84%) were in the same range than that of the established markers (CA 19-9: 93%, CEA: 89%).

PAI-1, CATB and UPA demonstrated a better diagnostic accuracy than CEA, CATL or CA 19-9, PAI-1 showing the highest accuracy (88%) (Table 4).

Sensitivity and specificity values during multiparametric tumor marker analysis are given in Table 5. When two markers were determined in identical blood samples, combined sensitivity values disclosed the superiority of the combination of PAI-1 together with CATB or UPA (both markers correctly positive in 78% and 64%, respectively; one of two markers correctly positive in 98%) as compared to the combinations of all other markers, including proteases with CEA or CA 19-9, or CEA with CA 19-9. The sensitivity of CEA or CA 19-9 in combination with a protease antigen level was more indicative for CRC than CEA or CA 19-9.
alone (when one of two markers was correctly positive). The combined use of three markers (one protease in combination with CEA and CA 19-9) did not lead to a further increase in sensitivity.

The specificity of CEA or CA 19-9 in combination with a protease antigen level or the combination of CEA with CA 19-9 was more indicative for correctly negative patients than CEA or CA 19-9 alone (when one of two markers was correctly negative). When two protease levels were considered, combined specificity values (on of two markers correctly negative) were also higher than specificity of CATB, CATL, UPA or PAI-1 as a single marker. The combined use of three markers (one protease in combination with CEA and CA 19-9) did not lead to a further increase in specificity (Table 5).

Table 6 demonstrates associations between proteases and CEA/CA 19-9, determined in identical blood samples obtained from CRC patients. When standard linear regression analysis was assessed between proteases, CATB significantly correlated with CATL, UPA and PAI-1. Significant correlations were also found between the antigen levels of PAI-1 and CATL, and finally PAI-1 and UPA. CEA and CA 19-9 also significantly correlated in the group of CRC. When standard linear regression analysis was performed in CRC between CEA, CA 19-9 and proteases, CEA significantly correlated with CATB and PAI-1, while CA 19-9 significantly correlated with CATB, CATL and PAI-1.

The CRC patients were enrolled in a follow-up protocol. The follow-up ended in the event of death or, when the patient was still alive, at the last follow-up date. Patients were followed either directly or through their attending physicians. Thirty-five patients (62.5%) died of tumor recurrence. Their median survival was 32 months (95% CL, 23-38; range, 7-78 months). At the end of follow-up period, 21 patients (37.5%) were still alive; their median follow-up was 86 months (95% CL, 85-91; range 76-96 months). The median survival time calculated for all patients was 45 months (95% CL, 43-61; range 7-96 months). The median
survival for the subgroup of patients who underwent curative resection (Dukes A-C) was 77 months (95%CL, 56-73, range 7-96 months), whereas it was 14 months in the remaining patients with Dukes D tumors (95%CL, 11-19; range, 8-36 months; \( P < 0.001 \)).

No statistically significant difference was observed in association with tumor location (median survival period for patients with colon cancer and rectal cancer: 43 months, 95%CL, 40-62; range, 8-96 months, and 55 months, 95%CL, 37-70, range, 7-94 months, respectively).

The association of proteolytic enzymes and CEA, CEA-19 and survival was tested using their median values in the group of CRC (CATB: 8.75 ng/ml; CATL: 1.1 ng/ml; UPA: 0.29 ng/ml; PAI-1: 52.45 ng/ml; CEA: 2.40 ng/ml; CA 19-9: 9.15 ng/ml).

In a univariate survival analysis the following parameters were significantly correlated with survival: CATB antigen levels \( (P=0.0004) \) (Figure 1); CATL antigen levels \( (P=0.02) \) (Figure 2); PAI-1 antigen levels \( (P=0.01) \) (Figure 3); CA 19-9 \( (P=0.004) \); presence of metastases \( (P=0.0001) \); Dukes classification \( (P=0.0001) \). No significant correlation was observed with respect to UPA antigen levels, CEA, tumor grade, tumor location, age and gender (data not shown).

In a multivariate statistical analysis including CATB, CATL, UPA, PAI-1 antigen levels, CEA, CA 19-9, Dukes classification, presence of metastases, tumor grade, tumor location, age and gender, CATB was found to be the only Dukes-independent prognostic variable, high levels of CATB identified patients with poor prognosis, low levels identified patients with good prognosis \( (\text{CATB: } P=0.02; \text{ Hazard ratio, HR: } 1.1, 95\% \text{ CL: } 1.01-1.13; \text{ Dukes classification: } P=0.03, \text{ HR: } 2.83, 95\% \text{ CL: } 1.08-7.39) \).
Discussion

Among several tumor markers, that are suggested to correlate with the presence and prognosis of CRC, CEA and CA 19-9 are the most widely accepted [3-7]. Increased preoperative serum levels of CEA and/or CA 19-9 in CRC patients were suggested to correlate with poor prognosis [8-12]. However, the rather low sensitivity of serum CEA or CA 19-9, their secretion rates from individual tumors and nonspecific elevations reduce their tumor marker impact and indicate the need for additional more reliable markers for CRC.

Proteolytic mechanisms, such as those that depend on cysteine (CATB, CATL) and serine proteases (UPA, PAI-1) are recognized as crucial factors in tumor invasion and metastasis. We have previously suggested that proteolytic enzymes involved in processes of gastrointestinal tissue remodelling and angiogenesis, may have a role in the process of gastrointestinal carcinogenesis and invasion, and finally, tissue antigen concentrations have a major prognostic impact in patients with gastric cancer and CRC [22-27].

Some studies have suggested the potential impact as tumor markers, of proteases in CRC [51-56]. Given the lack in the literature of a comparison of the tumor marker role and possible prognostic relevance of cathepsins (CATB, CATL) and the UPA/PAI-1 system in the same experimental setting, in the current study, we surveyed the behaviour of CATB, CATL, UPA, PAI-1 in CRC and compared with commonly used gastrointestinal tumor markers CEA and CA 19-9, and then evaluated any correlation between these parameters and clinicopathological staging of CRC.

We confirm the previous observations that preoperative serum CATB [51, 53, 56], UPA [52] or PAI-1 [54, 55] plasma concentrations are significantly higher in the CRC than those found in control non-cancer patients. In addition, with respect to cathepsins, we demonstrated that not only CATB but also CATL is elevated in sera of CRC patients. Interestingly enough, no statistically significant differences were seen in association with CEA and CA 19-9.
In our series, antigen levels of CATB, CATL, UPA, PAI-1 and CEA, CA 19-9 did not reach a statistically significant difference between colon and rectal cancer.

We demonstrate for the first time that antigen levels of CATB, CATL and PAI-1 were significantly higher in blood samples from patients with colorectal adenomas than from controls. Thus our results with previous results obtained in colorectal tissues [42, 60, 61] confirm that CATB, CATL and PAI-1 may be involved in the progression from premalignant colorectal adenoma into CRC.

A trend toward higher antigen levels of CATB, CATL, UPA and PAI-1 were also found in patients with UC than in healthy controls. Elevation of protease levels in patients with UC might be caused by the inflammatory process itself. Indeed, CATB, CATL and UPA have been detected immunohistochemically in tissues obtained from patients with chronic inflammatory bowel disease [62, 63]. Furthermore, patients with inflammatory bowel disease could be shown to have increased UPA levels in the inflamed mucosa as well as in plasma [52, 64, 65]. Our data, however, might indicate that tumor-associated mediators induce a more significant increase in protease production and/or release as compared to mediators associated with inflammatory processes alone [66].

With respect to the correlation between the proposed parameters and clinicopathological staging of CRC, our data are in agreement with previous published results. The finding in our study of significantly higher antigen levels in blood samples from patients with lymph node or liver metastases confirmed that CATB (more significantly so) on the one hand and PAI-1 on the other are involved in CRC progression [51, 54, 55].

When proteases, CEA and CA 19-9 were used as single markers, we found that sensitivity of PAI-1 (94%), CATB (82%), UPA (69%) and CATL (41%) were more indicative for CRC than CEA or CA 19-9 (30% and 18%, respectively). Our data are in agreement with results by
Huber et al. [52]. They reported that sensitivity of UPA was superior to that of the established markers (75.5% vs. 51.5% of both CEA and CA 19-9).

Specificity of CATB and PAI-1 were in the same range than that of CA 19-9 or CEA. PAI-1, CATB and UPA demonstrated a better diagnostic accuracy than CEA or CA 19-9, PAI-1 showing the highest accuracy.

It has been suggested that a combined use of different tumor-associated antigens might be of better clinical value for the detection and follow-up of various cancers [67]. The simultaneous determination of several markers led to a greater sensitivity in our group of CRC patients: PAI-1 combined with CATB or UPA was superior compared to the combination of all other markers, including proteases with CEA or CA 19-9. Furthermore, the sensitivity of CEA or CA 19-9 in combination with a protease antigen level was more indicative for CRC than CEA or CA 19-9 alone. As also shown by others [52], we confirm that the combined use of CEA and CA 19-9 did not lead to further increased sensitivity compared to the exclusive use of CEA or CA 19-9 in the detection of CRC. Furthermore, during the multiparametric tumor marker analysis, the combined use of two markers in all combinations led to a further increase in specificity.

This observation is confirmed by the data we obtained by examining the correlation of the investigated parameters with Dukes staging, an established predictor of prognosis. While CATL and UPA did not show any correlation with the stage, CATB, PAI-1, CEA and CA 19-9 did show a significant increase in patients with advanced stage. Interestingly enough CATB showed the most clear-cut increase.

Increased pre-operative serum levels of CEA and CA 19-9 have been already shown to correlate with shorter disease free and overall survival [8-12]. It was also previously reported that higher serum levels of CATB [51, 53, 56] and plasma levels of PAI-1 [54, 55] are correlated with advanced tumor stage and shorter survival.
In our experience, in a univariate survival analysis not only CATB and PAI-1, but also CATL antigen levels were significant in prediction of survival. High serum CATB, CATL and plasma PAI-1 antigen levels indeed identified patients with shorter survival and those who are at higher risk of death. In addition, CATB proved the only Dukes-independent predictor variable in a multivariate statistical analysis. With respect to the commonly used tumor markers, only CA 19-9 correlated significantly with survival. In our series, no significant correlation was found in association with tumor location.

The data in the literature and our own on patients’ survival suggest that serum CATB, CATL and plasma PAI-1 levels might be more useful than the traditionally used tumor markers.

According to our results, in agreement with Huber et al. [52], the determination of UPA might not be useful as a prognostic tool. UPA, together with CATB and PAI-1, however, might be of relevance for the screening of asymptomatic patients at risk for development of CRC, given that they present an overall diagnostic accuracy higher than those of CEA and CA 19-9.

We report for the first time that serum CATB antigen levels significantly correlate with plasma UPA and PAI-1 levels in CRC, and that a significant correlation is also found between the antigen levels of CATL and PAI-1. This confirms previous data obtained in gastrointestinal cancerous tissues on a concomitant activation of these systems [21, 24, 26, 27, 68, 69], while the simultaneous up-regulation of cysteine and serine proteases in CRC strongly confirms the role of cathepsins and the UPA/PAI-1 system in the biology of CRC.
Conclusion

In summary, our data provide evidence for possible clinical application of the determination of CATB, CATL, UPA and PAI-1 in addition to CEA and CA 19-9 in identical blood samples in patients with CRC. At the time of clinical presentation, serum or plasma protease levels are more sensitive indicators of diagnosis than the most commonly used markers CEA and CA 19-9. Even though the benefits of multiparametric tumor markers analyses are highly questionable, the levels of sensitivity and specificity reached in our experience seem to open the door to such an approach in CRC. On the other hand, we demonstrate the clear-cut prognostic impact of serum CATB, CATL and plasma PAI-1 antigen levels for patients with CRC, which together with Dukes stage, are identified as relevant prognostic factors. Finally, our results suggest that CATB, CATL and PAI-1 may have a crucial role not only in the invasive process of cancer, but also in the progression of colorectal precancerous lesions into cancer.
List of abbreviations

CATB: cathepsin B; CATL: cathepsin L; CEA: carcinoembryonic antigen; CA 19-9: carboanhydrate antigen 19-9; UPA: urokinase-type plasminogen activator; PAI-1: plasminogen activator inhibitor type-1

Competing interests

The author(s) declare that they have no competing interests. There is no conflict of interest, financially or personally, with other people or organization that could inappropriately influence our work.

Authors’ contributions

LH had the initial idea for the study, participated in the study design of the study, performed statistical analysis, generated experimental data and drafted the manuscript.

FF participated in the review and commentary of documents relative to the study and manuscript editing.

RC, GI, MDP and MP generated experimental data.

LDM performed statistical analysis.

ZT participated in the design and coordination of the study.

Each author participated in the study to a significant extent.

All authors read and approved the final manuscript.
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**Figure legends**

**Figure 1. Survival curves stratified by cathepsin B in colorectal cancer**

The association of preoperative serum cathepsin B (CATB) antigen levels and overall survival in patients with primary colorectal carcinoma (n=56). Group-oriented curves for survival were calculated according to the Kaplan-Meier method. By using the median value of 8.75 ng/ml the patients were divided into two groups. High serum CATB antigen levels identified patients with shorter survival. The $P$ value is shown in the figure.

**Figure 2. Survival curves stratified by cathepsin L in colorectal cancer**

The association of preoperative serum cathepsin L (CATL) antigen levels and overall survival in patients with primary colorectal carcinoma (n=56). Group-oriented curves for survival were calculated according to the Kaplan-Meier method. By using the median value of 1.1 ng/ml the patients were divided into two groups. High serum CATL antigen levels identified patients with shorter survival. The $P$ value is shown in the figure.

**Figure 3. Survival curves stratified by plasminogen activator inhibitor type-1 in colorectal cancer**

The association of preoperative plasma plasminogen activator inhibitor type-1 (PAI-1) antigen levels and overall survival in patients with primary colorectal carcinoma (n=56). Group-oriented curves for survival were calculated according to the Kaplan-Meier method. By using the median value of 52.45 ng/ml the patients were divided into two groups. High plasma PAI-1 antigen levels identified patients with shorter survival and those who are at higher risk of death. The $P$ value is shown in the figure.
Table 1. Proteolytic enzymes, CEA and CA 19-9 in patients with colorectal cancer, ulcerative colitis, adenoma and controls

Cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 serum or plasma levels in patients with colorectal cancer (n=56), ulcerative colitis (n=25), colorectal adenoma (n=26) and controls (n=35) expressed in ng/ml (median values and range)

<table>
<thead>
<tr>
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<th>CATB</th>
<th>CATL</th>
<th>UPA</th>
<th>PAI-1</th>
<th>CEA</th>
<th>CA 19-9</th>
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</thead>
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<tr>
<td>Colorectal cancer</td>
<td>8.75*</td>
<td>1.10*</td>
<td>0.29*</td>
<td>52.45*</td>
<td>2.40</td>
<td>9.15</td>
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<td>(n=56)</td>
<td>(2.4-39.3)</td>
<td>(1.0-35.3)</td>
<td>(0.1-0.79)</td>
<td>(13.5-138.6)</td>
<td>(0.4-235.0)</td>
<td>(1.0-540.0)</td>
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<tr>
<td>Ulcerative colitis</td>
<td>4.37</td>
<td>1.10</td>
<td>0.20</td>
<td>12.60</td>
<td>3.15</td>
<td>9.00</td>
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<td>(n=25)</td>
<td>(2.8-7.2)</td>
<td>(1.0-1.7)</td>
<td>(0.18-0.23)</td>
<td>(8.8-17.6)</td>
<td>(0.8-11.5)</td>
<td>(4.5-32.0)</td>
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<tr>
<td>Colorectal adenoma</td>
<td>4.45#</td>
<td>1.25#</td>
<td>0.20</td>
<td>14.05#</td>
<td>3.55</td>
<td>10.27</td>
</tr>
<tr>
<td>(n=26)</td>
<td>(4.2-10.3)</td>
<td>(1.0-6.5)</td>
<td>(0.16-0.53)</td>
<td>(10.5-65.5)</td>
<td>(0.7-5.7)</td>
<td>(3.7-41.0)</td>
</tr>
<tr>
<td>Controls</td>
<td>3.80</td>
<td>1.00</td>
<td>0.19</td>
<td>10.70</td>
<td>2.90</td>
<td>8.90</td>
</tr>
<tr>
<td>(n=35)</td>
<td>(1.4-5.8)</td>
<td>(1.0-2.1)</td>
<td>(0.16-0.27)</td>
<td>(1.87-23.8)</td>
<td>(0.8-18.9)</td>
<td>(1.27-47.4)</td>
</tr>
<tr>
<td>Kruskall-Wallis</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P=NS</td>
<td>P=NS</td>
</tr>
<tr>
<td>analysis of variance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P value)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics:

* Colorectal cancer:  \( P<0.05 \) vs Ulcerative colitis and Colorectal adenoma
# Colorectal adenoma:  \( P<0.001 \) vs Controls

Abbreviations: CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carbohydrate antigen 19-9; NS: not significant
Table 2. Proteolytic enzymes, CEA and CA 19-9 in correlation with metastases in patients with colorectal cancer

Cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 serum or plasma levels in association with the presence or absence of metastases in colorectal cancer expressed in ng/ml (median values and range)

<table>
<thead>
<tr>
<th></th>
<th>With Metastases (n=37)</th>
<th>No Metastases (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td></td>
</tr>
<tr>
<td>CATB</td>
<td>17.80 (3.6-39.3)</td>
<td>6.70 (2.4-37.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CATL</td>
<td>1.20 (1.0-35.2)</td>
<td>1.00 (1.0-35.3)</td>
<td>NS</td>
</tr>
<tr>
<td>UPA</td>
<td>0.26 (0.1-0.79)</td>
<td>0.30 (0.1-0.79)</td>
<td>NS</td>
</tr>
<tr>
<td>PAI-1</td>
<td>59.73 (13.8-138.66)</td>
<td>38.87 (13.5-108.19)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CEA</td>
<td>2.90 (0.4-235.0)</td>
<td>2.10 (0.6-21.1)</td>
<td>&lt;0.1, &gt;0.05</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>14.00 (1.0-540)</td>
<td>7.5 (1.0-35.0)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Abbreviations: With Metastases: presence of metastases; No metastases: absence of metastases; CL: confidence limits; CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carboanhydrate antigen 19-9; NS: not significant
Table 3. Proteolytic enzymes, CEA and CA 19-9 in correlation with Dukes classification

Cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 serum or plasma levels in association with Dukes classification of colorectal cancer expressed in ng/ml (median values and range)

<table>
<thead>
<tr>
<th>Stage</th>
<th>CATB</th>
<th>CATL</th>
<th>UPA</th>
<th>PAI-1</th>
<th>CEA</th>
<th>CA 19-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUKES A</td>
<td>4.50</td>
<td>1.10</td>
<td>0.30</td>
<td>32.81</td>
<td>3.00</td>
<td>6.80</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(2.4-37.3)</td>
<td>(1.0-35.3)</td>
<td>(0.2-0.62)</td>
<td>(17.2-66.7)</td>
<td>(0.6-3.4)</td>
<td>(3.0-11.5)</td>
</tr>
<tr>
<td>DUKES B</td>
<td>6.75</td>
<td>1.00</td>
<td>0.29</td>
<td>39.09</td>
<td>2.00</td>
<td>7.65</td>
</tr>
<tr>
<td>(n=12)</td>
<td>(3.2-23.8)</td>
<td>(1.0-12.3)</td>
<td>(0.1-0.79)</td>
<td>(13.5-108.1)</td>
<td>(0.6-21.1)</td>
<td>(1.0-35.0)</td>
</tr>
<tr>
<td>DUKES C</td>
<td>8.60</td>
<td>1.10</td>
<td>0.26</td>
<td>52.54</td>
<td>2.20</td>
<td>9.20</td>
</tr>
<tr>
<td>(n=23)</td>
<td>(3.6-35.7)</td>
<td>(1.0-34.1)</td>
<td>(0.1-0.79)</td>
<td>(13.8-126.4)</td>
<td>(0.4-235.0)</td>
<td>(1.0-78.6)</td>
</tr>
<tr>
<td>DUKES D</td>
<td>24.25</td>
<td>4.55</td>
<td>0.29</td>
<td>82.03</td>
<td>8.30</td>
<td>35.85</td>
</tr>
<tr>
<td>(n=14)</td>
<td>(9.2-39.3)</td>
<td>(1.0-35.2)</td>
<td>(0.12-0.42)</td>
<td>(38.7-138.6)</td>
<td>(1.9-74.6)</td>
<td>(4.6-540.0)</td>
</tr>
</tbody>
</table>

**Kruskall-Wallis analysis of variance (P value)**

<table>
<thead>
<tr>
<th></th>
<th>P=0.0002</th>
<th>P=NS</th>
<th>P=NS</th>
<th>P=0.01</th>
<th>P=0.003</th>
<th>P=0.001</th>
</tr>
</thead>
</table>

**Abbreviations:** DUKES A: Dukes stage A; DUKES B: Dukes stage B; DUKES C: Dukes stage C; DUKES D: Dukes stage D; CL: confidence limits; CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carboanhydrate antigen 19-9; NS: not significant
Table 4. Diagnostic accuracy of proteolytic enzymes, CEA and CA 19-9 in colorectal cancer

Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 in colorectal cancer

<table>
<thead>
<tr>
<th></th>
<th>CATB</th>
<th>CATL</th>
<th>UPA</th>
<th>PAI-1</th>
<th>CEA</th>
<th>CA 19-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>82</td>
<td>41</td>
<td>69</td>
<td>94</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>88</td>
<td>80</td>
<td>82</td>
<td>84</td>
<td>89</td>
<td>93</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>82</td>
<td>58</td>
<td>72</td>
<td>79</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>88</td>
<td>68</td>
<td>80</td>
<td>96</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>86</td>
<td>65</td>
<td>77</td>
<td>88</td>
<td>66</td>
<td>63</td>
</tr>
</tbody>
</table>

*Abbreviations: PPV: Positive predictive value; NPV: Negative predictive value; CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carboanhydrate antigen 19-9*
Table 5. Multiparametric tumor marker analysis in colorectal cancer

Sensitivity and specificity values of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 during multiparametric tumor marker analysis in colorectal cancer (n=56)

<table>
<thead>
<tr>
<th>Two markers</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Both positive</td>
<td>Either positive</td>
</tr>
<tr>
<td>PAI-1 + CATB</td>
<td>78%</td>
<td>98%</td>
</tr>
<tr>
<td>PAI-1 + UPA</td>
<td>64%</td>
<td>98%</td>
</tr>
<tr>
<td>PAI-1 + CATL</td>
<td>39%</td>
<td>96%</td>
</tr>
<tr>
<td>CATB + UPA</td>
<td>55%</td>
<td>95%</td>
</tr>
<tr>
<td>CATB + CATL</td>
<td>41%</td>
<td>84%</td>
</tr>
<tr>
<td>CATL + UPA</td>
<td>30%</td>
<td>80%</td>
</tr>
<tr>
<td>PAI-1 + CEA</td>
<td>30%</td>
<td>96%</td>
</tr>
<tr>
<td>CATB + CEA</td>
<td>28%</td>
<td>84%</td>
</tr>
<tr>
<td>UPA + CEA</td>
<td>20%</td>
<td>80%</td>
</tr>
<tr>
<td>CATL + CEA</td>
<td>20%</td>
<td>52%</td>
</tr>
<tr>
<td>PAI-1 + CA 19-9</td>
<td>18%</td>
<td>96%</td>
</tr>
<tr>
<td>CATB + CA 19-9</td>
<td>16%</td>
<td>84%</td>
</tr>
<tr>
<td>UPA + CA 19-9</td>
<td>14%</td>
<td>73%</td>
</tr>
<tr>
<td>CATL + CA 19-9</td>
<td>11%</td>
<td>48%</td>
</tr>
<tr>
<td>CEA + CA 19-9</td>
<td>16%</td>
<td>32%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Three markers</th>
<th>All positive</th>
<th>Either positive</th>
<th>All negative</th>
<th>Either negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 + CEA + CA 19-9</td>
<td>16%</td>
<td>96%</td>
<td>71%</td>
<td>98%</td>
</tr>
<tr>
<td>CATB + CEA + CA 19-9</td>
<td>14%</td>
<td>84%</td>
<td>80%</td>
<td>97%</td>
</tr>
<tr>
<td>UPA + CEA + CA 19-9</td>
<td>12%</td>
<td>80%</td>
<td>74%</td>
<td>97%</td>
</tr>
<tr>
<td>CATL + CEA + CA 19-9</td>
<td>9%</td>
<td>52%</td>
<td>74%</td>
<td>97%</td>
</tr>
</tbody>
</table>

CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carboanhydrate antigen 19-9;
Both: both markers correctly positive or negative; All: all three markers correctly positive or negative; Either: one of two/or three markers correctly positive or negative
Table 6. Correlation analysis of proteolytic enzymes, CEA and CA 19-9 in colorectal cancer

Correlation of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 in patients with colorectal cancer (n=56)

<table>
<thead>
<tr>
<th>Correlation of enzymes and biomarkers</th>
<th>P value, (correlation coefficient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATB/CATL</td>
<td>P=0.0001 (r=0.74)</td>
</tr>
<tr>
<td>CATB/UPA</td>
<td>P=0.03 (r=0.28)</td>
</tr>
<tr>
<td>CATB/PAI-1</td>
<td>P=0.01 (r=0.31)</td>
</tr>
<tr>
<td>CATL/UPA</td>
<td>P=0.25 (r=0.15)</td>
</tr>
<tr>
<td>PAI-1/CATL</td>
<td>P=0.02 (r=0.24)</td>
</tr>
<tr>
<td>PAI-1/UPA</td>
<td>P=0.03 (r=0.22)</td>
</tr>
<tr>
<td>CEA/CA 19-9</td>
<td>P=0.01 (r=0.31)</td>
</tr>
<tr>
<td>CEA/CATB</td>
<td>P=0.002 (r=0.39)</td>
</tr>
<tr>
<td>CEA/CATL</td>
<td>P=0.10 (r=0.22)</td>
</tr>
<tr>
<td>CEA/UPA</td>
<td>P=0.86 (r=0.02)</td>
</tr>
<tr>
<td>CEA/PAI-1</td>
<td>P=0.002 (r=0.39)</td>
</tr>
<tr>
<td>CA 19-9/CATB</td>
<td>P=0.0001 (r=0.51)</td>
</tr>
<tr>
<td>CA 19-9/CATL</td>
<td>P=0.0001 (r=0.53)</td>
</tr>
<tr>
<td>CA 19-9/UPA</td>
<td>P=0.77 (r=0.03)</td>
</tr>
<tr>
<td>CA 19-9/PAI-1</td>
<td>P=0.0001 (r=0.51)</td>
</tr>
</tbody>
</table>

Abbreviations: CATB: cathepsin B; CATL: cathepsin L; UPA: urokinase-type plasminogen activator, PAI-1: plasminogen activator inhibitor type-1; CEA: carcinoembryonic antigen; CA 19-9: carboanhyrdate antigen 19-9; r: correlation coefficient
Figure 1

Survival analysis showing the probability of survival over time. The blue line represents CATB ≤ 8.75, while the purple line represents CATB > 8.75. The log-rank test yielded a significance level of P=0.0004.
Figure 2

Survival Time (months)

Probability

CATL ≤ 1.1

CATL > 1.1

log-rank $P=0.02$
Figure 3

Survival time (months) vs. Probability

PAI-1 ≤ 52.45
PAI-1 > 52.45

log-rank $P=0.01$