

Platelet Count, ADAMTS13 Activity, von Willebrand Factor Level and Survival in Patients with Colorectal Cancer: 5-Year Follow-up Study

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Abstract

Distant metastasis is a major cause of colorectal cancer-related death, but the mechanism of tumour progression is not fully understood. There is growing evidence of an interaction between tumour cells and platelets which may influence tumour progression and metastasis formation. Quality and quantity of von Willebrand factor may regulate the interaction between tumour cells and platelets. Our aim was to measure the platelet count, von Willebrand factor antigen (VWF:Ag) levels and ADAMTS13 activity in a large ($n = 232$) cohort of colorectal cancer patients and to examine their relationships with the stage of the disease and 5-year survival without thrombotic complications using multivariable models. Significantly higher platelet counts ($p = 0.005$), VWF:Ag levels ($p = 0.008$) and decreased ADAMTS13 activity ($p = 0.006$) were observed in patients with metastatic disease. Results of the Kaplan–Meier analysis showed that lower platelet counts ($p < 0.0001$), lower VWF:Ag ($p = 0.0008$) levels and higher ADAMTS13 activity ($p < 0.0001$) were associated with better event-free survival. Finally, to investigate the association between overall event-free survival and the three study variables, multivariate Cox proportional hazard models were generated. All models were adjusted for age, gender and disease stage. Platelet count, ADAMTS13 activity or VWF:Ag level were incorporated and all of these variables turned out to be age-, gender- and stage-independent predictors of mortality (all hazard ratio > 1.7 , $p < 0.05$). In summary, this is the first observational study reporting association between higher mortality or thrombotic complications and increased platelet count, increased VWF:Ag levels and decreased ADAMTS13 activity in colorectal cancer.

Keywords

- ▶ ADAMTS13
- ▶ platelet
- ▶ von Willebrand factor
- ▶ mortality
- ▶ colorectal cancer

* Nóra Garam and Éva Maláti have equally contributed to this study.

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Introduction

Colorectal cancer is one of the leading causes of mortality all over the world. Based on the WHO World Cancer Report 2014, colorectal cancer is the third most prevalent cancer type in men and the second in women. It is the third most common cause of cancer-related mortality in men and fourth in women.

The mortality decreased in the last decades due to colorectal screening and therapeutic improvements,¹ but in spite of that the presence of distant metastasis is a major cause of colorectal cancer-related death.² The survival of patients with metastatic colorectal cancer is even worse than the localized form of the disease.³

It is important to find new reliable markers that can predict the prognosis of colorectal cancer patients.

The contribution of platelets in metastasis formation has been known for a long time. First, it was observed in the mid-19th century that venous thromboembolism and superficial thrombophlebitis occur together with malignancy.⁴ There is growing evidence of an interaction between tumour cells and platelets, which promote tumour progression.⁵ On the one hand, tumour cells can induce platelet activation; on the other hand, platelets can also promote tumour growth and metastasis formation with various mechanisms reviewed recently.⁴ Multiple studies investigated the role of platelets in several cancer types, such as ovarian,⁶ lung^{7,8} and colorectal cancer.^{9–13} Preoperative thrombocytosis was associated with poor survival in surgically resected colorectal cancer patients¹⁴ and was identified as independent predictor of survival.^{11,12} Paraneoplastic thrombocytosis was shown to be associated with decreased median time to disease progression in ovarian cancer patients.¹⁵

The interaction between tumour cells and platelets could be mediated by multiple factors,^{4,5} among which the von Willebrand factor (VWF) belongs to the most widely studied group. This multimeric glycoprotein is expressed in and released from platelets and endothelial cells, and can bind to platelet surface receptors facilitating the formation of the platelet thrombus. In clinical studies, an increased plasma level of VWF antigen (VWF:Ag) was observed in various cancer types such as ovarian, bladder, gastric and colon cancer, and increased levels were associated with poor prognosis.^{16,17}

The most adhesive form of the VWF is the ultra-large VWF, which is released from the endothelial cells. Under physiological circumstances, it is cleaved by ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type-1 repeats 13) protease into the multimer form in a shear-dependent manner.¹⁸ Significantly decreased ADAMTS13 serum levels were found in patients with localized or disseminated tumours compared to healthy individuals.¹⁹ In another study, an increased amount of ultra-large VWF multimers and decreased ADAMTS13 activity were observed in blood samples of patients with malignancies.^{20,21}

Only few studies were performed in connection with VWF:Ag level and ADAMTS13 activity in tumours. In patients with disseminated metastasis, a reduced plasma level of ADAMTS13 was measured with the presence of unusually large VWF multimers.²² Similar to this observation, a lower mean plasma

level of ADAMTS13 was found in disseminated malignancies compared with the localized form. The ADAMTS13 activity and VWF:Ag level correlate inversely.¹⁹ Although the epidemiological evidence supporting the potential association of increased platelet count and VWF:Ag level, or decreased ADAMTS13 activity with poor survival in cancer patients, is accumulating, none of the large studies evaluated these factors simultaneously in colorectal cancer patients. Therefore, our aim was to measure the platelet count, VWF:Ag level and ADAMTS13 activity and examine its association with the presence of ultra-large VWF in a large ($n = 232$) cohort of colorectal cancer patients, and to examine their relationships with the presence of metastatic disease and 5-year survival without thrombotic complications using multivariable models. Because the higher prothrombotic risk is associated with increased platelet count and VWF:Ag level, or decreased ADAMTS13 activity, we also compared these parameters in relation to the thrombotic complications during the disease.

Materials and Methods

Patients

A total of 232 patients diagnosed with colorectal cancer were enrolled in the study between January 2011 and June 2013 in the Oncology Department of the 3rd Department of Internal Medicine, Semmelweis University. The study was approved by the Medical Research Council Scientific and Research Committee and was performed at the 3rd Department of Internal Medicine, Semmelweis University, based on a study protocol approved by the highest Ethical Committee of Hungary. Patients' consent was obtained consecutively after the confirmation of colorectal cancer with any stage, which was based on the TNM staging system. Blood samples were taken after 6 hours of fasting between 8 and 10 AM by antecubital venipuncture into native, EDTA, or sodium citrate–anticoagulated tubes before starting any anticancer therapy. The samples were aliquoted and stored at -70°C for further analysis. Clinical data were collected. Baseline demographics and clinical characteristics of patients are summarized in **Table 1**. The mean age of patients was 68.5 years, with a male:female ratio of 138:94. After diagnosis and adequate surgery, patients were treated and followed up at the Oncology Department according to the stage of their disease and to the relevant guidelines. Patients with rectal cancer received radiochemotherapy before definitive surgery from cT3 or N+ disease. Twenty-two patients who had irresectable and/or metastatic disease received upfront primary systemic treatment without definitive surgery. The data of thrombotic complications, which were defined as occurrence of deep vein thrombosis, pulmonary embolism, arterial occlusion with infarction and superficial thrombophlebitis,²³ were collected from the disease onset until the last visit (median follow-up: 42.36 months). Patients were followed up for 5 years (median: 46.42 months) in the same institution and survival and thrombotic data were recorded and defined as events. Most of the patients died due to progressive malignant disease. Since there was no autopsy in the majority of cases, the cause of death, whether it was cardiovascular or cancer related, was not differentiated; therefore, mortality was defined as

Table 1 Clinical and laboratory characteristics of patients with colorectal cancer

	Patients who had an event within 5 y ^a	Patient who had no event ^a	p-Value ^c	All patients
Number of patients	105	127	–	232
Males/Females	64/41	74/53	0.607	138/94
Age at diagnosis, y	70.3 (65–76.2) ^b	65.2 (58–72)	<0.0001	68.5 (59.6–74.1)
Platelet G/L	309 (240–413)	271 (217–326)	0.013	286 (224.5–364.50)
ADAMTS activity, %	71.17 (59.33–88.45)	83.95 (70.71–104.66)	<0.0001	78.40 (65.02–96.79)
VWF antigen, ng/mL	665.74 (369.75–1,433.4)	414.75 (254.37–764.54)	0.001	521.83 (286.10–1,067.97)
Carcinoembryonic antigen, µg/L	4.45 (2–55)	1.8 (1–3.4)	<0.0001	2.50 (1.20–7.40)
CA19–9, U/mL	11 (6.8–116.9)	7.75 (4.15–14.2)	<0.0001	9.00 (5.0–20.30)
Body mass index	26.27 (24.2–29.74)	26.59 (24.54–29.4)	0.749	26.45 (24.22–29.40)
Stage				
1	1 (1.0%)	8 (6.3%)	<0.0001	9 (3.9%)
2	29 (27.6%)	72 (56.7%)		101 (43.5%)
3	32 (30.5%)	41 (32.3%)		73 (31.5%)
4	43 (41%)	6 (4.7%)		49 (21.1%)

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type-I repeats 13; VWF, von Willebrand factor.

^aNonfatal thromboembolic event and/or death of any cause.

^bMedian and interquartile range or number and percentages.

^cChi-square test or Mann–Whitney *t*-test.

all-cause mortality in this study. In the follow-up period, 97 patients died and/or 26 had thrombotic complication.

Laboratory Measurements

ADAMTS13 enzyme activity was determined in citrated plasma samples using the fluorogenic substrate FRET5-VWF73 as previously described.²⁴

VWF:Ag level was measured in EDTA plasma by ELISA as described.²⁵ Primary antibodies and horseradish peroxidase-labeled polyclonal rabbit anti-human VWF antibodies were used for the detection (both from Dako, Glostrup, Denmark).²⁴ Determination of the additional laboratory parameters including complete blood counts, clinical chemistry and tumour markers were completed by Roche Integra 800 analyser, and a Cell-Dyn 3500 haematology analyser.

VWF multimer analysis was performed with patients' plasma samples which were normalized to VWF:Ag concentration based on VWF:Ag ELISA results. VWF multimer gels (1.2% (w/v) type VIIA agarose [Sigma-Aldrich] running gel and SeaKem HGT(P) agarose [Lonza] stacking gel) were loaded with denatured normalized plasmas diluted in sample buffer. Gels were run overnight and blotted into a nitrocellulose membrane (Whatman Protran). Membranes were blocked in 1.5% milk shaking and probed with rabbit polyclonal anti-human VWF (DAKO) at 1:2,000, conjugated with goat anti-rabbit HRP antibody (Biorad) and developed using chemiluminescent substrate (Sigma-Aldrich). Digital images were acquired, densitometry waveforms were generated using Alpha Innotech (Fluorchem Ltd) and multimer structures of patients were compared with normal pooled plasma (NPP). A sample was defined as positive for the presence of ultra-large von Wil-

lebrand factor (ULVWF) if bands with more than 15 multimers were present.

Statistical Analysis

The continuous variables reported in this study showed skewed distribution according to the results of the Shapiro–Wilk test deviated from the normal distribution. Therefore, for descriptive purposes, the values of each measurement are given as median and 25th to 75th percentile or as numbers (percent) and non-parametric tests (Spearman's rank correlation or Mann–Whitney U-test) were used for group comparisons. For categorical variables, Pearson's chi-square test was applied. The correlation between the different variables was determined by using Spearman's correlation analysis.

Multivariable Cox proportional hazard models were fitted to assess the effect of platelet count, VWF:Ag level and ADAMTS13 activity on the event-free survival in patients. Survival times were measured from the diagnosis of colorectal cancer and patients were censored at the time of death, at the time of non-fatal thromboembolic event, or at the end of the follow-up period if still alive (month 60). Potential predictors of events and confounders were entered into the models as categorical variables, and high or low predictor groups were formed based on the results of receiver operating characteristic (ROC) curve analysis. All of the survival models were adjusted to age, gender and stage of disease. The proportional hazards assumption was confirmed graphically by Kaplan–Meier analysis, survival curves were compared with log-rank (Mantel–Cox) test. The results of the Cox regression models are presented as hazard ratios (HRs) with corresponding 95% confidence intervals (CIs). With a sample size of 232 and a predicted event rate around

0.5, the precision of Cox regression models are considered satisfactory, as reflected by the sufficiently narrow CI ranges. The presence of collinearity in the multivariable models was inferred by calculating variance inflation factors (VIF). IBM SPSS Statistics 20 and Graph Pad Prism 5 software were used for the statistical analysis and presentation of data. Two-tailed *p*-values were calculated and significance level of *p* < 0.05 was used, if not otherwise stated.

Results

A total of 232 patients with colorectal cancer, with a mean age of 68.5 years and slight male predominance (► **Table 1**), were enrolled in the study and followed up for 5 years. Over the 5-year-long follow-up period, 96 patients died and/or 26 had a thromboembolic event. Clinical and laboratory variables of the cohort are summarized in ► **Table 1**. Among these 26 patients, there were 8 patients who had only thromboembolic complications but did not die within the follow-up period, whereas 2 patients had a fatal thromboembolic event, and 18 patients died within the follow-up period after surviving thromboembolic event. Accordingly, during follow-up, 105 patients suffered nonfatal thromboembolic events, or died.

As presented in ► **Table 1**, mortality and/or thromboembolic events were associated with significantly higher platelet count (*p* = 0.013), VWF:Ag level (*p* = 0.001), carcinoembryonic antigen- (CEA) and CA19-9 (cancer antigen 19-9) levels (all *p* < 0.0001), and lower ADAMTS13 activity (*p* < 0.0001). Similarly, advanced disease stage was also associated with poor outcome (*p* < 0.0001). In 26 cases, thromboembolic complication occurred during the follow-up period. We found that patients with thromboembolism had lower ADAMTS13 activity (63.23%; 55.14–68.97%; *p* < 0.0001) and higher VWF:Ag (665.74 ng/mL; 387.56–2,092.92 ng/mL; *p* = 0.048) levels, when compared with patients without events (ADAMTS13 activity: 80.27% [67.58–98.92%]; VWF:Ag level: 514.43 ng/mL [280.73–964.27 ng/mL]), which also supports the hypothesis that the observed decrease of ADAMTS13 activity in colorectal cancer patients is clinically relevant. There was no correlation between VWF:Ag levels, ADAMTS13 activity and platelet count using Spearman's correlation (ADAMTS13-VWF:Ag: *r* = -0.114, *p* = 0.08; ADAMTS13-platelet count: *r* = 0.043, *p* = 0.526; VWF:Ag-platelet count: *r* = 0.022, *p* = 0.746).

Increased VWF:Ag levels and decreased ADAMTS13 activity were observed for many of our patients. There were 49 patients with high VWF:Ag level and low ADAMTS13 activity which is 21.6% of our patients (the other groups were as follows: low ADAMTS13-low VWF:Ag: *n* = 16 [7%]; high ADAMTS13 activity-low VWF:Ag: *n* = 46 [20.3%]; high ADAMTS13 activity-high VWF:Ag: *n* = 116 [51.1%]). Decreased ADAMTS13 activity with the presence of ultra-large VWF multimers was observed in several diseases such as TTP, HUS, preeclampsia, liver cirrhosis, chronic renal insufficiency and acute inflammatory states,²⁶ and was found to be linked to higher risk of thromboembolism.²⁷ Therefore, by analysing the presence of ultra-large VWF (ULVWF) oligomers in a selected subset of patients (in 55 plasma samples obtained from patients in the highest VWF:Ag level group associated with the lowest

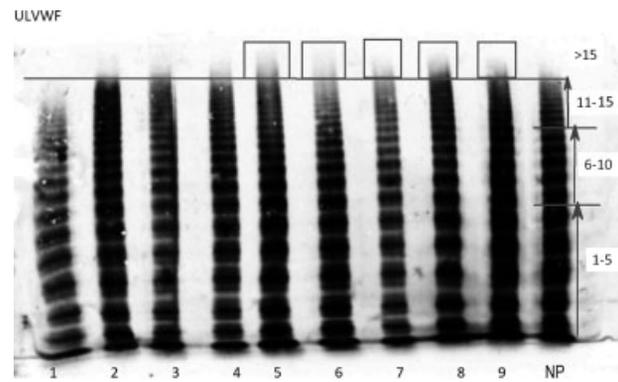


Fig. 1 Multimeric analysis of plasma von Willebrand factor (VWF). Oligomeric structure of VWF was determined by agarose electrophoresis, ULVWF was defined if bands representing more than 15-mer oligomers were present in patients' plasma samples. Oligomeric structures of nine representative samples are shown, samples 1 to 4 are negative, whereas samples 5 to 9 are positive for ULVWF (marked by squares). Normal human plasma (NP) is presented as negative control.

ADAMTS13 activity [*n* = 28] or vice versa [*n* = 27]), we further examined if the observed increase of VWF:Ag with the decrease of ADAMTS13 activity is clinically relevant or not. Oligomeric structure of VWF was determined by agarose electrophoresis, and a sample was considered positive for ULVWF if VWF with more than 15 multimers was present (► **Fig. 1**). A significantly (*p* = 0.01) increased frequency (67%) of ULVWF was observed in the high VWF:Ag and low ADAMTS13 activity group of colorectal cancer patients, when compared with the low VWF:Ag and high ADAMTS13 activity group (32%, ► **Supplementary Table S1** [online only]), indicating clinical relevance of this observation. Taken together, these data support that lower ADAMTS13 activity and higher VWF:Ag level could be useful information according to the prothrombotic state in the patients.

Next, we examined whether baseline platelet count, VWF-antigen level and ADAMTS13 activity are associated with disease stage (► **Fig. 2**). We merged stages 1 and 2 because of the small number of cases. Significantly higher platelet counts (*p* = 0.005), VWF:Ag levels (*p* = 0.008) and decreased ADAMTS13 activity (*p* = 0.006) were observed in the group of patients with metastatic disease (stage 4) when compared with patients in stages 1 and 2 or when compared the patients with lymph node metastasis (stage 3, all *p* < 0.05, except for VWF:Ag where this difference was nonsignificant). We also analysed the association between platelet counts, ADAMTS13 activity and VWF:Ag levels in the total cohort and no correlation was observed between any of the markers.

To examine in multivariable models whether platelet count, VWF:Ag level or ADAMTS13 activity could influence or have any relation with event-free survival, patients were categorized as those with "high" or "low" marker levels. The cut-off values (indicated in ► **Tables 2–4**), with optimum sensitivity and specificity for all-cause mortality of the above-mentioned variables were defined by ROC curve analysis. The distribution of the clinical and laboratory characteristics of patients with high or low platelet count, VWF:Ag level and ADAMTS13 activity is shown in ► **Tables 2 to 4**.

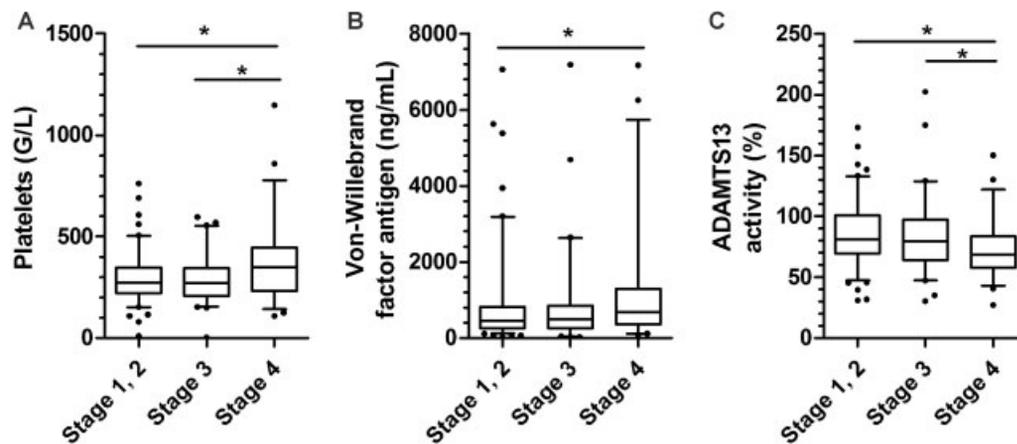


Fig. 2 Box plot of the studied three variables according to different tumour stages. (Panel A) Platelet count; (Panel B) von Willebrand factor antigen level; (Panel C): ADAMTS13 activity. The difference between stages 1, 2 and 4 is significant ($p < 0.05$) in all panels. In Panels A and C, there is a significant difference between Stages 3 and 4 as well (both $p < 0.05$). Horizontal lines show medians. The boxes enclose interquartile range and whiskers indicate the 5th to 95th percentiles of the groups. * $p < 0.05$.

In addition, we examined the levels of different tumour markers in these groups (► Tables 2–4). Higher CA19–9 levels were associated with elevated levels of VWF:Ag and decreased ADAMTS13 activity, whereas increased platelet count was associated with higher CEA levels (► Table 2).

By examining the association between TNM classification and the above markers, we found that advanced stages were significantly associated with high platelet count ($p = 0.001$) and lower ADAMTS13 activity ($p = 0.011$), and a borderline association ($p = 0.074$) with high VWF:Ag level was also observed.

The cumulative event-free survival of patients with different platelet counts, ADAMTS13 activity and VWF:Ag levels is displayed in ► Fig. 3. Results of the Kaplan–Meier analysis showed that lower platelet counts ($p < 0.0001$), lower

VWF:Ag ($p = 0.0008$) levels, and higher ADAMTS13 activity ($p < 0.0001$) were associated with better event-free survival. Overall event-free survival at the end of follow-up was 61.68% in the low and 38.57% in the high platelet count group, 32.8% in the low and 64.02% in the high ADAMTS13 activity group and 62.5% in the low and 49% in the high VWF:Ag group.

Finally, to investigate the association between overall event-free survival and the three studied variables, multivariate Cox proportional hazard models were generated, and the results are shown in ► Table 5. The baseline model (Model 1) included age, gender and disease stage, and all subsequent models were adjusted for these three variables. Next, platelet count, ADAMTS13 activity and VWF:Ag level (as binary variables) were incorporated (Models 2, 3 and 4) and all of these variables

Table 2 Clinical and laboratory characteristics of patients with high or low platelet count

	Platelet count ≤ 348.5 G/L ^b	Platelet count > 348.5 G/L	p -Value ^c
Number of patients	154	70	–
No event/event ^a	95/59	27/43	0.001
Males/Females	103/51	30/40	0.001
Age at diagnosis, y	68.35 (59.60–73.30)	68.70 (60.40–74.90)	0.407
ADAMTS13 activity, %	79.31 (65.04–98.69)	75.89 (65.8–94.18)	0.473
VWF antigen, ng/mL	501.28 (262.72–1,048.03)	576.60 (348.14–1011.30)	0.405
Carcinoembryonic antigen, μ g/L	2.00 (1.10–3.50)	5.80 (2.20–19.70)	< 0.0001
CA19–9, U/mL	9.10 (5.30–16.70)	8.30 (4.20–63.50)	0.754
Body mass index	27.17 (24.84–29.74)	26.11 (22.31–29.75)	0.111
Stage			
1	9 (5.8%)	0 (0.0%)	0.001
2	73 (47.4%)	26 (37.1%)	
3	50 (32.5%)	19 (27.1%)	
4	22 (14.3%)	25 (35.7%)	

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type-I repeats 13; VWF, von Willebrand factor.

^aNonfatal thromboembolic event and/or death of any cause.

^bMedian and interquartile range or number and percentages.

^cChi-square test or Mann–Whitney t -test.

Table 3 Clinical and laboratory characteristics of patients with high or low ADAMTS13 activity

	ADAMTS13 activity \leq 66.53% ^b	ADAMTS13 activity $>$ 66.53%	p-Value ^c
Number of patients	67	164	–
No event/event ^a	22/45	105/59	<0.0001
Males/Females	47/20	90/74	0.032
Age at diagnosis, y	72.40 (67.80–78.60)	65.45 (58.10–71.80)	<0.0001
Platelet G/L	266 (214–353)	291 (229–367)	0.343
VWF antigen, ng/mL	635.40(318.0–1,286.51)	502.62 (280.73–904.66)	0.207
Carcinoembryonic antigen, μ g/L	3.20 (1.10–28.90)	2.30 (1.20–5.20)	0.090
CA19–9, U/mL	12.40 (7.10–49.40)	8.10 (4.50–16.70)	0.008
Body mass index	26.11 (24.78–29.08)	27.04 (23.67–29.40)	0.734
Stage			
1	2 (3.0%)	7 (4.3%)	0.011
2	20 (29.9%)	81 (49.4%)	
3	23 (34.3%)	50 (30.5%)	
4	22 (32.8%)	26 (15.9%)	

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type-I repeats 13; VWF, von Willebrand factor.

^aNonfatal thromboembolic event and/or death of any cause.

^bMedian and interquartile range or number and percentages.

^cChi-square test or Mann–Whitney *t*-test.

Table 4 Clinical and laboratory characteristics of patients with high or low von Willebrand factor antigen

	von Willebrand factor antigen \leq 305.6 ng/mL ^b	von Willebrand factor antigen $>$ 305.6 ng/mL	p-Value ^c
Number of patients	62	165	–
No event/event ^a	45/17	81/84	0.002
Males/Females	40/22	94/71	0.303
Age at diagnosis, y	65.55 (58.50–73.00)	68.80 (60.60–74.70)	0.067
Platelet G/L	282 (223–339.5)	291 (227–368)	0.298
ADAMTS13 activity, %	83.88 (66.42–98.45)	77.74 (65.02–95.12)	0.377
Carcinoembryonic antigen, μ g/L	2.15 (1.10–4.20)	2.70 (1.20–8.50)	0.134
CA19–9, U/mL	7.45 (4.20–14.20)	9.50 (5.60–21.90)	0.03
Body mass index	26.20 (22.59–28.23)	26.93 (24.39–29.76)	0.164
Stage			
1	2 (3.2%)	7 (4.2%)	0.074
2	33 (53.2%)	67 (40.6%)	
3	21 (33.9%)	50 (30.3%)	
4	6 (9.7%)	41 (24.8%)	

Abbreviation: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type-I repeats 13.

^aNonfatal thromboembolic event and/or death of any cause.

^bMedian and interquartile range or number and percentages.

^cChi-square test or Mann–Whitney *t*-test.

turned out to be age-, gender- and stage-independent predictors of mortality or thromboembolic complications (all HR $>$ 1.7, $p <$ 0.05, ► **Supplementary Table S2** [online only]). When we further adjusted Models 2 to 4 also for the well-known tumour markers of colorectal cancer (CEA, CA19–9), which were associated with mortality or thromboembolic complications

(► **Table 1**), all of the examined variables remained significant predictors of event-free state except ADAMTS13 activity (platelet count in Model 1 adjusted for CEA: HR 1.908, CI 1.214–3; ADAMTS13 activity in Model 2 adjusted for CA19–9: HR 1.519, CI 0.955–2.416; VWF:Ag level in Model 3 adjusted for CA19–9: HR 2.021; CI 1.169–3.494). We also examined the relationship of

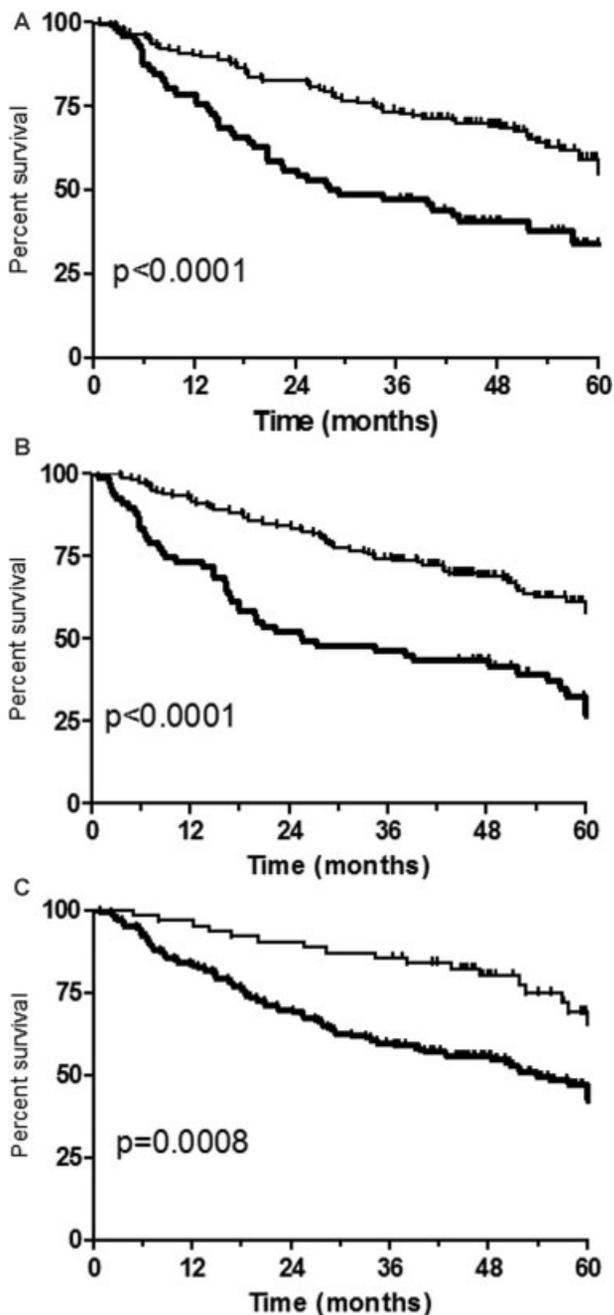


Fig. 3 Kaplan–Meier analysis of patient groups with higher and lower levels of the studied variables. (Panel A) Platelet count, thick line: >348.5 G/L, thin line: ≤348.5 G/L; (Panel B) von Willebrand factor antigen level, thick line: >305.6 ng/mL, thin line: ≤305.6 ng/mL; (Panel C): ADAMTS13 activity, thick curve: ≤ 66.53%, thin curve: >66.53%. *p*-Value of log-rank test is indicated.

these variables for an aggregate effect on event-free survival (Model 5, ► **Table 5**) and observed that platelet count (HR: 1.967, CI: 1.257–3.077), ADAMTS13 activity (HR: 1.903, CI: 1.22–3.577) and VWF:Ag level (HR: 2.089, CI: 1.169–3.651) remained significant predictors of mortality or thromboembolic complications in the final multivariable model. Multiple collinearity was not present in the final multivariable model, since all VIF scores were in the range 1 to 10.

Table 5 Model 5: Relationship between platelet count, ADAMTS13 activity and von Willebrand factor antigen and event-free survival of 232 patients with colorectal cancer during the 5-year-long follow-up period

	HR	CI	<i>p</i> -Value
Age (by 10 y of increase)	1.277	1.034–1.576	0.023
Gender			
Female	1	0.562–1.366	0.559
Male	0.876		
Stage			
1, 2, 3	1	2.345–5.645	<0.0001
4	3.638		
Platelet count			
≤ 348.5 G/L	1	1.257–3.077	0.003
> 348.5 G/L	1.967		
ADAMTS13 activity			
> 66.53%	1	1.22–3.577	0.003
≤ 66.53%	1.903		
von Willebrand factor antigen			
≤ 305.6 ng/mL	1	1.169–3.651	0.007
> 305.6 ng/mL	2.089		

Abbreviation: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type-I repeats 13.

Note: The effect of different other demographic and clinical variables was calculated by multivariable Cox regression analysis.

Based on all of the above observations, careful analysis and interpretation of platelet count, ADAMTS13 activity and VWF:Ag level may yield clinically important information in colorectal cancer, since these markers are associated with increased prothrombotic risk and independently predict worse outcome.

Discussion

In this cohort study with a 5-year follow-up, we report that platelet count, ADAMTS13 activity and VWF:Ag levels are related to different stages of colorectal cancer and have prognostic significance. Higher platelet count, VWF:Ag level and lower ADAMTS13 activity are associated with advanced stage and independently and significantly predicts poor overall event-free survival. The novelty of this study is the demonstration of a clinically relevant decrease of ADAMTS13 activity with increased thromboembolic risk in colorectal cancer, and the independent association of higher platelet count, higher VWF:Ag levels and lower ADAMTS13 activity with poor thromboembolic event-free survival in colorectal cancer patients. Higher occurrence of ultra-large VWF oligomers in colorectal cancer patients with increased VWF:Ag and decreased ADAMTS13 activity strongly supported clinical relevance of this observation. Taken together, previous literature results^{20,21} and our observations in colorectal cancer patients are convincingly supporting the link between increased VWF:Ag, decreased ADAMTS13 activity and increased prothrombotic risk.

An important observation of the current study is the demonstration of association between thrombocytosis with poor 5-year event-free survival in colorectal cancer, confirming previous results.^{6–13,15} Elevated platelet count was associated with metastatic (stage 4) disease, whereas patients with loco-regional disease (stage 3) had similar platelet counts as patients with stage 1 to 2 diseases (► **Fig. 1**). The association between metastatic disease and thrombocytosis is intriguing. During metastasis formation, cancer cells escape from the primary tumour and get to distal sites while the cells are hidden from the tumour surveillance mechanisms.²⁸ There is growing evidence of an interaction between tumour cells and platelets which may promote tumour cell survival and tumour progression. The surface receptors of platelets (adhesion receptors such as GP IIb/IIIa, GP Ib-IX-V and integrins $\alpha v\beta 3$) can directly interact with tumour cells leading to the formation of heteroaggregates.⁵ The interaction of platelets with tumour cells may also lead to platelet activation, aggregation⁵ and degranulation,²⁸ a process called tumour cell-induced platelet aggregation (TCIPA).^{4,28} Factors released from the α -granules include PDGF, VEGF, IGF-1 and TGF β , which may contribute to tumour growth, spread and neovascularization.

Both the thrombin- and the VEGF-dependent pathways induce endothelial cell activation followed by Weibel-Palade body exocytosis and the release of VWF.²⁹ Von Willebrand factor is a key regulator of primary haemostasis,¹⁶ as the released multimer can bind to platelet surface receptors and promote the formation of the platelet thrombus.^{17,30} Recent observations obtained from human clinical samples support a VWF-mediated mechanism in tumour progression and angiogenesis, where tumour-derived VEGF plays a pivotal role. Analysis of human blood samples and tumour tissue revealed that VWF release combined with a local inhibition of proteolytic activity and protein expression of ADAMTS13 accounted for a tumour propagating procoagulatory milieu.³¹ Increased plasma levels of VWF:Ag were associated with poor prognosis in different types of cancer (ovarian, bladder, gastric and colon).^{16,17,32,33} Increased expression of VWF was observed in the tumour compared with the non-tumoural parenchyma in gastric cancer, and late gastric cancer showed significantly higher levels of VWF:Ag.¹⁶ The GPIIb α receptor can be expressed by various tumour cells, such as human breast cancer cells, and it was suggested that the function of this receptor on tumour cells differs in its adhesive interaction with VWF from that of platelets.^{34,35} The most active, ultra-large form of VWF is released from the endothelial Weibel-Palade bodies,³⁶ and ADAMTS13-mediated proteolytic processes may be decreased. Hence, the cumulative action of these processes may lead to the formation of firm tumour-platelet aggregates linked by fibres of ultra-large VWF.³¹ Our observations are in line with this mechanism, since we were able to show in this study the independent association of decreased ADAMTS13 activity (72.28% on average) and increased VWF:Ag levels (677.86 ng/mL on average) with poor event-free survival of patients with colorectal cancer. The analysis of the oligomeric form of VWF showed that ultra-large oligomers occurred more frequently in patients with high VWF:Ag levels and low ADAMTS13 activity, when compared with

those with low VWF:Ag levels and high ADAMTS13 activity group (► **Supplementary Table S1** [online only]). Decreased ADAMTS13 activity with increased VWF:Ag levels was associated with increased occurrence of thromboembolic events which support the clinical relevance of this observation (► **Supplementary Table S1** [online only]). Decreased activity of ADAMTS13 was observed in clinical studies in the plasma of patients with different malignancies mostly in advanced, disseminated stages;^{19,22} however, this is the first study providing epidemiologic evidence for the association between decreased ADAMTS13 activity and poor event-free survival in a 5-year-long follow-up study. Our current observations are in line with results of previous studies and may support the important role of ADAMTS13 and VWF in the pathomechanism of cancer-related thromboembolism and mortality.

In summary, this is the first observational study reporting platelet count, ADAMTS13 activity and VWF:Ag level simultaneously in colorectal cancer. We showed that thrombocytosis, elevated VWF:Ag levels and decreased VWF-processing protease activity independently associate with poor event-free survival. Therefore, these epidemiological data may support prior observations obtained from animal clinical studies on the contribution of tumour cell-platelet heteroaggregates, held together by VWF-fibres, in the propagation of tumour progression. These observations may therefore provide further support to plan novel therapies targeting endothelial cell activation or microthrombi formation in cancer treatment.

What is known about this topic?

- Metastasis formation is the leading cause of cancer-related death. There is growing evidence of an interaction between tumour cells and platelets which may influence tumour progression and metastasis formation and may be regulated by the VWF.
- Preoperative thrombocytosis and increased VWF:Ag level are associated with poor prognosis in different types of cancer. In disseminated malignancies, decreased ADAMTS13 serum levels were measured.
- It is important to find new reliable markers which can predict prognosis in colorectal cancer.

What does this paper add?

- We measured ADAMTS13 activity in a large cohort of colorectal cancer patients at the first time and observed the association of decreased activity—together with increased platelet count and VWF:Ag level—with poor survival without thrombotic complications.
- We report that increased platelet count, VWF:Ag level and decreased ADAMTS13 activity are all independently associated with advanced stage and poorer event-free survival.
- These observational data may fuel further research on tumour cell-platelet heteroaggregates, to explore the role of VWF-fibres in tumour progression and metastasis formation and may finally highlight novel targets of therapy.

Conflicts of interest

None declared.

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