

# Von Willebrand factor in the plasma and in the tumor tissue predicts cancer-associated thrombosis and mortality

Cancer-associated thrombosis (CAT) is a well-known complication of malignant tumors and is associated with faster tumor progression and worse prognosis.<sup>1-3</sup> A promising candidate to serve as thrombogenic marker in tumor progression is von Willebrand factor (VWF). VWF multimers mediate intravascular platelet adhesion and activation and might be therefore responsible not only for thrombotic vessel occlusion, but also for the development of metastases.<sup>4,5</sup> The aim of the study was the assessment of the prognostic value of VWF and ADAMTS-13, an enzyme that cleaves and inactivates VWF multimers, in patients with malignant tumors. To this end, we analyzed VWF and ADAMTS-13 in blood samples and explored the association between venous thromboembolism (VTE), disease progression and tumor-related death. In order to substantiate an impact of VWF in platelet binding and vessel occlusion, we compared intratumoral VWF distribution and VWF-dependent platelet accumulation with peritumoral regions. There was an evident correlation between increased plasmatic VWF levels and VWF-mediated platelet aggregations in the tumor microenvironment, CAT and cancer-related mortality. The novelty of this study is the correlation of blood-based markers with the occurrence of ultra-large VWF multimers mediating platelet aggregation in tumor and peritumoral tissue. This opens a novel field of investigations for the development of prognostic biomarkers and innovative therapeutic concepts.

This study (clinicaltrials.gov. Identifier: 2012-293N-MA, date: June 19, 2012) included 194 patients diagnosed with cancer between January 2013 and September 2018 at the University Medical Center Mannheim. Healthy volunteers and patients with non-metastatic basal-cell carcinoma (BCC) were used as control. None of the included BCC patients developed metastases or VTE. All procedures were performed with written informed consent from all participants in accordance with the Declaration of Helsinki. The protocols received approval by the ethics committee. Inclusion criteria were: (i) a confirmed diagnosis of cancer according to the American Joint Committee on Cancer tumor staging and classification, (ii) histologic confirmation of diagnosis, and (iii) at least 18 years of age. Adult patients undergoing surgery in curative intent for colorectal, esophagogastric and lung cancer were included. Exclusion criteria were the presence of an autoimmune disease, hepatitis B or C and previous therapeutic anti-coagulation.

VWF levels and ADAMTS-13 activity were analyzed in sodium citrate plasma samples as described.<sup>4</sup> Local conformation changes of VWF in tumor tissue compared with peritumoral regions, defined by a board-certified pathologist (TG) and separated from the tumor by at least 5-10 mm, were analyzed by immunofluorescence. Rabbit anti-human VWF (DakoCytomation, RRID:AB\_2315602), mouse anti-human thrombospondin (Laboratory Vision, RRID:AB\_61137), mouse anti-human CD31 (DakoCytomation, RRID:AB\_2114471), FITC-conjugated goat anti-rabbit (BD Pharmingen, RRID:AB\_395212) and Alexa 555-conjugated goat anti-mouse (IgG; Invitrogen, RRID:AB\_141822) were used. For comparison of two groups the two-sided Student's *t*-test, and for multiple groups one-way ANOVA (Kruskal-Wallis H test) with the Wilcoxon *post hoc* test was used. Correlation coefficients were calculated with Spearman analysis. Survival was analyzed using Kaplan-Meier analysis and compared using log-rank (Mantel-Cox) test. Results of the Cox models are displayed as hazard ratio (HR) and the 95% confidence interval (95% CI). The cutoff values were selected by optimizing the log-rank statistics. \* $P \leq 0.05$  was considered as significant difference.

Baseline characteristics of the study population are shown in Table 1. There was no significant correlation between plasmatic VWF concentrations and age neither in the control group ( $R=0.18$ ;  $P=0.13$ ) nor in tumor patients ( $R=0.13$ ;  $P=0.14$ ). Plasma VWF levels did not differ between female and male study participants. In the healthy control population, the mean VWF level (ng/mL) was  $11,519.4 \pm 6,261.3$  and in patients with BCC it was  $14,730.2 \pm 10,537.4$ . Patients with gastric, esophageal, colorectal, and pancreatic cancer had significantly higher VWF values compared with the control group (Figure 1A). Mean plasmatic VWF levels (ng/mL) were  $19,551.5 \pm 14,029.1$  for patients with colorectal,  $27,236.1 \pm 14,476.8$  for patients with gastric cancer,  $25,555.5 \pm 10,340.7$  for esophageal tumors and  $37,041.5 \pm 14,641.2$  for patients with pancreatic tumors (Table 1). Among patients diagnosed with colorectal, gastric, esophageal, and pancreatic cancer, a significant reduction of ADAMTS-13 activity was observed (Figure 1A; Table 1). Consequently, a significantly inverse correlation (Spearman's  $\rho = -0.41$ ;  $P \leq 0.001$ ) between VWF values and ADAMTS-13 activity was reflected by a high VWF / ADAMTS-13 ratio (Figure 1B). In contrast, blood VWF was not elevated in patients with lung tumors associated with increased ADAMTS-13 activities (Figure 1A;  $P \leq 0.05$ ).

**Table 1.** Demographic, clinical and laboratory characteristics of the study population, plasma levels of von Willebrand factor and ADAMTS-13 activity.

	Control (N=56)	BCC (N=39)	Colorectal (N=39)	Stomach (N=37)	Esophagus (N=52)	Pancreas (N=13)	Lung (N=14)	Total (N=250)
Age in years Mean (SD)	46.4 (13.5)	70.3 (11.6)	67.0 (12.2)	63.0 (14.1)	63.8 (10.4)	71.9 (8.1)	66.6 (8.8)	62.8 (14.3)
Sex, N (%)								
F	24 (42.9)	18 (46.2)	17 (43.6)	12 (32.4)	9 (17.3)	2 (15.4)	7 (50.0)	89 (35.6)
M	32 (57.1)	21 (53.8)	22 (56.4)	25 (67.6)	43 (82.7)	11 (84.6)	7 (50.0)	161 (64.4)
UICC, N (%)								
na					2 (3.8)	1 (7.7)	1 (7.1)	4 (2.6)
0	na	na	0 (0.0)	4 (10.8)	5 (9.6)	0 (0.0)	0 (0.0)	9 (5.8)
I-II	na	na	19 (48.7)	12 (32.4)	8 (15.4)	11 (84.6)	7 (50.0)	57 (36.8)
III-IV	na	na	20 (51.3)	21 (56.8)	37 (71.2)	1 (7.7)	6 (42.9)	85 (54.8)
VTE, N (%)								
0	na	na	33 (84.6)	31 (83.8)	33 (63.5)	9 (69.2)	8 (57.1)	114 (73.5)
1	na	na	6 (15.4)%	6 (16.2)	19 (36.5)	4 (30.8)	6 (42.9)	41 (26.5)
VWF, ng/mL, mean (SD)	11,519.4 (6,261.3)	14,730.2 (10,537.4)	19,551.5 (14,029.1)	27,236.1 (14,476.8)	25,555.5 (10,340.7)	37,041.5 (14,641.2)	16,976.4 (13,326.8)	20,151.6 (13,337.2)
ADAMTS-13, %, mean (SD)	100.0 (18.1)	92.3 (26.7)	87.5 (20.8)	77.5 (19.6)	60.8 (33.4)	75.7 (31.1)	111.7 (24.6)	84.8 (29.1)

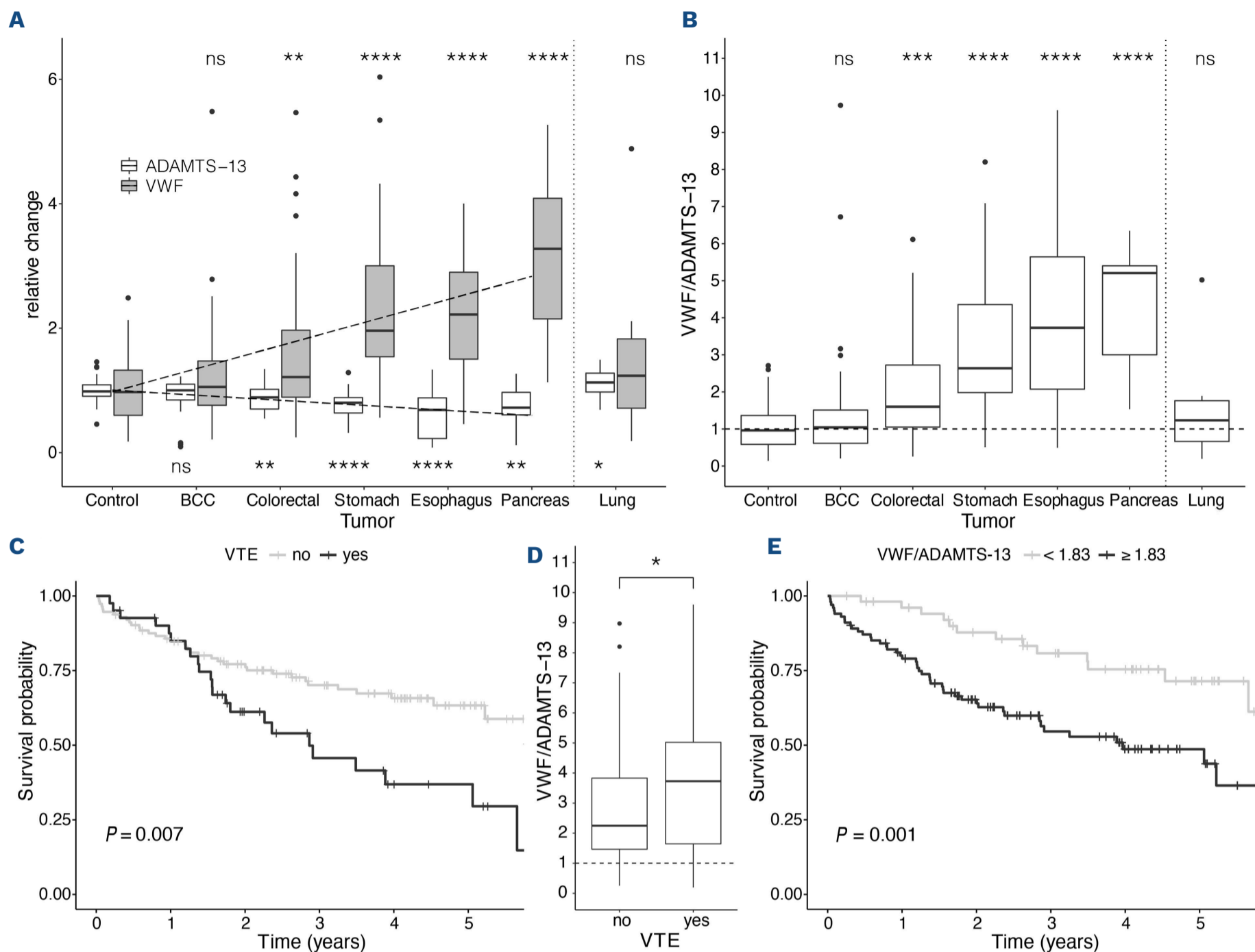
ADAMTS-13: a disintegrinlike and metalloproteinase with thrombospondin type I repeats 13; SD: standard deviation; VWF: von Willebrand factor; M: male; F: female; VTE: venous thromboembolism.

Next, the VWF / ADAMTS-13 ratio was analyzed in tumor patients of all tumor stages. Patients were divided into three groups depending on the stage of the disease - advanced disease (stage III and stage IV), localized disease (stage I and stage II) and neoplastic *in situ* tumors (stage 0). As shown in the *Online Supplementary Figure S1*, VWF levels in stage I and II and stage III and IV patients were significantly elevated. Importantly, the highest levels of the VWF / ADAMTS-13 ratio were measured in patients with *in situ* lesions (stage 0) and reveal increased systemic VWF levels combined with reduced plasmatic ADAMTS-13 activity in early disease stages (*Online Supplementary Figure S1*).

A telephone-based follow-up period of 6 years revealed that 26.5% (41/155) of the patients with cancer had developed a thromboembolic complication. In particular, VTE occurred in 15.4% of patients with colorectal cancers, in 16.2% with gastric tumors, in 30.8% with pancreatic tumors, in 36.5% of the patients with esophageal malignancies and in 42.9 % of patients with lung cancer (Table 1). In Cox regression analyses, patients with VTE were at higher risk of death compared with patients without thrombotic complications (Figure 1C; HR: 2.027; 95% CI: 1.2-3.424;  $P=0.007$ ). The VWF / ADAMTS-13 ratio was higher in patients diagnosed with VTE when compared to cancer patients without the occurrence of VTE (Figure 1D). For analysis of overall survival (OS) according to plasmatic VWF levels or ADAMTS-13 activity patients were separated into two groups according to their plasmatic VWF concentrations or ADAMTS-13 activity. Both, elevated VWF levels and reduced ADAMTS-13 activity (*Online Supplementary Figure S2*) were clearly associated with shorter OS. Con-

sequently, as determined by the Kaplan-Maier method shown in Figure 1E the 25<sup>th</sup> survival percentile of patients with a low VWF / ADAMTS-13 ratio (VWF / ADAMTS-13 <1.83) was 1,656 days compared to 448 days of patients with a high VWF / ADAMTS-13 ratio (VWF / ADAMTS-13  $\geq$ 1.83). Thus, elevated plasmatic VWF concentrations and reduced ADAMTS-13 activities may serve as prognostic marker for tumor patients (HR: 2.712; 95% CI: 1.459-5.04;  $P=0.001$ ). Finally, we evaluated whether elevated plasmatic VWF was related to intratumoral VWF networks mediating platelet aggregation (Figure 2; *Online Supplementary Figure S3*). Histological analyses demonstrated that VWF is mainly restricted to endothelial cells within the blood vessel wall in non-activated endothelial cells (Figure 2A). Blood vessels with endothelial cell activation and subsequent VWF secretion were identified by intraluminal VWF fibers ( $\geq 5 \mu\text{m}$ ) mediating platelet binding and aggregation (Figure 2B to D). As displayed in Figure 2E we calculated the relative increase of intratumoral vessels with VWF fibers in relation to blood vessels within peritumoral tissue. We detected that tumor entities with a low micro-environmental VWF network formation, such as colorectal (13.5%) or lung (10.4%) cancer, were associated with relatively low plasmatic VWF / ADAMTS-13 values (Figure 1). A gradual increase in luminal VWF network formation occurring in esophageal (18.4%), gastric (25.3%), and pancreatic cancer (26.4%) was statistically significant and was increased in patients with VTE (Figure 2F). Importantly, increased levels of VWF fibers in the tumor tissue were associated with a reduced OS (Figure 2G;  $P=0.013$ ).

The first aim of the current study was to evaluate if systemic VWF levels and ADAMTS-13 activity could serve as

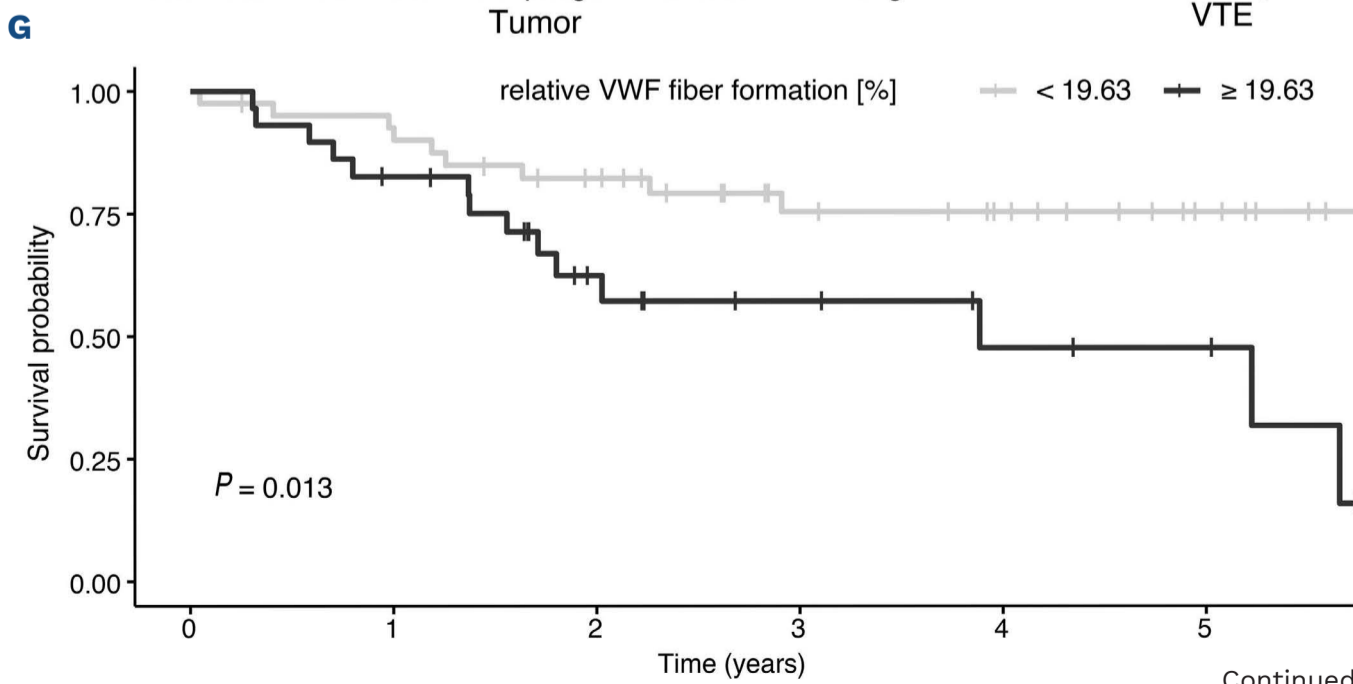
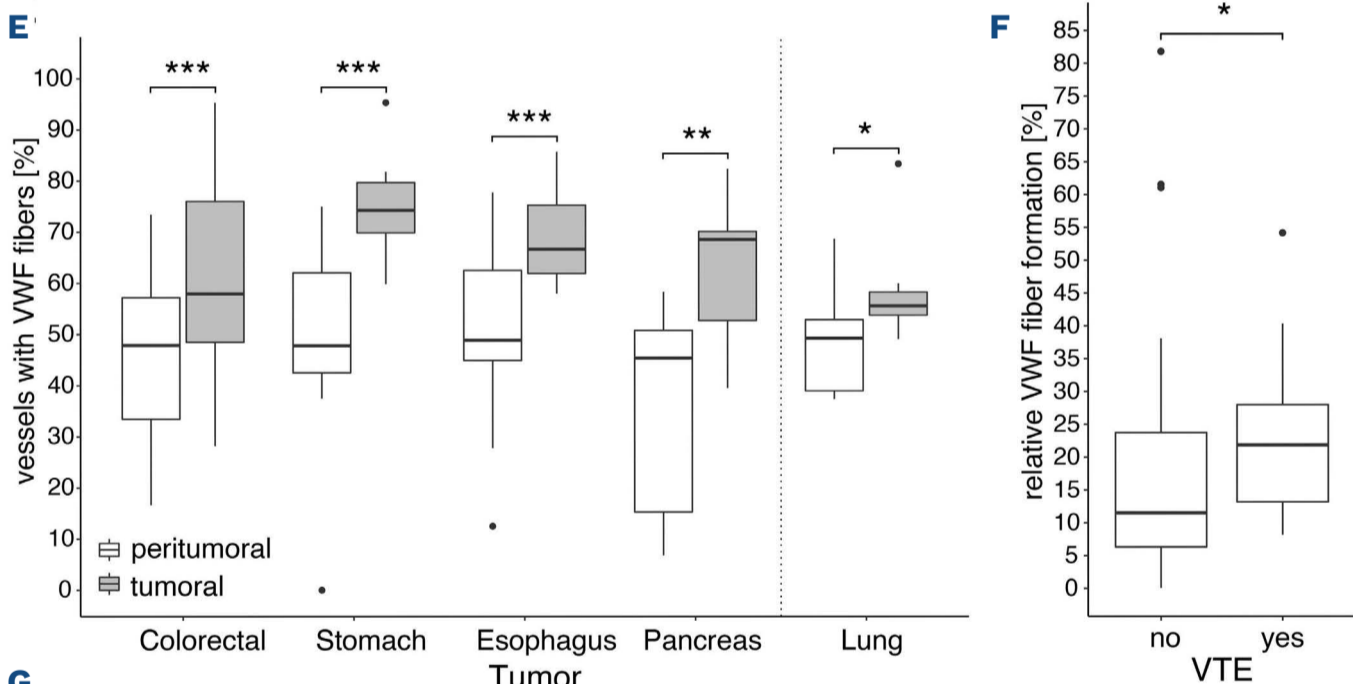
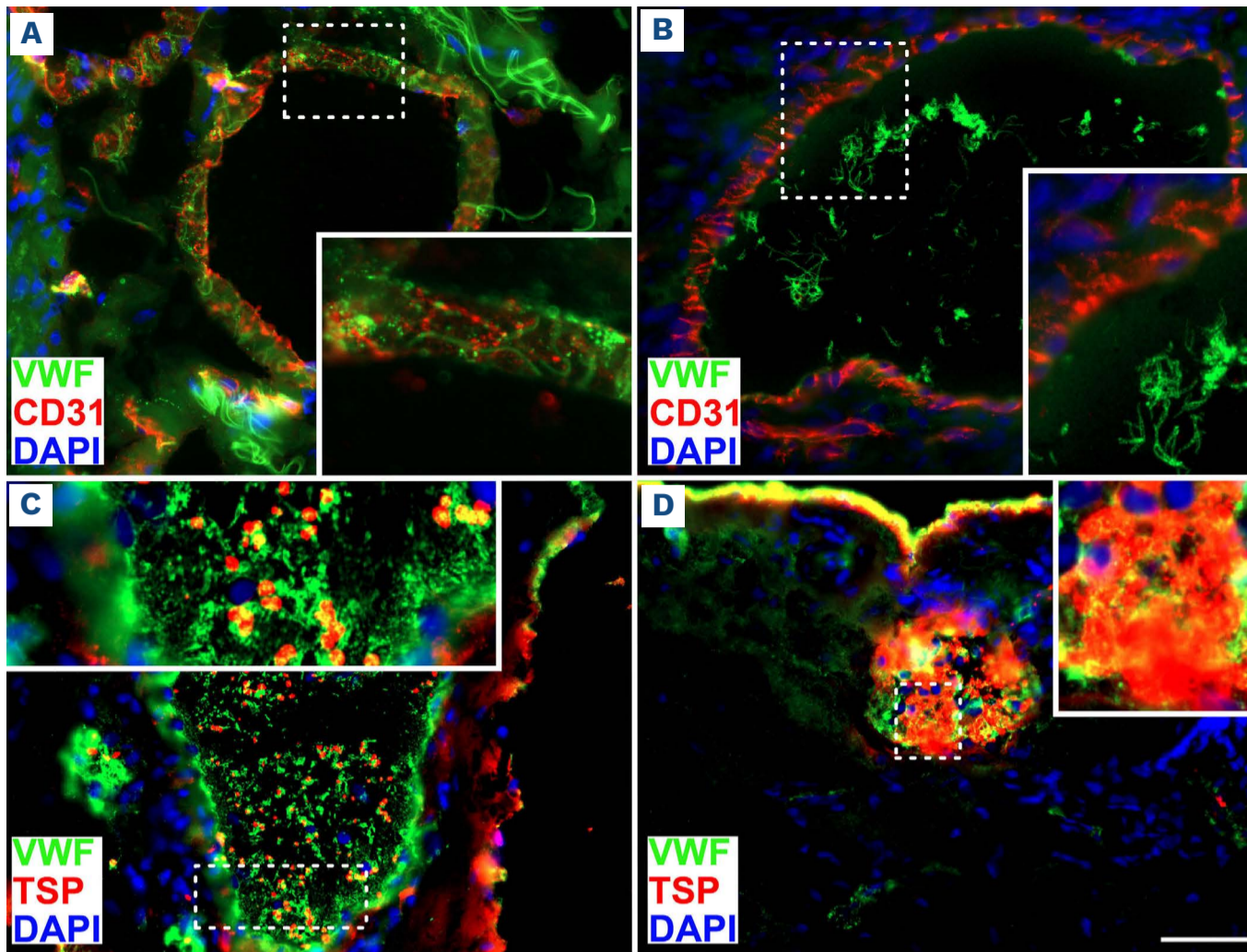


**Figure 1. Plasmatic von Willebrand factor levels and ADAMTS-13 activity correlate with the occurrence of venous thromboembolism and a reduced overall survival in tumor patients.** (A) Comparison of tumor patients with healthy controls and basal-cell carcinoma (BCC) according to relative plasmatic von Willebrand factor (VWF) levels and ADAMTS-13 activity and (B) the VWF / ADAMTS-13 ratio. Data are presented as box blots showing the median; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ . (C) Kaplan-Meier analysis for overall survival (OS) in years of 155 tumor patients according to the occurrence of venous thromboembolism (VTE) (with VTE  $n=41$ , without VTE  $n=114$ ). (D) Comparison of plasmatic VWF / ADAMTS-13 ratio of tumor patients according to the occurrence of VTE. Data are presented as box blots showing the median of 155 tumor patients; \* $P \leq 0.05$ . (E) Kaplan-Meier analysis for OS in years of 155 tumor patients according to elevated and non-elevated plasmatic VWF / ADAMTS-13 ratio (cutoff, 1.83).

prognostic biomarkers in malignancy. A significant increase of both the plasmatic VWF and the VWF / ADAMTS-13 ratio was observed in tumor patients, in contrast to healthy control or BCC. Data obtained from tumor patients are in line with our observations showing that elevated serum concentrations of VWF are associated with decreased ADAMTS-13 activities resulting in a high VWF / ADAMTS-13 ratio.<sup>6-8</sup> Interestingly enough, we detected no change in the systemic VWF concentration and VWF / ADAMTS-13 ratio in subjects with lung cancer compared to control. While this observation is in agreement with a previous study in patients with non-small-cell lung cancer,<sup>9</sup> other studies report elevated VWF levels,<sup>10</sup> an increased VWF / ADAMTS-13 ratio, and reduced ADAMTS-13

activities.<sup>11</sup> It remains to be explored whether VWF and its degradation by ADAMTS-13 contribute to lung cancer progression.

Next, we analyzed changes in the VWF / ADAMTS-13 ratio to examine the association between VWF and tumor progression. In our study, the VWF / ADAMTS-13 ratio was not directly correlated with the disease stages. Interestingly, patients with precancerous lesions, demonstrated even higher values compared to patients with metastatic disease. Imbalances of VWF and ADAMTS-13 have been associated with the development of hepatocellular carcinoma in cirrhotic patients<sup>12</sup> and could thus imply the use as a non-invasive biomarker for early detection of malignancy.



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**Figure 2. Von Willebrand factor fibers in the tumor tissue promote platelet aggregation and correlate with the occurrence of venous thrombosis and reduced overall survival.** Consecutive cryosections (10  $\mu\text{m}$ ) of primary colorectal tumors and peritumoral regions were stained for von Willebrand factor (VWF) (green), CD 31 (red) or thrombospondin (TSP) (red) and DAPI (blue). (A) Representative images demonstrate that VWF is located in the vessel wall of quiescent endothelium. (B) Representative images demonstrate that the secretion of VWF from activated endothelial cells correlates with luminal VWF fibers. (C) Luminal VWF fibers promote platelet binding and (D) platelet aggregation (scale bar: 50  $\mu\text{m}$ ). (E) The relative percentage of vessels with luminal VWF fibers (defined as fibers with a length of  $\geq 5 \mu\text{m}$ ) in peritumoral tissue was plotted against VWF fiber-containing blood vessels within the primary tumor. (F) Comparison of the relative percentage of vessels with luminal VWF fibers (peritumoral – tumoral) in patients with and without the occurrence of venous thromboembolism (VTE). Data are presented as box blots showing the median; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ . (G) Kaplan-Meier analysis for overall survival (OS) in years of tumor patients according to the relative VWF fiber formation (%) in the tumor tissue (cutoff, 19.63).

In order to analyze whether there is an association between a high VWF / ADAMTS-13 ratio and thrombotic complications, we performed a follow-up of 6 years after the initial blood sampling. Consistent with previous examinations indicating that coagulation is a prognostic parameter for cancer-related mortality,<sup>13-15</sup> our data show that a high VWF / ADAMTS-13 ratio is associated with VTE and a worse prognosis for patients. The procoagulant VWF is a key candidate for cancer-related VTE and metastasis, because in the blood stream stretched VWF multimers mediate platelet binding and aggregation.<sup>4,5</sup> We found a higher amount of VWF multimers in tumor samples promoting platelet binding and thrombotic vessel occlusions. In summary, our data support the concept that VWF multimers represent the pathophysiological link between the thrombotic risk and tumor progression. Thus, plasma VWF and the ADAMTS-13 activity in combination with histological VWF changes in the tumor microenvironment could help to predict tumor progression and CAT.

This is the first prospective study on the prognostic significance of VWF in cancer patients. As it was shown that ABO blood group and anti-tumor therapy are predictive for the development of VTE,<sup>3,16</sup> the correlation with VWF should be assessed in larger scale studies in order to establish the link between cancer and thrombosis.

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### Disclosures

No conflicts of interest to disclose.

### Contributions

IK, KN and JK performed experiments and generated data. CM performed statistical analyses, prepared figures and contributed to writing of the manuscript. LS, TG, FTM, LG and SWS discussed and analyzed data. ATB contributed to all experiments and wrote the manuscript.

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### Data-sharing statement

Original data will be made available upon request.

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