Published in final edited form as:

Thromb Haemost. 2017 January 5; 117(1): 90–98. doi:10.1160/TH16-02-0123.

Decreased platelet reactivity in patients with cancer is associated with high risk of venous thromboembolism and poor prognosis

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Summary

Platelets are suggested to play a crucial role in cancer progression and the prothrombotic state of cancer patients. Here, we aimed to examine the activation status of platelets in cancer patients and investigate their association with risk of death and occurrence of venous thromboembolism (VTE) in a prospective observational cohort study. We measured platelet surface P-selectin, activated glycoprotein (GP) IIb/IIIa and monocyte-platelet aggregate (MPA) formation *in vivo* and platelet response to *ex vivo* stimulation with agonists of protease-activated receptor (PAR) -1, -4, and GPVI, by whole blood flow cytometry, before beginning of chemotherapy and repeatedly during the first six months thereafter (total number of samples analysed: 230). Endpoints of the study were occurrence of death or VTE during a two-year follow-up, respectively. Of 62 patients (median age [interquartile range, IQR]: 63 [54–70] years, 48 % female), 32 (51.6 %) died and nine (14.5 %) developed VTE. Association with a higher risk of death was found for lower platelet surface expression of P-selectin and activated GPIIb/IIIa *in vivo* and in response to PAR-1, -4 and GPVI activation, but not for MPA formation. Furthermore, reduced platelet responsiveness to PAR-1 and GPVI agonists was associated with higher risk of VTE (hazard ratio per decile increase

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Conflicts of interest

None declared.

of percentage P-selectin positive platelets: 0.73 [0.56–0.92, $p=0.007$] and 0.77 [0.59–0.98, $p=0.034$], respectively). In conclusion, cancer patients with a poor prognosis showed decreased platelet reactivity, presumably as a consequence of continuous activation. Our data suggest that decreased platelet reactivity is associated with increased mortality and VTE in cancer.

Keywords

Platelets; thrombosis; monocyte-platelet aggregates; cancer; mortality

Introduction

Platelets play a crucial role in several steps of cancer progression, such as tumour growth, angiogenesis and metastatic spread of malignancy (1). Many cancer cells have the ability to directly activate platelets (2, 3), and increased levels of platelet activation markers were found in cancer patients (4). Moreover, patients with cancer frequently have elevated platelet counts (5), which are linked to a poor prognosis of their disease (6). It has been recently demonstrated that thrombocytosis (i. e. elevated platelet counts) is not merely an epiphenomenon in cancer, but rather a paraneoplastic syndrome that is actively induced by the malignant disease to promote tumour growth (7). The adverse effects of platelets in cancer are underpinned by studies, which showed that platelet depletion in animal models leads to reduction in metastasis formation (8). In addition, in large clinical trials investigating the effect of pharmacological platelet inhibition, a reduced risk of cancer, metastatic spread, and cancer-related mortality was observed upon aspirin intake (9, 10).

Platelets mediate both the physiologic process of haemostasis and pathologic thrombosis. In cancer patients, platelets are thought to be involved in the development of venous thromboembolism (VTE), a frequent complication associated with high morbidity and mortality (4). Although the VTE risk of cancer patients is influenced by multiple patient-, tumour- and therapy-related factors (11–13), several studies have shown that platelet characteristics, such as high platelet counts, low mean platelet volume (MPV) and high levels of the platelet and endothelial cell activation marker soluble P-selectin (sP-selectin) are associated with risk of cancer-associated VTE (14–17). These observations suggest an important contribution of platelet activation to the prothrombotic state in cancer.

Despite this relatively large body of evidence pointing to an important role of platelets in cancer progression and development of cancer-associated VTE, to the best of our knowledge, no prospective study has so far investigated platelet function in detail in patients with cancer in order to elucidate the association with disease progression, or, at least, with the risk for VTE. In this study, we determined platelet activation in cancer patients by whole blood flow cytometry before and during anti-tumour therapy and investigated the association of platelet activation with risk of mortality and VTE.

Material and methods

Patients and control individuals

The Vienna Cancer and Thrombosis Study (CATS)—Patients were recruited within the framework of the Vienna Cancer and Thrombosis Study (CATS), an on-going prospective, single-centre, observational cohort study that started in 2003 at the Medical University of Vienna. The primary aim of CATS is to investigate risk factors for VTE in patients with cancer. The study was approved by the Ethics Committee of the Medical University of Vienna and was performed in concordance with the Declaration of Helsinki.

Detailed study procedures, inclusion and exclusion criteria have been previously described (17, 18). In short, patients with a newly diagnosed cancer or progressive disease after complete or partial remission are included. At the day of study inclusion written informed consent is obtained, a blood draw is performed and patients are then prospectively followed in regular intervals of approximately three months for a maximum time period of two years for occurrence of VTE, death or loss-to-follow-up. No routine screening for VTE is performed. VTE is confirmed by objective imaging methods such as duplex sonography or venography for deep-vein thrombosis (DVT) or by computed tomography or ventilation/perfusion lung scan for pulmonary embolism (PE), respectively. Death certificates and, if available, autopsy findings are used for a diagnosis of fatal PE in patients who died during follow-up.

Outcome measure of the study: Endpoints of the study are death of any cause and development of objectively confirmed VTE within an observation period of two years.

A longitudinal sub-study within CATS for the investigation of platelet function during chemotherapy—The present sub-study was performed between March 2013 and July 2014, and special inclusion criteria were as follows: patients who were scheduled for chemotherapy and who were diagnosed with a cancer of the brain, lung, pancreas, stomach or colon (these cancer entities are known to be associated with a high risk for VTE). Exclusion criteria were intake of aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) within the last 10 days before study inclusion or current medication with oral anticoagulants or low-molecular-weight heparin (LMWH) at any dosage. Other in- and exclusion criteria resemble those of the main CATS (17, 18).

In the current longitudinal study serial blood draws were performed. Blood was taken at study inclusion and, if possible, before the beginning of each new chemotherapy cycle, approximately once per month, during the first six months of therapy. A maximum of seven blood draws was performed in each patient.

Healthy controls—Thirty healthy individuals without a history of VTE or cancer, who had not taken aspirin or any NSAID within the last 10 days before blood sampling, were recruited from the same geographical region and served as controls for the comparison of platelet activation markers.

All participants gave written informed consent.

Blood sampling

Blood was collected by clean venipuncture into plasma vacuum tubes (Vacuette®; Greiner Bio One, Kremsmünster, Austria) containing one-tenth volume sodium citrate stock solution at 0.129 mM. To avoid artificial platelet activation during blood draw only a light tourniquet was applied, which was immediately released and the first 5 ml of blood were collected in EDTA containing blood collection tubes (Vacutainer K3-EDTA tubes, Vacuette®; Greiner-Bio One) for measurement of complete blood counts on a Sysmex XE-2100 haematology analyser. All samples were mixed adequately by gently inverting the tubes. The time span between blood sampling and start of platelet function assays by flow cytometry did not exceed 30 minutes (min). To avoid procedural deviations, the same physician processed all blood samples.

Additionally, at each blood draw citrated blood was centrifuged to obtain platelet-poor plasma and samples were stored for measurement of soluble biomarkers of coagulation at – 80 °C until testing was performed in series. D-dimer levels were measured by a quantitative latex assay (STA-LIatest D-DI; Diagnostica-Stago, Asnieres, France) on an STA-R analyzer (Diagnostica-Stago). Prothrombin fragment 1+2 (F1+2) and soluble P-selectin (sP-selectin) levels were measured by enzyme-linked immunoassays (Enzygnost F1+2; Dade-Behring, Marburg, Germany and Human sP-selectin ELISA kit, Quantikine®, R&D Systems, Minneapolis, MN, USA; respectively) according to manufacturer's instructions.

P-selectin expression and detection of activated GPIIb/IIIa by flow cytometry

Platelet reactivity was determined by assessing binding of the monoclonal antibody PAC-1 to activated GPIIb/IIIa and by the platelets' surface expression of P-selectin, as previously published (19). In short, blood was diluted in phosphate-buffered saline (PBS) to obtain approximately $2 \times 10^4/\mu\text{l}$ platelets and incubated for 10 min without agonists or with suboptimal concentrations of the following three platelet agonists: PAR-1 agonist SFLLRN (Thrombin-receptor activating peptide [TRAP] –6; final concentration 14.25 μM ; Bachem, Bubendorf, Switzerland), PAR-4 agonist AYPGKF (final concentration 0.714 mM; Verum Diagnostica, Munich, Germany) and the GPVI agonist collagen-related peptide (CRP; final concentration 0.04 $\mu\text{g}/\text{ml}$; a generous gift from Dr. R. W. Farndale, Department of Biochemistry, University of Cambridge, Cambridge, UK). The platelet population was identified by staining with anti-CD42b (clone HIP1, allophycocyanin labelled, Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA), while P-selectin and activated GPIIb/IIIa expression were determined by the binding of the monoclonal antibodies anti-CD62P (P-selectin; clone CLB-Thromb6, phycoerythrin-labelled, Immunotech, Beckman Coulter Fullerton, CA, USA) and PAC-1 (fluorescein isothiocyanat-labelled, Becton Dickinson, Immunocytometry Systems). Isotype-matched control antibodies were used in separate vials for the determination of non-specific binding. After 15 min of incubation in the dark, their reaction was stopped by adding 500 μl cold PBS (4 °C) and samples were acquired after 10 min on a FACS Calibur flow cytometer (Becton Dickinson). Standard Becton Dickinson Calibrite beads were used for daily calibration of the cytometer.

A total number of 10,000 CD42b positive events were recorded. Positive analysis regions for P-selectin and activated GPIIb/IIIa were set with appropriate nonspecific controls.

Percentage positive platelets and mean fluorescence intensity (MFI) of anti-CD62P and PAC-1 were evaluated.

Detection of monocyte-platelet aggregates (MPA) by flow cytometry

Monocytes with adhering platelets were identified by previously published methods (20). In brief, 5 μ l whole blood were diluted in 55 μ l HEPES buffer and then incubated without platelet agonists or with platelet agonists (SFLLRN, final concentration 7.1 μ M; or AYPGKF, final concentration 0.357 mM; or CRP final concentration 0.04 μ g/ml) for 10 min. Thereafter, monoclonal antibodies (anti-CD45, clone 2D1, peridinin chlorophyll protein-labelled, Becton Dickinson; anti-CD41, clone P2, phycoerythrin-labelled, Immunotech, and anti-CD14, clone M Φ P9, allophycocyanin-labelled, Becton Dickinson) were added. After 15 min of incubation, samples were diluted with FACSlysing solution. A total of 10,000 CD45 positive events were acquired and within these events, lymphocytes, granulocytes, and monocytes were identified, based on their CD14 versus side scatter characteristics. Monocytes were identified as CD14⁺ and the CD45⁺CD14⁺ events were subjected to further analyses for CD45⁺CD41⁺ and CD45⁺CD41⁻ events. The percentage of CD14⁺CD41⁺ events (percentage monocytes with adhering platelets) was determined.

Statistical analyses

Continuous variables were described with the median and the interquartile range (IQR). Categorical variables were described by absolute numbers and percentages. The Wilcoxon rank sum test was applied to test for differences in platelet activation parameters between tumour patients and healthy controls and the Kruskal-Wallis test was used to test for differences between the different tumour sites.

Survival analyses—Cox proportional hazards regression models were performed for analysis of a possible influence of the platelet activation variables on survival. Each variable was considered in an univariate sense. At first, only the baseline variable values were considered in the regression models. Estimated hazard ratios (HR) with 95 % confidence intervals (CI) and p-values describe the strength of the prognostic variables measured at the first (baseline) blood sampling. Second, Landmark analyses were used to evaluate updated values after two months with regard to prognosis. In a third step, all values measured in the course of time were included (updated at every time point of the consecutive blood sampling) by considering the platelet activation variables as time-dependent covariates. The respective results describe the prognostic strength of the most recent value of each consecutively updated platelet activation variable.

Since most of the platelet activation variables were not normally distributed, each variable was categorised according to its deciles. Therefore, only the sign of the parameter estimate β can be interpreted, and a significant HR (= $e\beta$) greater than 1.0 describes an increasing risk at higher variable values, whereas a significant HR less than 1.0 stands for a decreasing risk at higher variable values. Parameter estimates for the different variables are all based on variables standardised to the same range (0–9). All p-values are results of two-sided tests and p-values < 0.05 were considered as statistically significant. The SAS software (version 9.4, SAS Institute Inc. (2002–2012), Cary, NC, USA) was used for statistical analyses.

VTE analyses—Univariable Cox proportional hazards regression models were performed to evaluate the possible influence of platelet activation variables on the risk of VTE.

Results

Study patients

In total, 62 cancer patients were included between March 2013 and July 2014. Cancer was newly diagnosed in 57 patients (91.9 %). Of the remaining five patients with recurrent disease after complete or partial remission, two had received a previous radio- and chemotherapy (in these patients the last chemotherapy had been administered 7 months and 15 months before inclusion into the current study, respectively), two patients had been treated with surgery only at the time of first diagnosis of cancer and one patient had been treated with surgery and a tyrosine-kinase inhibitor at initial diagnosis of cancer.

Thirty healthy individuals served as controls. Detailed characteristics of study patients and control individuals are shown in ►Table 1.

Pre-chemotherapy platelet P-selectin surface expression, activated GPIIb/IIIa and MPA formation in cancer patients and in healthy controls

Blood platelet counts did not differ between cancer patients and healthy controls (median [IQR], G/I: 249 [188–313] vs 240 [220–257], $p=0.779$).

We observed similar levels of the percentage of surface P-selectin and activated GPIIb/IIIa positive platelets *in vivo* and in response to *ex vivo* platelet activation with PAR-1 or GPVI agonists in the total population of cancer patients compared to healthy controls (►Table 2). MFI of platelet surface P-selectin and activated GPIIb/IIIa expression *in vivo* was higher in cancer patients compared to healthy controls (►Table 2). P-selectin expression in response to the PAR-4 agonist AYPGKF was higher in cancer patients compared to healthy controls. MPA formation *in vivo* and in response to PAR-1, PAR-4 and GPVI agonists was significantly higher in cancer patients than in healthy controls (►Table 2).

In subgroup analysis, however, those patients with a poor prognosis (i. e. patients who died within the first year of follow-up) showed lower activated GPIIb/IIIa expression on platelets in response to PAR-1 and PAR-4 agonists in comparison to healthy controls. In contrast, those patients who survived the first year of follow-up had higher levels of surface P-selectin and activated GPIIb/IIIa positive platelets without activation and in response to PAR-4 activation in comparison to healthy controls (Suppl. Table 2, available online at www.thrombosis-online.com).

Neither P-selectin, nor activated GPIIb/IIIa, nor MPA formation differed between patients with cancers of different sites (all p -values >0.05).

Association of pre-chemotherapy platelet P-selectin surface expression, activated GPIIb/IIIa and MPA formation with risk of death

Median overall survival was 465 days (25th percentile: 286 days, 75th percentile: not estimable). Median levels of P-selectin surface expression and activated GPIIb/IIIa *in vivo*

and upon *ex vivo* activation with PAR-1, PAR-4 and GPVI agonists were lower in patients who died during the first year of follow-up compared to patients who survived the first year of follow-up (►Figure 1).

In Cox regression analyses, patients with lower surface expression of platelet P-selectin and activated GPIIb/IIIa *in vivo* were at higher risk of death; the hazard ratio [HR] for death per one decile increase in percentage P-selectin positive platelets was 0.88 (0.78–0.99; $p=0.046$). Furthermore, lower surface expression of platelet P-selectin and activated GPIIb/IIIa in response to PAR-1, PAR-4 and GPVI agonists were associated with an increased risk of death. In contrast, MPA formation *in vivo* and in response to the three platelet agonists was not associated with an increased risk of death. Results of associations between all parameters and risk of death are shown in ►Table 3.

We divided patients into two groups to compare patients with high platelet P-selectin and GPIIb/IIIa (cut-off value was set at the median of the distribution of each parameter in the total population of cancer patients) to those with lower levels (< median). High levels (>median) of platelet P-selectin and GPIIb/IIIa in response to the three platelet agonists were associated with a decreased risk of death and this association was most pronounced for PAR-4 induced expression of P-selectin and activated GPIIb/IIIa, which is illustrated in ►Figure 2.

Platelet P-selectin surface expression, activated GPIIb/IIIa and MPA formation, measured over a period of time, and risk of death

Repetitive blood draws were performed during the first six months of chemotherapy (between 1 and 7 blood draws were taken per patient, median: 3.7) and updated values of platelet P-selectin and activated GPIIb/IIIa and MPA formation were analysed for their association with risk of death. Table 3 shows Landmark analyses of updated measurements at two months after study inclusion and associations between most recently measured parameters and risk of death. Higher levels of platelet P-selectin and activated GPIIb/IIIa upon PAR-4 activation, determined at several time points during the study, were constantly associated with a decreased risk of death, while associations of other parameters with risk of death were not statistically significant (►Table 3).

Association of pre-chemotherapy platelet P-selectin surface expression, activated GPIIb/IIIa and MPA formation with risk of VTE

Nine patients had VTE during the follow-up period (14.5 %). Levels of platelet P-selectin surface expression and activated GPIIb/IIIa *in vivo* were not associated with risk of VTE. Higher levels of platelet P-selectin surface expression and activated GPIIb/IIIa in response to PAR-1 activation were associated with a decreased risk of VTE (HR per decile increase: 0.73 [95 % CI: 0.56–0.92, $p=0.007$] and 0.76 [0.57–0.97, $p=0.025$], respectively). Furthermore, higher P-selectin surface expression in response to GPVI activation was associated with a lower risk of VTE, while P-selectin and GPIIb/IIIa expression in response to PAR-4 activation were not associated with risk of VTE.

Higher levels of MPA in response to PAR-1 activation were associated with a decreased risk of VTE (HR per decile increase: 0.78 [0.59–1.00], $p=0.046$), while *in vivo* and PAR-4 and GPVI induced MPA formation were not associated with risk of VTE.

Detailed results of associations between all parameters and their respective risks of VTE are shown in ►Table 4.

Correlations of platelet P-selectin surface expression, activated GPIIb/IIIa and MPA formation with plasma levels of D-dimer, F1+2 and sP-selectin

A weak inverse correlation (r between -0.2 and -0.3 , all $p<0.05$) was observed for plasma levels of D-dimer with platelet surface expression of P-selectin upon stimulation via GPVI, as well as with platelet surface expression of activated GPIIb/IIIa upon stimulation with PAR-1, PAR-4 and GPVI agonists. A weak positive correlation (r between 0.2 and 0.3 , all $p<0.05$) was observed between plasma levels of sP-selectin and MPA formation *in vivo* and upon stimulation with PAR-4 and GPVI agonists, as well as between plasma levels of F1+2 and MPA formation upon stimulation with PAR-1 and PAR-4 agonists. All data are shown in Suppl. Table 1 (available online at www.thrombosis-online.com).

Discussion

In this prospective observational and longitudinal study on patients with cancer we found that decreased platelet reactivity was associated with an increased risk of mortality and VTE. In detail, decreased platelet surface expression of P-selectin and activated GPIIb/IIIa *in vivo* and in response to PAR-1, PAR-4 and GPVI activation was associated with poor overall survival of cancer patients. Furthermore, a decreased platelet surface expression of P-selectin and activated GPIIb/IIIa, as well as decreased MPA formation in response to PAR-1 activation predicted a higher risk of developing VTE.

When we compared cancer patients to healthy controls, higher MFI levels of P-selectin and activated GPIIb/IIIa positive platelets were observed in cancer patients. While increased platelet activation has been previously described in cancer patients (21–23), the effect of platelet responsiveness and clinical outcome has never been investigated. Of note, we performed repetitive blood draws during the follow-up period and constantly found evidence of decreased platelet surface P-selectin and activated GPIIb/IIIa expression in those patients who had a poor prognosis compared to cancer patients with a more favourable outcome, suggesting a steadily reduced platelet response to agonists in advanced cancer. One previous study described the occurrence of “exhausted” platelets in cancer patients, as indicated by low levels of adenosine diphosphate (ADP) and serotonin, which is in accordance with our data (24).

Interestingly, the association of lower platelet surface expression of P-selectin and activated GPIIb/IIIa with an increased risk of death was most pronounced for a reduced response to the PAR-4 agonist AYPGK. This reduced responsiveness was already seen at the time of patients’ inclusion into the study, well before the start of chemotherapy. Furthermore, this reduced responsiveness persisted throughout the observation period. These latter findings are of particular interest, as chemotherapy strongly influences platelet production and function.

It should be emphasized, however, that the biological role of the platelet PAR-4 receptor is not fully elucidated. In particular, in comparison to PAR-1 the required concentration of the specific agonist for platelet activation in humans is many times higher. Some studies proposed that platelet granules, storage pool of multiple bioactive molecules, are differentially released upon stimulation of PAR-1 and PAR-4: They found that PAR-4 agonists primarily induce the release of anti-angiogenic factors, like thrombospondin-1 (TSP-1), while PAR-1 agonists induce the release of proangiogenic factors, including vascular endothelial growth factor (VEGF) (25). Platelets are known to be a major source of circulating VEGF (26) and a main storage pool for TSP-1 (27). The reduced response of platelets to PAR-4 in our study may indicate a failure of platelets to release anti-angiogenic factors, which could be related to the patients' poor prognosis. We have previously shown that cancer patients at risk of VTE, had increased circulating levels of VEGF (28), while we found no association between plasma levels of TSP-1 and VTE in our study cohort (29). This observation further argues for potential selective platelet release defects or changes in platelet α -granule composition in cancer patients.

However, further studies are needed to confirm our results and to investigate underlying mechanisms of the association between reduced platelet activation, especially in response to PAR-4, and increased mortality.

Another major finding of our present study is that platelets that were less responsive to the thrombin receptor PAR-1 agonist SFLLRN (in terms of surface expression of P-selectin and activated GPIIb/IIIa, as well as MPA formation) were associated with an increased risk of VTE. Interestingly, also in patients with stroke less active, so-called "exhausted", platelets were documented by failure to respond to activation by thrombin (30). Thrombin is one of the most potent platelet agonists. It was hypothesised that platelet exhaustion in stroke might be caused by continuous platelet activation due to high thrombin generation during the thromboembolic event, leading to cleavage of thrombin receptors (30). Michelson et al. showed in an experimental study, in which platelets were *ex vivo* activated by thrombin and re-infused into baboons, that these platelets still circulate even though they lost surface P-selectin (31). Based on our current data we suggest that also in patients with cancer continuous platelet activation *in vivo* leads to a reduced response to activating agonists *ex vivo*. It is known that in patients with cancer high levels of thrombin are generated, leading to a high risk for VTE (32). In our study a weak, but significant inverse correlation between platelet surface expression of activated GPIIb/IIIa in response to agonists and D-dimer levels was observed, suggesting at least a partial relationship between high levels of D-dimer, a marker which reflects activation of blood coagulation and fibrinolysis, and reduced activation of platelets. However, no association between F1+2 and platelet surface P-selectin and activated GPIIb/IIIa expression was found, arguing against this hypothesis. Alternatively, in cancer patients the malignancy might directly lead to a down-regulation of the thrombin receptors. It has been previously demonstrated that in patients with cancer differences of α -granule packaging occur (33) and that tumour progression leads to an accumulation of VEGF in platelet granules (34). Moreover, inflammation can modulate megakaryopoiesis (35), indicating that differences in platelet receptor expression could occur in pathologies that effect megakaryocyte development.

In our study we found strongly enhanced *in vivo* MPA formation and elevated levels of MPA in response to PAR-1, PAR-4 and GPVI activation in cancer patients compared to a group of healthy controls. MPA formation is considered to be a very sensitive surrogate parameter of *in vivo* platelet activation (36). We hence conclude that platelets of most cancer patients are activated continuously *in vivo*, leading to the formation of MPA. It is known that cancer cells can directly activate platelets (37), which might contribute to the high levels of MPA in cancer patients. However, although in our study MPA were significantly elevated in cancer patients compared to healthy controls, MPA formation was not associated with mortality of cancer patients. MPA formation might therefore be present reactively in cancer disease, but seems to have no prognostic potential for patient outcome in cancer. Interestingly, MPA formation but not platelet surface expression of P-selectin was positively correlated to sP-selectin, suggesting that sP-selectin in part reflects MPA formation.

Our study has some limitations and strengths. First of all, our study has an exploratory character, includes only a relatively small number of patients and has been considered to be hypothesis-generating. However, to the best of our knowledge, it is the first study to evaluate the prognostic relevance of platelet function, measured by flow cytometry, in cancer patients. Second, as this was an explorative study parameters assessed to characterise platelet activation were not corrected for multiple statistical testing. Third, in our study patients with different cancer entities were included and due to the limited sample size and event rates we were not able to perform subgroup analyses to evaluate the effects of platelet activation on mortality and VTE risk in each single cancer entity. In this respect, we would like to point out that the study aimed at globally evaluating platelet function in a more general setting of cancer, and that all included cancer entities are known to be associated with a high risk of VTE. Fourth, diagnoses of fatal PE might have been missed in some patients as autopsy is rarely performed and therefore occurrence of fatal PE might be underestimated in our study. Moreover, we did not measure reticulated platelets, a parameter that allows estimating half-life of platelets, which should be considered in future studies. The major strength of our study is its prospective design that was specifically chosen to investigate risk factors for VTE and mortality in cancer patients. Also the standardised laboratory techniques and repetitive measurements of platelet function at several times during chemotherapy are strengths of our study.

In summary, we show that cancer patients had higher levels of MPA compared to healthy controls and that decreased surface expression of platelet P-selectin and activated GPIIb/IIIa *in vivo* and in response to agonist-induced activation, especially upon PAR-4 activation, were associated with a poor overall survival of cancer patients. Furthermore, decreased platelet reactivity in response to PAR-1 activation was associated with a higher risk of developing VTE.

Our study delivers novel insights into the behaviour of platelets in cancer patients and provides the first evidence that decreased platelet activation in response to agonists is associated with a poor prognosis and a higher risk of VTE in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank all the patients who participated in this study for donating their time and their blood. We thank Dr. Farndale for kindly providing collagen-related peptide (CRP). We thank Tanja Altreiter for proof-reading the manuscript.

Financial support:

This study was supported by funds of the Austrian National Bank (Anniversary Fund; project number 14744), Funds of the Mayor of Vienna (Bürgermeister-Fonds; project number 14056) and by the Austrian Science Fund (FWF), Special Research Program (SFB) 54.

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What is known about this topic?

- Venous thromboembolism (VTE) is a frequent complication in patients with cancer.
- High platelet counts are associated with an increased risk of VTE and poor prognosis of patients with cancer.
- Platelets and their activation were suggested to be involved in cancer-associated VTE and cancer progression.

What does this paper add?

- In this prospective and longitudinal study decreased platelet reactivity upon agonist-induced activation *ex vivo* was associated with increased mortality in cancer patients.
- Furthermore, low platelet response upon thrombin receptor protease-activated receptor (PAR)-1 activation was associated with high risk of VTE.

Figure 1. Pre-chemotherapy platelet surface expression of P-selectin and activated GPIIb/IIIa in patients who died and those who survived one year of follow-up.

Platelet surface expression of P-selectin and activated GPIIb/IIIa without stimulation and in response to different platelet agonists were lower in cancer patients who died within one year after study inclusion compared to those who survived the first year, respectively (* $p < 0.05$; ** $p < 0.01$).

Figure 2. Kaplan-Meier estimates for cumulative survival probability of cancer patients with PAR-4 induced platelet surface expression of P-selectin (A) and GPIIb/IIIa activation (B) at study inclusion above and below the median, respectively.

Survival rates were significantly higher in patients with high P-selectin surface expression and GPIIb/IIIa activation (> median) compared to patients with levels below the median ($p=0.009$ and $p=0.001$, respectively).

Table 1
Clinical characteristics of cancer patients and healthy controls and number of events (VTE events and deaths) during follow-up.

	N	Median age Years (IQR)	Female, n (%)	Stage IV cancer [*] , n (%)	Died, n (%)	VTE, n (%)
Cancer patients	62	63 (54–70)	30 (48.4)	45 (72.6)	32 (51.6)	9 (14.5)
Lung	18	69 (64–72)	7 (38.9)	10 (55.6)	10 (55.6)	3 (16.7)
Pancreas	19	65 (54–72)	12 (63.2)	13 (68.4)	12 (63.2)	4 (21.1)
Brain	14	56 (41–65)	4 (28.6)	12 (85.7)	6 (42.9)	2 (14.3)
Colon	8	58 (41–61)	6 (75.0)	8 (100)	2 (25.0)	0 (0.0)
Stomach	3	63 (51–70)	1 (33.3)	2 (66.7)	2 (66.7)	0 (0.0)
Healthy controls	30	53 (50–61)	15 (50)	-	-	-

^{*} In solid tumours: presence of distant metastasis, in brain tumours: WHO grade IV tumours.

Table 2
Comparison of pre-chemotherapy platelet surface expression of P-selectin and activated GPIIb/IIIa and MPA formation between cancer patients (n=62) and healthy controls (n=30).

Percentage positive platelets/ percentage of monocytes carrying platelets as well as mean fluorescence intensity (MFI) of surface P-selectin and activated GPIIb/ IIIa expression is shown.

	Activated via		Cancer patients (n=62), Median (IQR)	Healthy controls (n=30), Median (IQR)	P-value
P-selectin	-	MFI	885 (773–1031)	790 (744–897)	0.033
		%	5.2 (3.4–10.4)	4.6 (3.0–6.5)	0.461
	PAR-1	MFI	3762 (2921–4418)	2832 (1769–4209)	0.069
		%	92.2 (84.4–95.9)	89.6 (70.4–95.8)	0.330
	PAR-4	MFI	2015 (1314–3620)	1691 (1211–2944)	0.185
		%	67.4 (41.7–89.4)	45.1 (30.8–74.7)	0.037
	GPVI	MFI	4694 (3410–5720)	4752 (2341–5951)	0.813
		%	91.1 (77.1–94.9)	89.8 (71.0–94.2)	0.871
Activated GPIIb/IIIa	-	MFI	160 (141–178)	144 (127–164)	0.016
		%	2.5 (1.4–4.1)	2.4 (1.7–5.5)	0.554
	PAR-1	MFI	232 (200–281)	280 (215–354)	0.023
		%	35.7 (19.5–50.3)	45.7 (28.6–67.2)	0.053
	PAR-4	MFI	310 (235–529)	335 (247–577)	0.549
		%	43.3 (18.5–73.1)	49.0 (25.3–73.5)	0.692
	GPVI	MFI	721 (436–1147)	756 (485–1087)	0.883
		%	86.9 (62.4–94.6)	87.8 (72.2–94.2)	0.916
MPA	-	%	21.6 (10.4–33.7)	12.0 (8.4–16.9)	0.001
	PAR-1	%	74.6 (51.3–92.2)	49.8 (26.8–80.8)	0.005
	PAR-4	%	44.9 (24.7–67.7)	23.2 (12.5–35.4)	<0.001
	GPVI	%	71.2 (44.2–86.1)	36.9 (17.8–59.2)	<0.001

IQR: interquartile range; MFI: mean fluorescence intensity; PAR: protease-activated receptor; GP: glycoprotein; MPA: monocyte-platelet aggregates.

Table 3
Platelet surface expression of P-selectin and activated GPIIb/IIIa, MPA formation and risk of death in 62 cancer patients

Analyses of baseline levels as well as Landmark analyses of updated measurements at two months after study inclusion and analyses of the most recently measured values are given. Hazard ratios (HR) and 95 % confidence intervals (CI) for the increase per decile in each parameter are given.

	Activated via	Pre-chemotherapy value		Value measured 2 months after inclusion		Most recently measured value	
		HR (95 % CI)	P-value	HR (95 % CI)	P-value	HR (95 % CI)	P-value
P-selectin (%)	-	0.88 (0.78–1.00)	0.046	0.88 (0.77–1.00)	0.050	0.94 (0.84–1.06)	0.304
	PAR-1	0.87 (0.77–0.98)	0.027	0.93 (0.82–1.05)	0.232	0.94 (0.83–1.05)	0.270
	PAR-4	0.83 (0.73–0.95)	0.007	0.85 (0.74–0.97)	0.015	0.87 (0.76–0.99)	0.041
	GPVI	0.84 (0.74–0.96)	0.011	0.89 (0.77–1.01)	0.078	0.89 (0.78–1.00)	0.056
Activated GPIIb/IIIa (%)	-	0.83 (0.73–0.95)	0.007	0.94 (0.82–1.07)	0.345	1.00 (0.89–1.12)	0.981
	PAR-1	0.89 (0.78–1.01)	0.067	1.02 (0.90–1.17)	0.730	0.97 (0.86–1.11)	0.681
	PAR-4	0.82 (0.72–0.94)	0.004	0.85 (0.75–0.98)	0.019	0.85 (0.74–0.98)	0.025
	GPVI	0.84 (0.73–0.97)	0.015	0.92 (0.81–1.06)	0.235	0.90 (0.79–1.02)	0.092
MPA (%)	-	1.02 (0.90–1.14)	0.811	1.04 (0.92–1.17)	0.585	1.10 (0.97–1.25)	0.160
	PAR-1	0.96 (0.85–1.09)	0.563	1.05 (0.93–1.19)	0.451	1.06 (0.94–1.19)	0.383
	PAR-4	1.00 (0.88–1.13)	0.951	1.03 (0.92–1.17)	0.598	1.06 (0.94–1.21)	0.331
	GPVI	1.01 (0.89–1.14)	0.927	0.96 (0.85–1.09)	0.559	1.05 (0.93–1.19)	0.415

PAR: protease-activated receptor; GP: glycoprotein; MPA: monocyte-platelet aggregates.

Table 4
**Pre-chemotherapy platelet surface expression of P-selectin and activated GPIIb/IIIa,
 MPA formation and risk of VTE in 62 cancer patients.**

Hazard ratios (HR) and 95 % confidence intervals (CI) for the increase per decile in each parameter are given.

	Activated via	Risk of VTE	
		HR (95 % CI)	P-value
P-selectin (%)	-	0.92 (0.72–1.15)	0.438
	PAR-1	0.73 (0.56–0.92)	0.007
	PAR-4	0.89 (0.70–1.12)	0.307
	GPVI	0.77 (0.59–0.98)	0.034
Activated GPIIb/IIIa (%)	-	0.97 (0.77–1.23)	0.817
	PAR-1	0.76 (0.57–0.97)	0.025
	PAR-4	0.90 (0.71–1.13)	0.355
	GPVI	0.79 (0.59–1.02)	0.065
MPA (%)	-	0.88 (0.69–1.10)	0.266
	PAR-1	0.78 (0.59–1.00)	0.046
	PAR-4	0.85 (0.66–1.07)	0.169
	GPVI	0.79 (0.59–1.00)	0.052

PAR: protease-activated receptor; GP: glycoprotein; MPA: monocyte-platelet aggregates.