Association of platelet activation markers with cancer-associated venous thromboembolism

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Abstract

Venous thromboembolism (VTE) is a frequent complication in cancer patients. Platelet activation is thought to be involved in cancer-associated VTE. Here, we determined the association between evolving markers of platelet activation (soluble P-selectin [sP-selectin], soluble CD40 ligand [sCD40L], thrombospondin-1 [TSP-1] and platelet factor-4 [PF-4]) and the development of cancer-associated VTE. A nested matched case–control study was applied within a cohort of 1779 patients with different types of cancer that had been included in the Vienna Cancer and Thrombosis Study (CATS), a prospective, observational study on patients with newly diagnosed or progressive cancer after remission. Primary endpoint is symptomatic VTE during a maximum follow-up of 2 years. Cases (patients who developed VTE during follow-up) were matched in a 1:2 ratio to controls without VTE during follow-up with respect to tumor type, stage and time of observation in the study. In total, 131 VTE cases were compared to 262 controls. In logistic regression analysis, only sP-selectin was associated with risk of VTE. The odds ratios (OR) per double increase of sP-selectin, sCD40L, TSP-1 and PF-4 were 1.66 (95% confidence interval: 1.17–2.35, \( p = 0.005 \)), 1.04 (0.89–1.21, \( p = 0.635 \)), 1.09 (0.90–1.32, \( p = 0.360 \)) and 1.03 (0.87–1.21, \( p = 0.737 \)), respectively. In conclusion, sP-selectin, but not sCD40L, TSP-1 or PF-4 were associated with risk of VTE in cancer patients in this nested case–control study.

Introduction

Venous thromboembolism (VTE) is a frequent complication in patients with cancer and occurs in up to 20% of cancer patients per year [1]. The risk of VTE varies considerably between individual patients. To better understand which patients have a particularly high risk of VTE, several studies in the past years have focused on the identification of risk factors for VTE. A multitude of clinical and biological risk factors could be identified [2]. Interestingly, among these risk factors several platelet parameters have been found to be associated with an increased risk of VTE in patients with cancer: high platelet count [3], low mean platelet volume (MPV) [4] and high levels of the cell adhesion molecule soluble P-selectin (sP-selectin) [5], which is a marker of platelet and endothelial cell activation. Interestingly, in patients with brain tumors, a group with a very high thrombotic risk, a contrary observation was made. In these patients, a low platelet count was identified as a risk factor for VTE [6]. Hence, platelets are considered to be involved in the development of cancer-associated VTE – although their specific role might be diverging in different types of cancer [7]. Interestingly, it is not known whether high levels of sP-selectin observed in cancer patients with a high thrombotic risk derive from activated platelets or endothelial cells.

Platelet activation is a major and well-known process contributing to the pathophysiology of thrombotic diseases, such as myocardial infarction or stroke. Moreover, anti-platelet therapy with aspirin was shown to effectively reduce risk of VTE after orthopedic surgery [8], as well as during combined chemo- and immunomodulatory therapy in patients with multiple myeloma [9]; and long-term secondary VTE prophylaxis with aspirin was shown to reduce risk of recurrence in patients with an unprovoked VTE who received initial standard treatment with low-molecular weight heparin followed by vitamin K antagonists [10, 11]. Hence, platelets are also considered to be involved in the development of venous thrombosis. Platelets might specifically play a role in cancer-associated VTE, as increased platelet activation markers have been found in a variety of cancer patients [7].

Upon their activation, platelets change their shape and release a multitude of bioactive molecules into the circulation. Platelet alpha-granules, containers of numerous proteins such as coagulation factors, angiogenic factors, growth factors or cytokines, fuse with the cell membrane and release their contents. During this process, P-selectin becomes translocated from the alpha granule membrane to the platelet surface from where it is cleaved and a soluble part is released into the circulation (sP-selectin). Furthermore, CD40 ligand (CD40L), a transmembrane protein...
also located on the platelet alpha-granules, becomes translocated to the platelet surface. Once on the surface, it is cleaved and a soluble form of CD40L (sCD40L) is released into the circulation. The CD40-CD40L axis has important functions in the adaptive immune system, such as activation of antigen-presenting cells, and the discovery of CD40L on platelets points to a role of platelets in adaptive immunity [12]. Elevated levels of sCD40L in patients with cancer have been found and an immunosuppressive effect of sCD40L in cancer patients was suggested [13]. Furthermore, high levels of sCD40L were observed in patients with atherosclerotic diseases and acute coronary syndrome. In this setting, sCD40L was suggested to be a biomarker of worse clinical outcome [14]. Thrombospondin-1 (TSP-1) and platelet factor-4 (PF-4), two proteins stored within the platelet alpha-granules, are released from platelets upon their activation [14]. TSP-1 has anti-angiogenic and also procoagulatory properties and has been found to be elevated in cancer patients in comparison to healthy controls in some studies [15, 16]. PF-4 is an abundant platelet alpha-granule protein that becomes released from activated platelets in large amounts. It was found to have procoagulatory functions in vitro, however, its biological function in vivo is not yet fully understood [17]. One study found high amounts of PF-4 to be associated with an increased risk of VTE in patients with pancreatic cancer [18].

In the current study, we aimed to evaluate whether, in addition to sP-selectin, other biomarkers of platelet activation such as sCD40L, TSP-1 and PF-4 are associated with an increased risk of VTE in patients with different types of cancer.

Material and methods

Study design and study population

A nested case–control study was performed within the Vienna Cancer and Thrombosis Study (CATS) to investigate the association of three platelet biomarkers with risk of cancer-associated VTE. The detailed study design of CATS can be found in previous publications [5, 19] and is briefly outlined in the following.

The Vienna Cancer and Thrombosis Study (CATS)

The Vienna Cancer and Thrombosis study (CATS) is an ongoing prospective, observational cohort study that started in 2003 at the Medical University of Vienna, Austria. CATS was designed with the primary aim to investigate risk factors for the development of VTE in patients with cancer. Patients with newly diagnosed cancer or progressive disease after remission are included and followed for a maximum period of 2 years.

Primary endpoint of the study is occurrence of symptomatic VTE. Patients with cancer of the brain, breast, lung, upper or lower gastrointestinal tract, pancreas, kidney, prostate or gynecological system; sarcoma and hematologic malignancies (lymphoma, multiple myeloma) are included in the study. Patients with venous or arterial thromboembolism or chemotherapy within the last 3 months; overt bacterial infection, radiotherapy or surgery within the last 2 weeks or patients receiving continuous anticoagulation are excluded from the study. Written informed consent was obtained from each patient, a structured interview about the patients’ medical history was performed and a single blood sample is drawn at the day of study inclusion. Follow-up was performed regularly approximately every 3 months per postal questionnaire. The observation period starts from the time of blood sampling and lasts for 2 years or until the occurrence of VTE, death, loss of follow-up or withdrawal of informed consent. Once a year, the Austrian death registry is searched for entries of study participants.

No routine screening for VTE is performed. Diagnosis of VTE had to be confirmed by duplex sonography or venography for deep vein thrombosis (DVT), and by computed tomography or ventilation/perfusion lung scan for pulmonary embolism (PE), respectively. In patients who died during follow-up, death certificates and, if available, autopsy findings have been checked for diagnosis of fatal PE. Ethical approval of the study was received from the institutional ethics committee and the study has been conducted in accordance with the Declaration of Helsinki.

A nested case–control study within CATS

From October 2003 until September 2013, 1990 cancer patients were included in CATS. Due to this large number of patients and the consequently large number of blood samples available for measurement of platelet biomarkers, for the current study, a nested case–control design was chosen to limit expenses and to optimize efforts. For this study design, all the patients who reached the primary endpoint of CATS, i.e. development of VTE, were evaluated and defined as cases. Controls were selected in a 1:2 ratio from the remaining cohort of cancer patients (i.e. patients who did not develop VTE during the observation period), which means that for each VTE case two control cancer patients were selected. The controls were matched on tumor type (cancer of the brain, breast, lung, stomach, colon, pancreas, kidney, prostate; lymphoma; multiple myeloma; or other cancer site), tumor stage (metastatic spread vs. localized disease [patients with locally advanced, inoperable pancreatic cancer patients were treated like patients with a metastatic tumor]) and duration of observation period in the study.

The original cancer cohort consisted of 1990 patients that had been included in CATS. Of these, 121 patients had to be excluded because they did not meet the exact in- and exclusion criteria after re-evaluation (n = 86) or because no information about the follow-up period was available (n = 35). Therefore, a cohort of 1779 patients remained for the current nested case–control study. Of these patients, in total 131 (7.4%) had developed VTE during the follow-up period of CATS and were consequently defined as cases. For each case, two matched controls without VTE were identified, resulting in a group of 262 matched cancer patients without VTE available as controls for the current nested case–control study.

Healthy subjects

A cohort of 65 sex- and age-matched healthy individuals from the same geographic region (30 [46%] women, median age 60 years) without a history of venous or arterial thrombosis and without cancer served as controls for comparison of levels of sCD40L, TSP-1 and sP-selectin.

Blood sampling and laboratory analyses

At the day of study inclusion, blood was collected by sterile venipuncture into plasma vacuum tubes (Vacuette®; Greiner Bio One, Kremsmünster, Austria) containing one-tenth volume sodium citrate stock solution at 0.129 mM and processed within a time period of 2 hours. Plasma was obtained by centrifugation at 3000 g for 10 minutes, and aliquotes were stored at −80°C until the time of analysis. TSP-1, sCD40L, PF-4 and sP-selectin were measured by using commercially available ELISA kits (Quantikine®, R&D Systems, Minneapolis, MN). TSP-1, sCD40L and sP-selectin were measured in samples from cancer patients and in samples from a cohort of 65 healthy subjects. PF-4 was measured in all the cancer patient samples. Due to limited sample material, PF-4 was not measured in samples from healthy control individuals.
Statistical analyses

Matching was performed by randomly drawing two control observations for each VTE patient from the whole set of controls with similar tumor type, tumor stage and observation period. With respect to tumor type and tumor stage, perfect 1:2 matching was possible. If no perfect match was possible regarding the observation period, then the controls observation period could be longer, but not >2 weeks shorter than the observation period of the related VTE-case.

Due to the skew distributions, log-2-transformed values of the platelet activation markers were used for all the statistical analyses. Differences between cancer patients and healthy controls were analysed by using the 2-sample t-test. Association between levels of platelet activation markers and risk of VTE were evaluated by performing univariate and multivariable logistic regression analyses conditioning on the matched data structure. The Pearson correlation coefficient was used to describe the degree of correlation between investigated platelet parameters.

Two-sided p values <0.05 were considered to indicate statistical significance. The SAS software [version 9.4, SAS Institute Inc. (2002–2012), Cary, NC] was used for statistical computations.

Results

Study population

During a median observation time of 152 days, out of 1779 cancer patients, 131 (7.4%) developed VTE. These cases were matched to 262 control patients who had not developed VTE during the observation period. Detailed characteristics of the study population are listed in Table I.

Thromboembolic events

Thromboembolic events (n = 131) consisted of 53 isolated DVTs of the lower extremity, 53 isolated PEs, 7 combined DVTs of the lower extremity and PEs; in 4 patients a thrombosis of either the portal vein or the jugular vein was diagnosed; 6 patients had an isolated DVT of the upper extremity and 1 patient each had a sinus vein thrombosis, combined DVT of the lower extremity and portal vein thrombosis, combined PE and DVT of the upper extremity, or a thrombosis of the inferior caval vein. PE was fatal in 4 cases (3.1% of all the thromboembolic events).

Distribution of platelet activation markers in cancer patients and healthy subjects

Median levels of TSP-1 were higher in the cancer patient cohort compared to healthy subjects (836 ng/ml [487–1347] vs. 583 ng/ml [430–975], p = 0.033). Median levels of sCD40L and sP-selectin were similar in the groups of all the cancer patients and healthy subjects (sCD40L: 264 pg/ml [25th–75th percentile: 148–473] vs. 225 pg/ml [115–326], p = 0.152; sP-selectin: 39.4 ng/ml [30.7–53.6] vs. 37.7 ng/ml [32.1–44.3], p = 0.183). The subgroup of cancer patients who developed VTE during follow-up had higher levels of sP-selectin and similar levels of sCD40L in comparison to healthy subjects (sP-selectin: 44.5 ng/ml [25th–75th percentile: 31.9–61.3] vs. 37.7 ng/ml [32.1–44.3], p = 0.012; sCD40L: 273 pg/ml [170–493] vs. 225 pg/ml [115–326], p = 0.129).

The distributions of platelet biomarkers according to the different types of cancer are shown in Supplementary Table I and Supplementary File I.

| Table I. Baseline characteristics of cases (cancer patients who developed VTE during follow-up), controls (cancer patients who did not develop VTE during follow-up) and healthy subjects. |
|--------------------------------------------------|-----------|-------------|
| **Median age at study entry, years (25th–75th percentile)** | Cases (n = 131) | Controls (n = 262) | Healthy subjects (n = 65) |
| 63 (54–69) | 61 (51–67) | 60 (58–66) |
| **Median observation time, months (25th–75th percentile)** | 3.5 (1.7–6.6) | 5.6 (3.3–11.5) | – |
| **Sex, n (%)** | – | – | – |
| Female | 55 (42) | 121 (46.2) | 30 (46.2) |
| Male | 76 (58) | 141 (53.8) | 35 (53.8) |
| **Site of cancer, n (%)** | – | – | – |
| Brain | 32 (24.4) | 64 (24.4) | – |
| Lung | 21 (16.0) | 42 (16.0) | – |
| Pancreas | 19 (14.5) | 38 (14.5) | – |
| Colon/rectum | 14 (10.7) | 28 (10.7) | – |
| Lymphoma | 12 (9.2) | 24 (9.2) | – |
| Stomach | 8 (6.1) | 16 (6.1) | – |
| Breast | 7 (5.3) | 14 (5.3) | – |
| Prostate | 3 (2.3) | 6 (2.3) | – |
| Multiple myeloma | 3 (2.3) | 6 (2.3) | – |
| Kidney | 1 (0.8) | 2 (0.8) | – |
| Others | 11 (8.4) | 22 (8.4) | – |
| **Dissemination of tumor at study entry, n** | – | – | – |
| Localized tumor | 36 (27.5%) | 72 (27.5%) | – |
| Distant metastasis | 49 (37.4%) | 98 (37.4%) | – |
| Not classifiable** | 46 (35.1%) | 92 (35.1%) | – |
| **Median level of sCD40L, pg/ml (25th–75th percentile)** | 273 (170–493) | 252 (146–474) | 225 (115–326) |
| **Median level of TSP-1, ng/ml (25th–75th percentile)** | 866 (561–1399) | 825 (468–1307) | 583 (430–975) |
| **Median level of PF-4, ng/ml (25th–75th percentile)** | 386 (202–601) | 333 (194–579) | – |
| **Median level of sP-selectin, ng/ml (25th–75th percentile)** | 44.5 (31.9–61.3) | 38.4 (29.8–49.3) | 37.7 (32.1–44.3) |
| **Median platelet count, g/l (25th–75th percentile)** | 235 (190–308) | 240 (186–303) | 218 (188–259) |
| **Median MPV, fl (25th–75th percentile)** | 10.2 (9.6–10.7) | 10.2 (9.4–10.9) | 10.3 (10.0–11.0) |

Cases and controls were matched on tumor site, stage and observation time in the study.

**Brain tumor, lymphoma and multiple myeloma.**

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Table II. Association of sCD40L, TSP-1, PF-4 and sP-selectin (sP-selectin data were in part available from a previous study) with risk of VTE in patients with cancer.

| Platelet Activation Marker | Univariable OR (95% CI) | p Value | Multivariable OR (95% CI) | p Value |
|---------------------------|-------------------------|---------|---------------------------|---------|
| sCD40L (per double increase) | 1.04 (0.89–1.21) | 0.635 | 0.95 (0.81–1.13) | 0.572 |
| sCD40L (>75th percentile [>473 pg/ml]) | 1.02 (0.63–1.67) | 0.934 | 0.80 (0.47–1.37) | 0.419 |
| TSP-1 (per double increase) | 1.09 (0.90–1.32) | 0.360 | 0.99 (0.80–1.22) | 0.935 |
| TSP-1 (>75th percentile [>1347 ng/ml]) | 1.22 (0.74–2.00) | 0.441 | 0.97 (0.56–1.67) | 0.916 |
| PF-4 (per double increase) | 1.03 (0.87–1.21) | 0.737 | 0.98 (0.82–1.16) | 0.797 |
| PF-4 (>75th percentile [>589 ng/ml]) | 1.15 (0.71–1.87) | 0.563 | 0.98 (0.59–1.63) | 0.951 |
| sP-selectin (per double increase) | 1.66 (1.17–2.35) | 0.005 | 1.64b (1.15–2.34) | 0.006 |
| sP-selectin (>75th percentile [>53.6 ng/ml]) | 2.33 (1.43–3.78) | 0.001 | 2.27b (1.39–3.70) | 0.001 |

Odds ratios (OR) are given per double increase in each marker and for the comparison between patients with high and low levels of each platelet activation marker (cut-off was set at the 75th percentile of each marker in the total cancer patient cohort).

a Adjusted for age, sex, platelet count and sP-selectin.
b Adjusted for age, sex, and platelet count.

Discussion

In our study, TSP-1 was significantly higher in cancer patients compared to healthy subjects. Inconsistencies between previous studies [5] and high levels of sP-selectin were associated with an increased risk of VTE in patients with cancer [5], we had hypothesized that an association might likewise exist between other platelet activation markers and risk of VTE. However, this hypothesis could not be confirmed in our present study.

To our knowledge, so far data about markers of platelet activation and their association with risk of cancer-associated VTE are very rare. sP-selectin was found to be associated with risk of VTE in previous studies, and this observation was also made in the current analysis. Only one other study of patients with pancreatic cancer reported that high levels of PF-4 increase the risk of VTE [18]. Interestingly, in subgroup analyses of our study, we observed a similar tendency. The OR for development of VTE in patients with pancreatic cancer and high levels of PF-4 (above the 75th percentile of all the cancer patients) was ~3 when compared to patients with lower PF-4 levels. This result was statistically not significant (OR 3.03 [95% CI: 0.88–10.43], p = 0.078), however, the sample size of this subgroup analysis was relatively small (n = 57), which could be the reason for the wide confidence interval.

In our study, TSP-1 was significantly higher in cancer patients than in healthy controls, which was also described by other authors [15, 20]. In contrast, although reported in previous studies [13, 21–25], in our study, cancer patients did not exhibit higher levels of sCD40L or sP-selectin in comparison to healthy individuals. Only the group of cancer patients who developed VTE during follow-up had significantly higher levels of sP-selectin compared to healthy subjects.
Platelet count

Table III. Correlation coefficients of platelet parameters with each other.

|             | sCD40L | TSP-1 | PF-4 | sP-selectin | Platelet count | MPV |
|-------------|--------|-------|------|-------------|----------------|-----|
| sCD40L Coeff. | 1.000  | 0.657 | 0.501| 0.243       | 0.263 – 0.090  |     |
| p Value     | <0.001 | <0.001| <0.001| <0.001      | <0.001         |     |
| TSP-1 Coeff. | 0.657  | 1.000 | 0.758| 0.319       | 0.329 – 0.124  |     |
| p Value     | <0.001 | <0.001| <0.001| <0.001      | <0.001         |     |
| PF-4 Coeff.  | 0.501  | 0.758 | 1.000| 0.162       | 0.263 – 0.130  |     |
| p Value     | <0.001 | <0.001| <0.001| <0.001      | <0.001         |     |
| sP-selectin Coeff. | 0.243 | 0.319 | 0.162| 1.000       | 0.105 – 0.004  |     |
| p Value     | <0.001 | <0.001| <0.001| <0.001      | <0.001         |     |
| Platelet count Coeff. | 0.263 | 0.329 | 0.263| 0.105       | 0.100 – 0.295  |     |
| p Value     | <0.001 | <0.001| <0.001| <0.001      | <0.001         |     |
| MPV Coeff.  | −0.090 | −0.124| −0.130| −0.004      | −0.295         | 1.00|
| p Value     | 0.080  | 0.015 | 0.011 | 0.942       | <0.001         |     |

Pearson’s correlation coefficients of log-2 transformed variables and 2-tailed significance levels are given.

Various studies might be related to differing characteristics of cancer patient populations and types of cancer. Our study population consisted of a heterogeneous group of patients comprising of cancers of different sites and stages, reflecting the general cancer population. Blood sampling in our study was performed prior to initiation of chemotherapy in all the patients.

As expected the three markers of platelet activation sCD40L, TSP-1 and PF-4 showed a moderate to strong mutual correlation. However, these biomarkers correlated only weakly with sP-selectin. PF-4 is a molecule that is almost solely found in megakaryocytes and platelets, from where it can be released into the circulation. Interestingly, sP-selectin can also be released from endothelial cells upon their activation. Although it was proposed that the vast amount of sP-selectin in plasma is platelet-derived [26], our data might rather suggest that high levels of sP-selectin in plasma, as they can be observed in cancer patients with a high thrombotic risk, might eventually derive from a distinct source other than platelets. Interestingly, in experimental studies platelets were found to be only secondarily involved in the development of VTE. Brühl et al. demonstrated in an in vivo mouse thrombosis model that endothelial cell activation and leukocyte binding to activated endothelial cells were the first steps for initiation of thrombosis. Platelets were only secondarily involved in the propagation of thrombosis, but not in the initial steps [27]. Our data might support the hypothesis that also in humans the prothrombotic state preceding a thrombotic event is not mainly mediated by platelets. As blood sampling in our study was performed at study inclusion, in median 152 days prior to occurrence of VTE events, we cannot exclude that at a later timepoint, closer to development of VTE, one of the biomarkers would indicate platelet activation. Unfortunately, we were not able to perform longitudinal blood sampling for biomarker measurements to analyse changes during follow-up. However, such a project is currently being prepared and will be performed as a further sub-study within the framework of the Vienna CATS.

A major aspect that needs to be discussed when interpreting the results of the current study is the fact that preanalytical conditions are a major issue when measuring platelet activity or surrogate markers of platelet activation in plasma [28]. Platelets get easily activated during blood draw procedures and sample processing, such as centrifugation steps, especially when citrated blood is used. Therefore, a major limitation of our study is that samples obtained with special anticoagulants that inhibit ex vivo platelet activation, such as blood collection tubes containing CTAD (citrate, theophylline, adenosine, dipyridamole), were not available for our study. However, all our samples were treated equally and processed within 2 hours from blood draw. We also believe that a clinically relevant and routinely applicable biomarker needs to be stable during routine blood draw procedures and measurable in generally available blood tubes. The robust association between sP-selectin and risk of cancer-associated VTE in previous investigations and in the current study may be given as an example. We would like to mention that the association between high levels of sP-selectin with an increased risk of VTE was first reported in a previous analysis from CATS including 687 patients [5]. However, since then the CATS patient cohort increased to almost 2000 patients, and approximately two-thirds of patients in the current analysis were newly recruited into CATS.

In conclusion, although platelets are considered to be involved in the development of VTE, the platelet activation markers sCD40L, TSP-1 and PF-4 do not seem to be promising candidates for predicting risk of future VTE in patients with cancer. Whether they might play a role in specific cancer entities needs to be clarified in further studies. Finally, in our study, we could confirm that sP-selectin is an independent predictive biomarker of VTE risk in patients with cancer. sP-selectin might be useful in risk stratification of patients with cancer into high or low VTE risk groups. The weak correlation between sP-selectin and other soluble markers of platelet activation might suggest that high levels of sP-selectin in patients with cancer who have a high thrombotic risk are likely to derive from other sources than platelets. This assumption needs to be verified in future studies.

Acknowledgements

We thank Tanja Altreiter (Clinical Division of Haematology and Haemostaseology, Department of Medicine I, Medical University of Vienna) for proof-reading this article. We thank the members of the adjudication committee: Andrea Willfort-Ehringer (Department of Angiology, Medical University of Vienna) and Sylvia Metz-Schimmerl (Department of Diagnostic Radiology, Medical University of Vienna).

Declaration of interest

The authors report no declarations of interest. This study was supported by the Anniversary Fund of the ‘‘Österreicherische Nationalbank’’ (project number 14744). Previous presentations of this article: Data from this study were presented in part as a poster presentation at ‘‘Platelets 2014: 8th International Symposium’’, in Ma’ale Rachamisha, Israel, and as an oral and poster presentation at the 7th International Conference on Thrombosis and Hemostasis Issues in Cancer (ICTHIC), in Bergamo, Italy.

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Supplementary material available online.
Supplementary Tables I and II and Supplementary File I.