Doctoral Thesis at the Medical University of Vienna

for obtaining the “PhD”-Degree

Analysis of longitudinal changes in hemostasis

biomarkers among cancer patients

Author: Eva-Maria Reitter, MD

Supervisor: Prof. Ingrid Pabinger-Fasching, MD

Affiliation:
Clinical Division of Hematology and Hemostaseology
Department of Medicine I, Comprehensive Cancer Center,
Medical University of Vienna
Waehringer Guertel 18-20
A-1090 Vienna, Austria

Vienna, April 26, 2017
Table of contents

List of Figures .................................................................................................................. 3
List of Tables .................................................................................................................... 3
Abstract in English ........................................................................................................... 4
Abstract in German .......................................................................................................... 5
Abbreviations in alphabetical order .................................................................................. 7
Acknowledgements ........................................................................................................... 7
1 Introduction ..................................................................................................................... 8
1.1 Aim of the study .......................................................................................................... 19
2 Results ............................................................................................................................ 19
2.1 Patient characterization .............................................................................................. 19
2.2 Longitudinal analysis of the investigated parameters according to tumor type ....... 23
2.3 Differences of the longitudinal investigated parameters according to disease stage and therapy response .................................................................................. 33
2.4 Differences of the investigated longitudinal parameters with respect to chemotherapy and radiotherapy .................................................................................. 36
2.5 Outcome venous thromboembolic events ................................................................. 36
2.6 Outcome survival ......................................................................................................... 40
3 Discussion ....................................................................................................................... 42
4 Methods .......................................................................................................................... 48
4.1 Study design ................................................................................................................ 48
4.2 Diagnosis of VTE ....................................................................................................... 49
4.3 Blood sampling ........................................................................................................... 50
4.4 Laboratory analysis .................................................................................................... 51
4.5 Statistical analysis ..................................................................................................... 52
Appendix 1 Patient questionnaire at study inclusion ...................................................... 61
Appendix 2 Patient questionnaire for follow-up .............................................................. 63
Curriculum vitae ............................................................................................................... 76
List of Figures

Figure 1 Virchow triad [7].................................................................................................................. 8
Figure 2 Mechanisms of the coagulation activation through tumor cells .................. 12
Figure 3 Thrombin generation curve ......................................................................................... 13
Figure 4 Risk factors for VTE in cancer patients ................................................................. 15
Figure 5 Ay et al. cancer-related thrombosis score – extended Khorana risk model 18
Figure 6 Boxplot of the FVIII levels at the various blood sampling time points .... 23
Figure 7 Boxplot of the sP-selectin levels at the various blood sampling time points 24
Figure 8 Boxplot of the thrombin (peak height) levels at the various blood sampling time points ................................................................................................................................................................................................................................. 25
Figure 9 Boxplot of the FGEN levels at the various blood sampling time points ...... 26
Figure 10 Boxplot of the F1+2 levels at the various blood sampling time points ..... 27
Figure 11 Boxplot of the D-dimer levels at the various blood sampling time points . 28
Figure 12 Boxplot of the ATIII levels at the various blood sampling time points .... 29
Figure 13 Boxplot of the hemoglobin levels at the various blood sampling time points ................................................................................................................................................................................................................................. 30
Figure 14 Boxplot of the leukocyte levels at the various blood sampling time points 32
Figure 15 Boxplot of the FGEN levels (I), as well as of F1+2 levels (II) and levels of D-dimer (III) in patients with remission vs. progression of disease .......................... 35
Figure 16 Boxplots of the various parameters in patient with and without thrombosis ................................................................................................................................................................................................................................. 38
Figure 17 Values of the respective parameters of each patient developing VTE within 250 days and the interquartile range of patients without VTE ........................................................................... 39
Figure 18 Values of the respective parameters for each patient dying within 250 days and the interquartile range of patients who survived ............................................................................. 41

List of Tables

Table 1 Wells score for DVT (Source: Streiff et al. [9]) ...................................................... 9
Table 2 Wells score for PE (Source: Streiff et al. [9]) ....................................................... 10
Table 3 Number of patients per blood sample time point for each tumor entity .... 21
Table 4 Baseline patient characteristics (n= 112) .................................................................. 22
Table 5 Trend of the various parameter levels over the investigated study period of 250 days according to tumor site .................................................................................................................. 33
Table 6 Univariate Cox-regression analysis – outcome VTE ........................................ 37
Table 7 Results of univariate Cox-regression analysis – outcome survival ............ 40
Abstract in English

Background: Hemostasis parameters are known predictors for VTE occurrence in patients with cancer. Furthermore, an association between hemostasis parameter levels and disease state, patients’ prognosis as well as survival in cancer patients has been described previously. VTE risk differs among various tumor entities, being highest in patients with pancreatic cancer. Data on the longitudinal behavior of hemostasis parameters in cancer patients are scarce. It is also not known if and how the various hemostasis parameters alter in cancer patients during chemotherapy and along the course of disease and how these alterations might correlate with VTE occurrence and survival.

Therefore, we aimed to investigate longitudinal changes of hemostasis parameters in cancer patients in order to evaluate their possible predictive role along time regarding VTE occurrence and survival, respectively.

Patients and Methods: The present longitudinal analysis was performed as a sub-study of the “Vienna Cancer and Thrombosis Study (CATS)”, an ongoing, prospective observational single-center study at the General Hospital of Vienna, which was approved by the local Ethics Committee. For the sub-study, patients with pancreatic cancer, glioblastoma (representing patients at high risk for VTE occurrence) as well as patients with lung and colorectal cancer (representing an intermediate risk for VTE occurrence), who have been included in CATS were followed longitudinally for a time period of 250 days. During this time period, hemoglobin, platelet and leucocyte count, FVIII, sP-selectin, peak height thrombin, fibrinogen, prothrombin fragment 1+2, D-dimer and ATIII were determined on a monthly basis. Study end points were death, VTE and study completion.

Results: Overall, 112 patients, thereof 39 with brain, 41 with lung, 15 with colorectal and 17 with pancreatic cancer, were enrolled. Their median age was 62.4 years. During the observation period, 14 patients (12.5 %) had a VTE and 17 (16.2 %) died. Patients with distant metastases had higher levels of sP-selectin and D-dimer than those without. Complete remission was associated with lower F1+2, D-dimer and fibrinogen levels. Regarding the four investigated tumor entities, the hemostasis parameter levels showed a different behaviour: Levels of thrombin (peak height), ATIII and hemoglobin decreased in the four tumor subgroups over time. VTE patients
had higher values of D-dimer, FVIII and sP-selectin before VTE occurred. Steadily increasing D-dimer, FVIII or sP-selectin levels correlated with a higher mortality.

Conclusion: Our results indicate that in cancer patients an association of hemostasis parameters with VTE risk, disease state and prognosis (mortality) exists - both, at diagnosis and thereafter.

Abstract in German


Patienten und Methoden: Die vorliegende Longitudinalstudie ist eine Substudie der “Vienna Cancer and Thrombosis Study (CATS)”, einer laufenden, prospektiven Beobachtungsstudie, welche am Allgemeinen Krankenhaus der Stadt Wien mit Zustimmung der lokalen Ethikkommission durchgeführt wird. Für die Substudie wurden PatientInnen mit Pankreaskarzinom und Glioblastom (welche HochriskopatientInnen in Bezug auf VTE-Entwicklung repräsentieren) sowie PatientInnen mit Lungen- und kolorektalem Karzinom (welche PatientInnen mit mittlerem Risiko in Bezug auf VTE-Entwicklung repräsentieren), die in CATS eingeschlossen wurde, über einen Zeitraum von 250 Tagen beobachtet. Während
Dieses Beobachtungszeitraumes wurden monatlich Hämoglobin, Thrombozyten- und Leukozytenzahl, FVIII, sP-Selektin, peak height thrombin, Fibrinogen, Prothrombinfragment 1+2, D-Dimer und ATIII bestimmt. Studienendpunkte waren Tod, Auftreten einer VTE oder Ende der Beobachtungsdauer von 250 Tagen.


Schlussfolgerung: Unsere Ergebnisse deuten auf einen Zusammenhang zwischen Hämostaseparametern und Erkrankungsstadium, Prognose (Mortalität) und VTE-Risiko hin, nicht nur zum Zeitpunkt der Diagnose, sondern auch während des Krankheitsverlaufs.
**Abbreviations in alphabetical order**

ANOVA  analysis of variance  
ATIII  antithrombin III  
BMI  body mass index  
CATS  Vienna Cancer and Thrombosis Study  
CI  confidence interval  
CHT  chemotherapy  
CT  computed tomography  
Δ abs  monthly absolute changes  
Δ rel  monthly relative changes  
DVT  deep vein thrombosis  
ELISA  enzyme-linked immunoassay  
F1+2  prothrombin fragment 1+2  
FGEN  fibrinogen  
FIGO  International Federation of Gynecology and Obstetrics  
FVIII  coagulation factor VIII  
FXa  activated factor X  
HR  hazard ratio  
i.e.  that is  
LMWH  low molecular weight heparin  
max  maximum  
min  minimum  
PE  pulmonary embolism  
RT  radiotherapy  
sP-selectin  soluble P-selectin  
TF  tissue factor  
TGA  thrombin generation assay  
TP  time point  
VEGF  vascular endothelial growth factor  
VTE  venous thromboembolism  

**Acknowledgements**

Special thanks to Tanja Altreiter for proof-reading of the thesis and to Silvia Koder for her support regarding the laboratory analyses.
1 Introduction

Venous thromboembolism is, besides myocardial infarction [1;2] and stroke [3], the most frequent cardiovascular disease. The VTE incidence ranges from 1 to 2 per 1000 persons per year in the general population. [4] Nowadays, various risk factors for VTE occurrence are known. [5] Already in 1865, the relationship between cancer and VTE was first described by Trousseau. [6] Years before, the so-called Virchow triad (see Figure 1) has been described as a mechanism of thrombosis aetiology. This triad comprises the following factors: persistence of hypercoagulability, hemodynamic changes (ie. turbulence or stasis) or dysfunction or endothelial injury. [7]

Figure 1 Virchow triad [7]

In malignancies, VTE does not only occur as pulmonary embolism and deep-vein thrombosis, but also as thrombosis of unusual sites, whereby the veins of the brain, neck or arms as well as the vena cava, portal vein or visceral veins can be affected. [8]

Depending on the localisation of VTE, characteristic symptoms at onset are as follows: pain, extremity oedema and erythema, dyspnoea or tachypnoe, tachycardia
as well as unspecific fatigue. [9] Testing of D-dimer levels is part of the diagnostic procedure of VTE. The respective assays show sensitivities of > 90 % range but only specificities of about 40 %. Therefore, this parameter can be used to rule out VTE in case of a negative test, but is not applicable to confirm the diagnosis. [10] A clinical probability scoring system for diagnosing DVT and PE, respectively, is the so-called “Wells score”. The probability for DVT can be predicted considering the following parameters: “present cancer, paralysis, paresis or immobilisation, a major surgery within the last 12 weeks, a localized tenderness along the distribution of the deep venous system, swollen extremity, oedema, newly developed collateral superficial veins and previous VTE” [9], see Table 1.

**Table 1 Wells score for DVT (Source: Streiff et al. [9])**

<table>
<thead>
<tr>
<th>Clinical characteristics according to the Wells score for DVT</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active malignancy (chemotherapy- currently or in the last 6 months)</td>
<td>1</td>
</tr>
<tr>
<td>Paresis, paralysis or recent immobilization of the lower limbs</td>
<td>1</td>
</tr>
<tr>
<td>Bedridden for ≥ 3 days or surgery within the last 12 weeks</td>
<td>1</td>
</tr>
<tr>
<td>Localized tenderness alongside the deep vein system</td>
<td>1</td>
</tr>
<tr>
<td>Swelling of the leg</td>
<td>1</td>
</tr>
<tr>
<td>Calf swelling (≥ 3 cm) compared to opposite side</td>
<td>1</td>
</tr>
<tr>
<td>Edema</td>
<td>1</td>
</tr>
<tr>
<td>Superficial collateral (non-varicose) veins</td>
<td>1</td>
</tr>
<tr>
<td>Previous DVT</td>
<td>1</td>
</tr>
<tr>
<td>Another diagnosis explaining the present symptoms</td>
<td>-2</td>
</tr>
</tbody>
</table>

**COMMENT:** If the score is lower than 2 points, DVT is unlikely.
Concerning the probability of a PE, the following clinical characteristics are used for its prediction: “active cancer, surgery or immobilisation for at least three days within the last four weeks, previous PE or DVT, hemoptysis, heart rate > 100 beats per minute and the presence of a specific DVT sign” (see Table 2). [9]

**Table 2 Wells score for PE (Source: Streiff et al. [9])**

<table>
<thead>
<tr>
<th>Clinical characteristics according to the Wells score for PE</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active malignancy (patient receiving antitumor treatment within six months or currently)</td>
<td>1</td>
</tr>
<tr>
<td>Bedridden for at least three days or surgery within the last four weeks</td>
<td>1.5</td>
</tr>
<tr>
<td>Previous DVT or PE</td>
<td>1.5</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>1</td>
</tr>
<tr>
<td>Heart rate &gt; 100 beats/minute</td>
<td>1.5</td>
</tr>
<tr>
<td>PE is the most likely diagnosis</td>
<td>3</td>
</tr>
<tr>
<td>Clinical signs/symptoms are suitable for DVT</td>
<td>3</td>
</tr>
</tbody>
</table>

COMMENT: If score is \( \leq 4 \), PE is unlikely.

To date, various diagnostic imaging modalities, which can be used to objectively confirm VTE, exist. Hereby, venous ultrasound, mainly duplex ultrasound, is the most useful diagnostic instrument to detect DVT in cancer patients. For determination of venous thrombosis proximal of the inguinal ligament, contrast-enhanced computed tomography or resource imaging should be used. The diagnosis of an intra-abdominal thrombosis is much more challenging and, therefore, CT contrast venography should be applied. When central venous catheter associated DVT is suspected, again, the use of duplex ultrasound is the diagnostic state of the art. Contrast CT venography is the best choice to identify isolated thrombosis of the proximal vena subclavia, as well as thrombosis of the vena brachiocephalica or the superior vena cava. For the diagnosis of a PE, the CT-angiography, which has a higher detection rate when compared to the pulmonary scintigraphy, is the primary imaging modality. [9]
Tumor-associated VTE aggravates the clinical course of disease, worsens the patients’ survival [11] and is in cancer patients the second most frequent cause of death. [12-14] Patients' prognosis seems to correlate with the time point of VTE occurrence, whereas patients diagnosed with cancer concurrently or shortly after a venous thromboembolic event have the poorest survival rates.

The reason therefore can possibly be due to the existence of an advanced and more aggressive disease. [11] Cancer-related VTE can occur at any time of disease with a peak within the first year after diagnosis or even as idiopathic VTE prior to tumor detection. [15] This is of paramount importance as every fifth patient with idiopathic VTE is later diagnosed with cancer. [16]

The incidence of VTE in cancer patients shows a wide variation in literature. Malignancies increase the risk to develop VTE 4- to 7-fold, up to a percentage of 7.8 % [17;18]. In total, one quarter of all venous thromboembolic events are associated with cancer. As autopsy studies have already demonstrated, in nearly every second cancer patient, VTE is found post-mortem. [19]

Nowadays, although the complete pathogenesis and the exact mechanisms of cancer-associated VTE remain unclear, it is known that cancer patients show both, a coagulation and fibrinolysis activation and an elevated consumption of clotting factors [20]. This results in a hypercoagulable state, which predisposes to the occurrence of VTE. Moreover, tumor cells express tissue factor, produce circulating TF-bearing microparticles and inflammatory cytokines and adhere to platelets (see Figure 2), leucocytes and endothelial cells. These are relevant mechanisms with respect to tumor growth and dissemination and also influence various hemostasis parameters, which are known to predict VTE, as well as survival in cancer patients. [21-25]
Figure 2 Mechanisms of the coagulation activation through tumor cells
(Source: Falanga et al. [14], adopted)

Factor VIII (FVIII), which is factor of the intrinsic coagulation system, dissociates from the vWF after activation by thrombin. Together with the activated factor IX, it contributes to the activation of factor X. Thus, thrombin generation proceeds more rapidly. [26] For this reason, increased FVIII activity correlates with a higher risk for both, primary and recurrent VTE. [27;28] Studies have already ascertained that patients with plasmocytoma, breast, or colorectal cancer show high FVIII levels. [29-31] In CATS significantly higher values of FVIII at study inclusion time (ie. prior to tumor therapy) were seen in patients who later developed VTE. Thereby, the rate for VTE occurrence increased continuously with the amount of FVIII. A correlation of FVIII with the patients’ age and blood group was also noted. [32]

SP-selectin, which is important for cell adhesion, is situated on endothelial cells and platelet, when they are activated. It is stored in so-called Weibel-Palade bodies of inactivated endothelial cells and in α-granules of inactivated platelets, respectively. An increase of sP-selectin levels was also found to correlate with the incidence of VTE in cancer patients. [33]
Upon completion of the coagulation pathway, thrombin is generated and fibrin is formed. The thrombin concentration can be measured over time and displayed in the so-called thrombin generation curve. This curve describes numerous parameters of thrombin activity: “the lag-phase (time until thrombin burst), the peak amount of generated thrombin (peak height) and the total amount of generated thrombin (area under the curve)”. [34]

Figure 3 displays the thrombin generation curve and shows the parameters which can be read from the curve.

**Figure 3 Thrombin generation curve**
(Source: www.genengnews.com)

Again, in CATS the development of VTE significantly correlated with elevated peak thrombin values. Also, a shorter lag phase, a shorter time to peak thrombin and a higher velocity index were seen in patients with cancer and VTE. After adjustment for the different tumor types and stages, high peak thrombin still correlated significantly with VTE occurrence. [35]
Fibrinogen, a hexadimeric molecule [36], represents the pre-stage and inactive form of fibrin. As an acute-phase protein, FGEN increases in case of inflammation or tissue injury. [37] A rise of FGEN levels promotes the development of cancer by impeding the clearance of cancer cells and, therefore, also boosts distant metastases. [38] Moreover, increased FGEN levels result in a higher risk for VTE. [39] Several studies pointed out that high FGEN levels are associated with reduced overall survival in cancer. [40-42] Also, a correlation of FGEN levels with white blood cell count and thrombocytes was described in females suffering from endometrial cancer. [43]

Prothrombin fragment F1+2 is released when FXa cleaves prothrombin to thrombin; high levels of this peptide were more likely in cancer patients with VTE than in those without. In addition, cancer patients with elevated levels of both, D-dimer and prothrombin fragment F1+2, were found to have the highest VTE incidence. [44]

In cancer patients, D-dimer, as a fibrin degradation product, was higher in those with venous thromboembolism. [44] Furthermore, D-dimer levels were seen to be higher in lung cancer patients with distant metastases and in those with worse survival, respectively. [45] In colorectal cancer patients, preoperative elevated D-dimer levels correlated with a higher rate of DVT during the first year after surgery. [46]

Antithrombin III (ATIII), an important inhibitor of the coagulation system, is a member of the serine protease inhibitors and is involved in anti-angiogenesis and inflammatory processes. In patients with lung cancer, ATIII seems to be predictive for survival with higher mortality being associated with low levels. [45]

Due to the presence of a chronic inflammation state and several myelotoxic treatments, ie. chemotherapy and radiotherapy, cancer patients frequently suffer from anemia. In addition, older age further predisposes to a so-called chemotherapy-associated anemia. [47] Next to hemoglobin, other routine blood parameters, such as leucocyte and platelet count prior to initial chemotherapy, seem to be further predictive markers for VTE occurrence and mortality in malignancy. [23;24]

Multiple additional factors increase the risk of developing VTE and can, therefore, lead to thrombotic events in cancer patients. [14;48;49] In general, these factors can be divided into three groups (see Figure 4); namely, the so-called demography-related risk factors (ie. age, BMI, immobility, previous VTE), the tumor-associated
(tumor stage, site of cancer) and last, but not least, the treatment-related risk factors (ie. surgery, hospitalization, chemotherapy, hormonal therapy, presence of a central venous catheter). [13;14]

**Figure 4 Risk factors for VTE in cancer patients**

- **Demography-related risk factors**
- **Tumor-related risk factors**
- **Treatment-related risk factors**

Elderly patients (≥ 65 years of age) develop VTE more frequently, especially females and patients with distant metastases. [50] Elder hospitalized cancer patients (≥ 65 years) had a higher VTE risk. Furthermore, those patients over 60 years, who have undergone surgery, showed a higher VTE incidence. On the contrary, in out-patients, age does not seem to be a risk factor, which is possibly due to the better performance status of these patients. [48] Regarding age as risk factor for VTE, it is worth mentioning that the rate of developing a VTE was found to decrease with higher age in lung cancer patients. [51]

Body mass index > 35 kg/m² also increases the risk for VTE. [24]

Concerning the tumor type, numerous studies have already shown that the highest incidence of VTE can be found in pancreatic, gastric or colorectal cancer as well as
in patients with lung cancer and in glioblastoma patients [50;52;53], whereas breast or prostate cancer patients show a relatively low risk. [23;54]

With regard to tumor stage, distant metastases double the risk for developing a VTE [55]. However, with the presence of a good performance status and chemotherapeutic treatment in ambulatory settings, respectively, the tumor stage no longer seems to be predictive for VTE occurrence. [48] Hereby, our study group investigated possible associations between local stage cancer and regional and distant metastases, respectively, and already found out that those patients with regional and distant metastases had a significantly higher risk for venous thromboembolism and also showed significantly higher levels of D-dimer, FVIII and platelet count. [56]

In addition, tumor histology apparently also plays a role in VTE development: in lung cancer patients with an adenocarcinoma, VTE occurred more often than in those with squamous cell carcinomas. [51] Interestingly, as further published by our study group, “tumor grade is also associated with the occurrence of venous thromboembolic events in cancer patients”. Patients with high grade tumors showed an elevated risk for VTE compared to low grade tumor patients. [57]

Among cancer patients, who previously had a VTE, the rate of recurrence is much higher compared to non-cancer patients with an earlier venous thromboembolic event. [58;59] Recurrence of VTE also seems to be related to certain tumor types and is most common in patients with leukemia, followed by cancer of the brain, bladder, ureter and testis. [55]

With relation to surgery, the risk of VTE occurrence varies not only among different types of surgery [60], but also depending on the patients’ age, their perioperative care (including mobility, fluid status and possible transfusions), and the used anesthesia. [61] The probability to develop DVT after surgery without adequate thromboprophylaxis is twice as high in cancer patients as in patients without malignancy. [62] Additionally, coexistent immobilization [63], as well as hospitalization itself [64], are known VTE risk factors.

Further, chemotherapy increases the risk of VTE considerably, depending on the duration of chemotherapy [65] and the particular chemotherapeutic agents, respectively. Hereby, in literature, a strong correlation between chemotherapeutic
treatment and VTE occurrence was found in patients treated with anthracyclines, platin-based drugs or nitrogen mustard analogues [66], with the highest VTE risk during the initial treatment period. [67] The VTE rate among cancer patients treated with platin-based cytostatic agents also shows a significant difference with much higher rates in those patients receiving cisplatin compared to patients with an oxaliplatin containing chemotherapeutic regimen. [68;69] The use of another chemotherapeutic agent of the nucleoside analogue group, namely gemcitabine, also correlates with a higher probability for VTE occurrence, when given alone or in combination with platin analogues. [69] An initial treatment with radiotherapy, as well as surgery, did not raise the rate of VTE occurrence. [55]

A study conducted on pancreatic cancer patients ascertained that during or after radiotherapy, the number of VTE remained the same. [70] There is also evidence that chemotherapy increases the risk for developing a fatal VTE up to 47-fold compared to the general population. [67] Especially patients receiving chemotherapy who are also treated with hormones [71-73] or who have a central venous catheter [74], show a further increase of VTE rates. The incidence of VTE caused by central venous catheters varies widely due to differences of the used catheter types, their position, the duration of their use, the cancer type of the patient or the applied chemotherapeutic agents. [75] Other treatment modalities like hormonal therapy (e.g. tamoxifen) [76] or the treatment with immunomodulatory agents (e.g. lenalidomid, thalidomid) were associated with a higher VTE incidence, especially when combined with chemotherapy. [66]

Nowadays, many cancer patients, including those with colorectal cancer or non-small cell lung cancer often benefit from the use of bevacizumab in conjunction with CHT. [77;78] Bevacizumab is an antibody “directed against the vascular endothelial growth factor and plays an important role in forming new blood vessels”. Therefore, it is of utmost importance for tumor growth and metastatic progression. [80] Moreover, VEGF inhibits endothelial cell apoptosis and reduces the entry of cytotoxic agents into the tumor mass. [79]

Many studies have already pointed out that bevacizumab is also associated with higher VTE rates. [80;81]
As cancer patients often suffer from anemia, leukopenia or thrombocytopenia, a so-called supportive treatment with erythropoiesis-stimulating factors [82], white blood cell growth factors [48] or platelet transfusions [83] are necessary in many cases, which then additionally increase the risk for VTE.

Today, scoring systems, which predict the risk of chemotherapy-associated thrombosis in out-patients, are well established. Hereby, in the so-called Khorana score, besides the tumor entity, an elevated BMI (≥ 35kg/m²), leucocytosis (ie. leucocyte count > 11 000/mcL) and thrombocytosis (ie. platelet count > 350 000/mcL) at baseline increase the risk for VTE. Moreover, patients with low hemoglobin levels (< 10g/dL) or those receiving erythropoiesis stimulating agents have a higher VTE risk. The risk for VTE occurrence in cancer patients can then be divided into three different risk groups: Patients with low risk for VTE, who have zero points in the cancer related thrombosis score, those with an intermediate risk with a maximum of two points and a high-risk patient group with more than 2 points.

The addition of two further VTE markers to the VTE risk score model in cancer patients; namely, sP-selectin with an elevation ≥ 53.1 mg/ml and D-dimer levels above 1.43 µg/ml, allows an even more precise prediction of VTE occurrence and significantly improves the risk stratification. [23;24]

Figure 5 shows the cancer-related thrombosis risk score.

**Figure 5 Ay et al. cancer-related thrombosis score – extended Khorana risk model**

<table>
<thead>
<tr>
<th>site of cancer</th>
<th>hemoglobin</th>
<th>leukocyte count</th>
<th>platelet count</th>
<th>BMI</th>
<th>D-dimer</th>
<th>sP-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>very high risk: stomach, pancreas, gliomas</td>
<td>&lt;10 g/dL or use of erythropoiesis-stimulating agents</td>
<td>≥11000/mcL</td>
<td>≥350000/mcL</td>
<td>≥35 kg/m²</td>
<td>&gt;1.43 µg/ml</td>
<td>≥53.1 mg/ml</td>
</tr>
<tr>
<td>high risk: lung, lymphoma, gynaecologic, bladder, testicular</td>
<td>1 point</td>
<td>1 point</td>
<td>1 point</td>
<td>1 point</td>
<td>1 point</td>
<td>1 point</td>
</tr>
</tbody>
</table>

As previously mentioned, the VTE incidence and mortality among cancer patients strongly depend on the tumor type: the highest VTE rates are observed in patients with brain, lung, pancreatic or colorectal cancer. [23;50;52;53]
Therefore, we included patients with one of these four tumor entities in the present longitudinal study.

Today, several treatment modalities, based on tumor type, histology and stage of disease, are applied. Different kinds of treatment can be distinguished, namely surgery, chemotherapy, radiotherapy, treatment with antibodies and angiogenesis inhibitors. In most cases, a combined therapy is used. Hereby, the use of novel drugs – especially the treatment with new antibodies and angiogenesis inhibitors, respectively, become more and more important. Brain tumors, especially glioblastomas, are mostly treated with temozolamid, which is an alkylating chemotherapeutic agent, in combination with concomitant radiotherapy. Lung cancer patients show the best response rates when treated with a combined chemotherapy, based on platin analogues. Patients with colorectal carcinoma often receive fluorouracil, a pyrimidine analogue, in combination with platin analogues. To date, most patients with pancreatic cancer are treated with gemcitabine, a substance belonging to the nucleoside analogue group. [84]

1.1 Aim of the study

We aimed to investigate whether hemostasis parameters, which are known to predict the risk of VTE, change during the course of disease, ie. during antineoplastic treatment with chemotherapy and radiotherapy. Furthermore, we evaluated if the levels of hemostaseologic parameters show a certain trend during the course of disease and if alterations of the various parameter levels (ie. an increase or decrease) can possibly be correlated to the probability of developing VTE.

Also, the predictive role of hemostasis parameters regarding survival and the correlation of their changes during the course of disease and survival were analyzed.

2 Results

2.1 Patient characterization

Overall, 112 patients with a newly-diagnosed malignancy of the brain (n = 39), lung (n = 41), with colorectal (n = 15) or pancreatic (n = 17) cancer were included between January 2011 and December 2012. Three patients died before the first follow-up time point and four patients were not able to return for a follow-up blood sample, due to
their bad performance status. Therefore, these seven patients (3 patients with brain cancer, 2 patients with pancreatic cancer and one each with colorectal and lung cancer) were only included in the analyses concerning the outcomes VTE and survival.

The time range between study entry of the first patient and the latest monthly taken blood sample of the last patient was 27 months.

The median age of the included patients was 62.4 years (range: 21.3 – 80.3 years). Among the enrolled patients, 64 (57.1 %) were males and 48 (42.9 %) were females, respectively.

From these patients, we collected a median of 6 blood samples including the baseline sample (min: 1; max: 8) within a median follow-up period of 250 days (min: 15 days, max: 250 days). Overall, 17 patients (16.2 %) died within the follow-up period, 44 patients (41.9 %) dropped out due to increased immobility and 14 patients (13.3 %) refused to participate during the full period. Some patients did not come consecutively for all blood samples – for these patients some samples are missing between the given intervals.

Blood sample collection was scheduled for each included patient at the following time intervals: (1) baseline blood sample, which was taken after diagnosis before any kind of anti-tumor treatment had been started (= TP0), (2) sample between day 14 and day 40 (= TP1), (3) between day 41 and day 70 (= TP2), (4) between day 71 and day 101 (= TP3), (5) from day 102 to day 131 (= TP4), (6) from day 132 to day 162 (= TP5), (7) between day 163 and day 200 (= TP6) and (8) between day 201 and day 250 (= TP7). Table 3 gives the number of patients per blood sample time point for each tumor entity.
### Table 3: Number of patients per blood sample time point for each tumor entity

<table>
<thead>
<tr>
<th>Time point (TP) – time interval</th>
<th>Tumor site (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP0 – day 0</td>
<td>39 brain</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>41 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>112</td>
</tr>
<tr>
<td>TP1 – day 14-40</td>
<td>30 brain</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>34 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>TP2 – day 41-70</td>
<td>22 brain</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>32 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>TP3 – day 71-101</td>
<td>25 brain</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>16 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>TP4 – day 102-131</td>
<td>23 brain</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>24 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>TP 5 – day 132-162</td>
<td>19 brain</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>22 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>TP 6 – day 163-200</td>
<td>22 brain</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>19 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>TP 7 – day 201-250</td>
<td>13 brain</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>16 lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 colorectal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38</td>
</tr>
</tbody>
</table>

n = number of patients

Regarding tumor type, 38 % of the patients had lung cancer (n = 40), 34 % had a malignancy of the brain (n = 36), 14 % had pancreatic cancer (n = 15) and 13 % colorectal cancer (n = 14). Forty-three percent of all patients (n = 45) had distant metastases at inclusion.

All patients received a chemotherapeutic treatment, following the tumor-specific regimens. Regarding treatment, 53 patients (50.5 %) received chemotherapy with platinum analogues.

In addition to chemotherapy, 50 patients (47.6 %) were also occasionally treated with radiation. Further, 5 patients (4.8 %) also received angiogenesis inhibitors during the follow-up period.

Table 4 summarizes the baseline characteristics of all patients.
Table 4 Baseline patient characteristics (n= 112)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>21.3 – 80.3</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>57.1</td>
</tr>
<tr>
<td>Female</td>
<td>48</td>
<td>42.9</td>
</tr>
<tr>
<td><strong>Follow-up period, days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>21 – 246</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>36</td>
<td>34.3</td>
</tr>
<tr>
<td>Lung</td>
<td>40</td>
<td>38.1</td>
</tr>
<tr>
<td>Colorectal</td>
<td>14</td>
<td>13.3</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>15</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Distant metastases</strong></td>
<td>45</td>
<td>42.9</td>
</tr>
<tr>
<td><strong>Specific treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platinum analogues</td>
<td>53</td>
<td>50.5</td>
</tr>
<tr>
<td>Radiation</td>
<td>50</td>
<td>47.6</td>
</tr>
<tr>
<td>Angiogenesis inhibitors</td>
<td>5</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Previous VTE</strong></td>
<td>8</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Abbreviations:  
VTE, venous thromboembolic event

Regarding the primary outcome variables, the median (quartile) coefficients of variation are as follows: 16.7 % [11.2 %; 25.4 %] for FVIII, 23.0 % [13.7 %; 35.1 %] for sP-selectin, 38.2 % [28.0 %; 52.0 %] for peak height thrombin, 15.1 % [9.8 %; 22.1 %] for FGEN, 26.1 % [18.6 %; 38.3 %] for F1+2, 47.5 % [26.7 %; 71.5 %] for D-dimer, 8.1 % [5.0 %; 11.9 %] for ATIII, 6.6 % [4.6 %; 9.5 %] for hemoglobin, 22.7 % [16.1 %; 31.3 %] for thrombocyte count and 31.0 % [18.9 %; 44.0 %] for leucocytes count.
2.2 Longitudinal analysis of the investigated parameters according to tumor type

During the follow-up period, FVIII decreased significantly in patients with brain cancer (Δ rel per month = 0.95; [95 % CI, 0.94 – 0.97], p < 0.001), whereas in patients with lung cancer, FVIII levels significantly increased (Δ rel per month = 1.02; [95 % CI 1.01 – 1.03], p = 0.002) over time. (Figure 6)

Figure 6 Boxplot of the FVIII levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: FVIII levels
Soluble P-selectin significantly declined in brain cancer patients during the study period ($\Delta$ rel per month = 0.96; [95% CI, 0.94 – 0.98], p < 0.001). In the other tumor entities, no significant change of sP-selectin levels over time was seen. (Figure 7)

Figure 7 Boxplot of the sP-selectin levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: sP-selectin levels
Levels of **peak height thrombin**, which is the most important parameter to determine thrombin generation, were decreasing by time in all tumor types (Δ abs per month = -12.9; [95 % CI, -17.0 – -8.8], p < 0.001).

Over the total course of time, a significant difference in peak height thrombin was seen between brain and lung cancer patients (Δ abs of means = -57.5; [95% CI, -105.9 – -9.2], p = 0.01) as well as between lung and pancreatic cancer patients (Δ abs of means: 75.7; [95% CI, 10.7 – 140.8], p = 0.02) respectively. (Figure 8)

**Figure 8 Boxplot of the thrombin (peak height) levels at the various blood sampling time points**

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: peak height thrombin levels
Regarding **FGEN** levels, no significant differences in time were found. Levels of FGEN differed significantly among the tumor types; namely, between brain and lung cancer patients ($\Delta$ abs of means = -111.2, [95 % CI, -161.5 – 61.0], p < 0.001), as well as between brain and colorectal cancer patients ($\Delta$ abs of means, = -100.3, [95 % CI, -170.8 – 29.8], p = 0.002) and between lung and pancreatic cancer patients ($\Delta$ abs of means = 91.6, [95 % CI, 22.9 – 160.2], p = 0.009).

The FGEN levels were seen to be highest in lung cancer patients whereby in brain cancer the lowest levels were found. (Figure 9)

**Figure 9** Boxplot of the FGEN levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: FGEN levels
With regard to F1+2 levels, no significant changes in time were found. Longitudinally measured levels of F1+2 showed a significant association with the tumor type. Brain cancer patients showed significantly lower levels compared to patients with pancreatic cancer (Δ rel of means = 0.68 [CI 0.49 – 0.95], p = 0.016). (Figure 10)

Figure 10 Boxplot of the F1+2 levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: F1+2 levels
We also analyzed D-dimer levels and found significant differences in the time effect between the tumor types. Hereby, in lung cancer patients, the levels of D-dimer increased over the follow-up period ($\Delta$ rel per month = 1.06; [95 % CI, 1.02 – 1.10], $p < 0.001$), whereas in patients with pancreatic cancer a significant decline in D-dimer levels over time was noted ($\Delta$ rel per month = 0.91; [95 % CI, 0.84 – 0.98], $p = 0.049$). (Figure 11)

**Figure 11** Boxplot of the D-dimer levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: D-dimer levels
With respect to ATIII, in our analysis, cancer patients showed a significant decrease over time (Δ abs per month = -1.40; [95 % CI, -2.10 – 0.71], p < 0.001). Brain cancer patients showed a significant elevation of ATIII levels over time course compared to lung cancer patients (Δ abs of means = 11.9; [95 % CI, 5.2 – 18.5], p < 0.001), as well as comparing brain to colorectal cancer patients (Δ abs of means = 15.8; [95 % CI, 6.6 – 25.0], p < 0.001) and comparing brain to pancreatic cancer patients (Δ abs of means = 17.4; [95 % CI, 8.3 – 26.5], p < 0.001), respectively. (Figure 12)

Figure 12 Boxplot of the ATIII levels at the various blood sampling time points

a) All patients

b) According to the tumor site

x-axis: blood sampling time points; y-axis: ATIII levels
In addition, we analyzed the routinely measured blood parameters (ie. hemoglobin, leucocytes and thrombocytes). Hemoglobin significantly decreased over the time course (Δ rel per month = 1.0; [95 % CI, 0.99 – 1.00], p = 0.030) and significant differences between brain and lung cancer patients (Δ rel of means = 1.11; [95 % CI, 1.04 – 1.17], p < 0.001), brain and colorectal cancer patients (Δ rel of means = 1.11; [95 % CI, 1.02 – 1.20], p = 0.012) and brain and pancreatic cancer patients (Δ rel of means = 1.15; [95 % CI, 1.06 – 1.24], p < 0.001) were found. (Figure 13)

**Figure 13 Boxplot of the hemoglobin levels at the various blood sampling time points**

a) All patients

![Boxplot of hemoglobin levels at various time points](image)

b) According to tumor site

![Boxplot of hemoglobin levels by tumor site](image)

x-axis: blood sampling time points; y-axis: hemoglobin levels
**Thrombocyte count** also significantly differed over time. Patients suffering from brain cancer (Δ rel per month = -15.6; [95 % CI, -20.3 – -10.8], p < 0.001) as well as lung cancer patients (Δ rel per month = -6.6; [95 % CI, -10.6 – -2.6], showed a significant decline within the follow-up period (p = 0.008). (Figure 14) No significant change in the time course was detected in the other tumor types (ie. colorectal and pancreatic neoplasia).

**Figure 14 Boxplot of the thrombocyte levels at the various blood sampling time points**

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: thrombocyte levels
Concerning leucocyte count, brain cancer patients had significantly decreased values within the study period (Δ rel per month = 0.89; [95 % CI, 0.87 – 0.90], p < 0.001) No significant change over time was found in the other tumor types. (Figure 15)

Figure 15 Boxplot of the leucocyte levels at the various blood sampling time points

a) All patients

b) According to tumor site

x-axis: blood sampling time points; y-axis: leucocyte levels
Table 5 Trend of the various parameter levels over the investigated study period of 250 days according to tumor site

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tumor site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>FVIII</td>
<td>↓</td>
</tr>
<tr>
<td>SP-selectin</td>
<td>↓</td>
</tr>
<tr>
<td>Peak height thrombin</td>
<td>↓</td>
</tr>
<tr>
<td>FGEN</td>
<td>↔</td>
</tr>
<tr>
<td>F1+2</td>
<td>↔</td>
</tr>
<tr>
<td>D-dimer</td>
<td>↔</td>
</tr>
<tr>
<td>ATIII</td>
<td>↓</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>↓</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>↓</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑ indicates a significant increase, ↓ a significant decrease of the parameter level over time; ↔ the parameter shows no significant increase or decrease over time

2.3 Differences of the longitudinal investigated parameters according to disease stage and therapy response

We investigated, whether the tumor stage had an impact on the various hemostasis parameters during the time course of disease. Only patients with solid tumor were included in this analysis. Among the 69 patients with cancer of the lung, colon or pancreas, 22 patients had already been diagnosed in an advanced stage, (i.e. presence of distant metastases at study inclusion), 24 patients suffered from lymph node metastases, and 23 patients were diagnosed without any metastases, respectively.

We found that over time, sP-selectin levels were significantly higher when distant metastases were present. (p = 0.005). Patients without metastases had lower levels than patients with lymph node metastases (p = 0.008). D-dimer levels also differed significantly among the various tumor types: In presence of distant metastases, higher levels of sP-selectin were found, compared to lymph node metastases (p = 0.002) or no metastases (p = 0.023).
Significance was also found with regard to ATIII levels, when patients without metastases were compared to patients suffering from distant metastases: distant metastases were associated with a decline of ATIII levels over time \( (p = 0.011) \). Regarding leucocyte count, patients with distant metastases showed higher values than patients without \( (p = 0.025) \) or just with lymph node metastases \( (p = 0.009) \), respectively.

We also evaluated if a complete remission within the observation time period changes the levels of the investigated parameters. Patients who reached a complete remission of disease after the observed time period \( (n = 36; 8 \) with brain cancer, 16 with lung cancer, as well as 8 with colorectal cancer and 4 pancreatic cancer patients)\), had significantly lower levels of FGEN \( (p = 0.014) \), as well as of F1+F2 \( (p = 0.017) \), and D-dimer \( (p \leq 0.001) \) over the investigated time course, compared to those without complete remission (see Figure 16).

The other investigated parameters (ie. sP-selectin, peak height thrombin, ATIII, hemoglobin, thrombocytes and leucocytes) showed no correlation regarding remission of disease (data not shown).
Figure 16 Boxplots of the FGEN levels (I), as well as of F1+2 levels (II) and levels of D-dimer (III) in patients with remission vs. progression of disease.

I) fibrinogen

II) F1+2

III) D-dimer

x-axis: blood sampling time points
y-axis: parameter levels
shaded boxplots: patients with remission within 250 days
unshaded boxplots: patients without remission within 250 days
2.4 Differences of the investigated longitudinal parameters with respect to chemotherapy and radiotherapy

Brain cancer patients, who did not receive platin-based drugs were excluded before analyzing possible interactions between platin-based drugs and hemostatic parameters. Further, as patients with colorectal cancer or pancreatic cancer were not treated with radiotherapy, these two tumor entities were excluded from analysing the influence of radiotherapy.

Regarding the different anti-tumor treatments, significantly higher FVIII levels were found in patients receiving platin-based drugs compared to patients, who received other chemotherapeutic agents (Δ rel per month = 1.13 [CI 1.06 – 1.20], p < 0.001).

Moreover, patients, who were treated with platin-based drugs, showed increased levels of F1+2 (Δ rel per month = 1.10 [CI 1.00 – 1.21], p = 0.050).

Concerning radiation, elevated F1+2 levels during radiotherapy were seen (Δ rel per month = 1.12 [CI 1.01 – 1.24], p = 0.021). No further correlations could be found between treatment modalities (chemotherapy and radiotherapy) and the investigated parameters.

2.5 Outcome venous thromboembolic events

In our cohort, 14 patients (12.5 %) had a venous thromboembolic event during the observation period of 250 days. Thereof, 7 patients (50.0 %) suffered from a DVT, 4 patients (28.6 %) developed a PE, and in three patients (21.4 %) other kinds of thrombosis types (one each, thrombosis of the jugular, subclavian vein and portal vein) were found. The venous thromboembolic events occurred after a median time of 110 days (min: 9 days, max: 346 days) following study inclusion. Hereby, the cumulative incidence after 3 months was 6 % (CI 3 – 13 %) and after 6 months 11 % (CI 7 – 20 %). Regarding tumor site, 10.3 % (n = 4) of all patients with brain cancer, 9.8 % (n = 4) of all patients suffering from lung cancer, 29.4 % (n = 5) of all pancreatic cancer patients, as well as 6.7 % (n= 1) of patients with colorectal cancer, developed VTE.

Using Cox-regression analyses, we found that an increase of FVIII levels was significantly associated with VTE occurrence during the follow-up time period (HR per 2-fold increase: 3.49 [95 % CI, 1.355 – 8.990], p < 0.01), see Table 6.
Furthermore, increased sP-selectin levels also correlated with VTE occurrence (HR per 2-fold increase: 2.44 [95 % CI, 1.309 – 4.529], p = 0.005) as well as increased levels of F1+2 (HR per 2-fold increase: 2.111 [95 % CI, 1.474 – 3.025], p < 0.001) and D-dimer (HR per 2-fold increase: 1.764 [95 % CI, 1.324 – 2.351], p < 0.001 (see Table 6).

In patients who developed VTE, the D-dimer levels at the latest blood sampling time point before VTE lay above the median value in 13 patients and thereof, in seven patients the levels even lay above the 75th percentile. In our cohort, the other investigated parameters (FVIII, TGA peak height, FGEN, ATIII, hemoglobin, thrombocytes and leucocytes) were not associated with VTE.

Table 6 Univariate Cox-regression analysis – outcome VTE

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome VTE</th>
<th></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95 % CI</td>
<td></td>
</tr>
<tr>
<td>FVIII*</td>
<td>3.49*</td>
<td>1.36 – 8.99</td>
<td>0.0096</td>
</tr>
<tr>
<td>sP-selectin*</td>
<td>2.44*</td>
<td>1.31 – 4.53</td>
<td>0.005</td>
</tr>
<tr>
<td>TGA peak height</td>
<td>1.00</td>
<td>0.99 – 1.00</td>
<td>0.60</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.00</td>
<td>1.00 – 1.002</td>
<td>0.50</td>
</tr>
<tr>
<td>F1+2*</td>
<td>2.11*</td>
<td>1.47 – 3.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>D-Dimer*</td>
<td>1.76*</td>
<td>1.32 – 2.35</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Log2-transformed, which implies that the corresponding HRs refer to a two-fold increase in these variables

Figure 17 depicts the boxplots of those parameters, which were significantly associated with VTE in Cox-regression analyses in patients with and without VTE within 250 days. Figure 18 shows these individual parameter levels over time in patients developing VTE within 250 days in comparison to the interquartile range of all included patients without VTE occurrence.

SP-selectin, F1+2 and D-dimer values were at all sampling time points higher in those patients developing VTE.
Figure 17 Boxplots of the various parameters in patient with and without thrombosis

I) FVIII

II) sP-selectin

III) F1+2

IV) D-dimer

x-axis: blood sampling time points; y-axis: parameter levels
shaded boxplots: patients with VTE within 250 days
unshaded boxplots: patients without VTE within 250 days
Figure 18 Values of the respective parameters of each patient developing VTE within 250 days and the interquartile range of patients without VTE

I) FVIII

II) sP-selectin

III) F1+2

IV) D-dimer

x-axis: blood sampling time points
y-axis: parameter levels
thin lines: individual values of each patient developing VTE within 250 days; lines end up with VTE occurrence
bold lines: median (continuous line) and quartile (dotted lines) levels of patients not developing VTE within 250 days
2.6 Outcome survival

Within the follow-up period of 250 days, 22 patients (19.6 %) died. Thereof, 7 (31.8 %) patients suffered from brain cancer, 7 (31.8 %) had pancreatic cancer, 7 (31.8 %) lung cancer and 1 (4.5 %) patient was suffering from colorectal cancer. In Cox-regression analyses (see Table 7), a higher mortality was associated with increased FVIII levels (HR per 2-fold increase: 4.432 [95 % CI, 1.852 – 10.604], p < 0.001), sP-selectin (HR per 2-fold increase: 3.085 [95 % CI, 1.676 – 5.680], p < 0.001) and D-dimer (HR per 2-fold increase: 1.597 [95 % CI, 1.231 – 2.071], p < 0.001).

Table 7 Results of univariate Cox-regression analysis – outcome survival

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td>FVIII*</td>
<td>4.43*</td>
</tr>
<tr>
<td>sP-selectin*</td>
<td>3.09*</td>
</tr>
<tr>
<td>TGA peak height</td>
<td>1.00</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.60</td>
</tr>
<tr>
<td>F1+2*</td>
<td>1.40*</td>
</tr>
<tr>
<td>D-Dimer*</td>
<td>1.00*</td>
</tr>
</tbody>
</table>

* Log2-transformed, which implies that the corresponding HRs refer to a two-fold increase in these variables

No statistically significant association regarding survival was found for the other investigated parameters, namely peak height thrombin, FGEN, F1+2, ATIII, hemoglobin, thrombocytes and leucocytes. Hereby, the patients, who died within the observation period, had higher levels of these parameters; however, not all patients who died showed elevated levels.

Figure 19 shows individual parameter levels of procoagulant factors in patients, who deceased within 250 days and the interquartile range of survivors, in whom a statistically significant elevation of FVIII, D-dimer or sP-selectin goes along with an increased mortality.
Figure 19 Values of the respective parameters for each patient dying within 250 days and the interquartile range of patients who survived

I) FVIII  
II) sP-selectin  
III) F1+2  
IV) D-dimer  

x-axis: blood sampling time points  
y-axis: parameter levels  
thin lines: individual values of each patient dying within 250 days; lines end up with death  
bold lines: median (continuous line) and quartile (dotted lines) levels of surviving patients
3 Discussion

The present longitudinal cohort study was conducted to investigate whether patients with malignant disease of the brain, the lung, the colon or the pancreas show significant alterations of hemostatic and fibrinolytic parameters during the course of disease. Besides, a possible association between anti-tumor treatment and the above-mentioned parameter levels was examined. Furthermore, a possible association between the various parameter levels on the one side and disease stage, individual prognosis and VTE occurrence on the other side within the follow-up period of 250 days was evaluated.

We investigated the influence of hemostasis parameters on occurrence of VTE and survival over time during the course of disease. This is, as far as we know, the first study, in which several hemostasis biomarkers; namely, FVIII, sP-selectin, peak height thrombin, fibrinogen, prothrombin fragment F1+2, D-dimer, and antithrombin III, as well as routine blood parameters; namely, hemoglobin, thrombocyte and leucocyte count, are followed longitudinally in cancer patients, which is a real strength of our study.

The discussion section takes into consideration mostly results of longitudinal research. Only few data on the course of hemostatic parameters in cancer patients can be found in literature. Further, longitudinal investigations of hemostasis biomarkers are often not done to the same extent as in our study as many studies focus on pre- and postoperative levels only. To date, numerous prospective studies which investigated patients at a single time point have been conducted and pointed out that a coagulation and fibrinolysis activation already exists at time of cancer diagnosis.

This systemic activation may be of great interest regarding the regulation of tumor growth, cancer dissemination and VTE development.

We studied the behavior of hemostasis biomarkers in four different tumor entities. Behavior of the various parameters was not uniform, but rather variable in the different cancer entities. For example, factor VIII decreased in patients with brain cancer, whereas it increased in those with lung cancer. Soluble P-selectin only declined in brain cancer and remained rather constant in the other three cancer entities. Interestingly, thrombin (peak height), which is important to monitor hypercoagulability, decreased over time in the investigated tumor entities.
D-dimer, which is a parameter that as well reflects activation of clotting system and fibrinolysis, increased over time in lung cancer patients, whereas it decreased in pancreatic cancer patients.

As expected, parameters of the blood count analysis, such as hemoglobin, thrombocyte count and leukocyte count, decreased over time in most of the tumor entities, most probably due to the chemotherapy that was given to every patient of our study population.

Anyway, we could not deduce a specific pattern for the various hemostatic parameters over time. However, what we may conclude is that there is not an excessive increase in overall clotting activation during chemotherapy.

Later in this section, we will discuss the possible influence of certain chemotherapeutic agents, such as platin analogues, on hemostasis parameters.

Comparing patients at different disease stages, elevated sP-selectin and D-dimer levels were seen in patients with a more advanced disease. As we could demonstrate, this was not only the case at diagnosis, as previously shown by our study group [56], but also remained to be present during the course of disease.

This supports previous findings by Ünsal et al. [45] who reported that in lung cancer patients, those with distant metastases showed higher D-dimer levels before receiving anti-tumor treatment. Seitz et al., who also compared lung cancer patients with extensive disease to those with limited disease, found significant differences in baseline D-dimer levels as well. In the study by Seitz et al., increased D-dimer levels at baseline were also predictive for tumor spread. [85]

Another study, which found differences of hemostasis parameters to be associated with disease stage, was a study by Walter et al. [49] conducted in patients with cancer of the breast, the lung or the kidney. Hereby, in patients with intracranial metastases, who had the primary tumor mass removed and/or had been treated for peripheral metastases, he showed a preoperative elevation of the coagulation factors II, VIII, IX, X and XI, compared to preoperative coagulation factor levels of control individuals who had a lumbar disk herniation surgery.

Moreover, in the tumor group, Walter et al. found higher ATIII activities at all three blood sampling time points; namely, pre- and postoperatively – on days one and four after surgery – respectively, whereas fibrinogen levels were lower in tumor patients than in control individuals.
Regarding fibrinogen levels, a study in ovarian cancer patients [86] showed no association between the FIGO stage and fibrinogen levels over time course.

Regarding remission and therapy response, our data suggest that patients, who once reach complete remission, then have lower levels of the hemostasis biomarkers. Thereby, cancer patients, who reached complete remission in our cohort, then had significantly decreased F1+2, D-dimer and fibrinogen levels. This is in accordance with a study conducted by Van Haren et al. [87] who detected changes of hemostasis and fibrinolysis parameters over time in patients who had surgical resection of thoracoabdominal tumors. In their study, all parameters were measured longitudinally – i.e. preoperative and postoperative on day 1 and between 6 to 12 months following surgery, respectively. The hereby detected increased hypercoagulability returned to baseline in long-term follow-up. In some patients a reversal of cancer-induced hypercoagulability occurred, varying with the histology and the location of the tumor. In the afore-mentioned study by Seitz et al., patients, who reached partial or complete remission, showed lower baseline D-dimer levels compared to non-responders. Therefore, Seitz et al. hypothesized that D-dimer seems to be predictive for the patients’ prognosis. [85] Another study by Beer et al. [88] supports these findings as well. It showed that patients with active tumor had clearly higher D-dimer levels at study inclusion than those having reached remission of disease. This is also in accordance with another study by Inal et al. [89], in which plasma D-dimer levels were measured longitudinally in lung cancer patients before, during and after receiving four cycles of chemotherapy. When compared to healthy controls, lung cancer patients had significantly higher D-dimer levels. Besides, therapy responders then showed significantly lower D-dimer levels than non-responders.

With regard to possible changes of hemostasis parameters during anti-tumor treatment, our study indicates that over time, brain cancer patients show a decrease of FVIII and sP-selectin levels, whereas levels of peak height thrombin declined over time in all four tumor entities. We also found significantly different levels of fibrinogen as well as of F1+2 and D-dimer between the four tumor entities within the observation period. We surmise that these changes are due to an at least temporary response to anti-tumor treatment.
With regard to the type of antineoplastic treatment, we analyzed, if patients, who received a platin-based chemotherapy showed significant differences of the investigated parameters over time compared to those patients, who never received a platin-based chemotherapy. Our findings showed higher levels of F1+2 and FVIII when treated with platin-based chemotherapy.

In summary, we cannot assume that anti-tumor treatment itself fuels procoagulatory mechanisms, except platin-based chemotherapeutic agents, which seemed to increase FVIII and, moderately, F1+2 levels. We observed a decrease in hemostatic biomarkers rather than an increase.

This is somewhat in contrast to findings by Byrne et al. [90], who observed in a study of localized esophageal cancer patients that both, the first cycle of chemoradiation as well as the second cycle of chemotherapy resulted in significantly increased levels of D-dimer and prothrombin fragment F1+2. Surgery itself was associated with an increase of fibrinogen, D-dimer, F1+2 and FVIII levels, respectively, for a time period of up to 6 months postoperatively. However, this might rather be the effect of surgery itself, which fuels procoagulatory mechanisms, than a result of chemotherapy.

Alterations of hemostatic parameters during treatment were also observed in children with acute lymphoblastic leukemia [91]. Herein, patients showed increased thrombin generation as well as higher D-dimer levels at diagnosis, before any chemotherapy had been started. Under treatment, thrombin generation decreased. This correlates with our finding of decreasing peak height thrombin over time in all tumor entities.

Regarding radiotherapy, we found increased F1+2 levels in patients who underwent radiotherapy during this treatment. According to this finding, Stender et al. [92] reported significantly increased F1+2 values in rectal cancer patients undergoing radiotherapy, when comparing levels after long-term radiotherapy to baseline. Besides, a significant fluctuation of F1+2 levels was seen in patients treated with long-term low-intensity radiotherapy.

Our observation of a decrease in hemostatic biomarkers rather than an increase correlates with data from a prospective study conducted by Oberhoff et al. [93] in which postmenopausal breast cancer patients had adjuvant antiestrogen treatment with tamoxifen. During the six-month observation, blood samplings were drawn four times (at baseline, after the first, third and sixth month of treatment), whereby initially
a decrease of D-dimer, fibrinogen, F1+2 and ATIII in comparison to the baseline levels, was found. After the third and sixth month of treatment, no further significant effect, caused by the blood collection time period, namely, maximum 14 days postoperatively, was seen. This is somewhat contrary to the findings of a study by Falanga et al. [94], in which prothrombin F1+2 levels were seen to increase significantly throughout the treatment with tamoxifen, whereby D-dimer levels did not change significantly during treatment with tamoxifen. Findings of van Marion et al. [95] showed no association between neither alterations of FVIII:C nor fibrinogen levels before and during treatment and VTE occurrence in patients with multiple myeloma.

Regarding VTE, single measurements of hemostatic parameters have been conducted in order to evaluate hemostatic biomarkers for cancer-associated VTE. Hereby, it has been described that levels of hemostatic and fibrinolytic parameters correlate with the risk of cancer patients for developing a VTE at a later time point. [32;33;35;44;48;96]

Previously, our study group demonstrated that in cancer patients with active tumor, before starting anti-tumor treatment, high platelet counts [92], high D-dimer and high prothrombin fragment 1+2 levels [44], respectively, predict VTE. Moreover, high sP-selectin levels [33], elevated peak thrombin [35] and high FVIII levels [32] showed an association with VTE occurrence.

This is in accordance with findings of Khorana et al. [24;48], who described an association of elevated pre-chemotherapeutic levels of platelet and leucocyte count, and low hemoglobin levels, with VTE occurrence.

Kroger et al. [66] already showed a relationship between elevated D-dimer levels at enrollment and later VTE occurrence in cancer patients.

We found elevated levels of FVIII, sP-selectin, D-dimer and F1+2 in patients, who developed a VTE, not only at inclusion (reflecting the predictive role of these parameters), but also during time course. Although changes of the median of these various biomarkers during the course of disease were associated with VTE occurrence, individual parameter courses were highly variable. Therefore, these parameters, in our opinion, do not allow individual risk prediction, neither for VTE occurrence, nor at the time point when VTE actually occurs.
Pertaining to the patients’ **prognoses**, our data reflect the association of a shorter survival with significantly higher values of D-dimer, FVIII and sP-selectin. This correlates with findings of the afore-mentioned study by van Marion et al. [95], which found high FVIII:C and fibrinogen levels before starting chemotherapy to be highly associated with mortality.

Another study on the prognostic significance of D-dimer levels in lung cancer patients [97] reported that elevated D-dimer levels are a strong predictor for survival, with high D-dimer levels at study inclusion correlating with an increased mortality.

Also, the above already mentioned study by Ünsal et al. found that high D-dimer levels as well as low ATIII levels correlated with a higher mortality in lung cancer patients. Inal et al.[89] also observed an association between high D-dimer levels and mortality in lung cancer patients, with significantly higher levels in the patients who died.

The present study has several limitations, mainly the number of patients included is rather small. Therefore, we were not able to do a separate analysis on anti-angiogenic treatment or surgery, for which the respective sub-populations were far too small. However, performance of such a study is rather cumbersome for the patients and for the investigators. These difficulties in consecutive collection of blood samples over time in cancer patients with accurate documentation of all treatment modalities and events might be the reason that only very few longitudinal studies are available in the literature, ours being by far the largest with regard to patient numbers and numbers of investigated time points.

In conclusion, the present longitudinal study provides data suggesting that in newly-diagnosed cancer patients, starting anti-tumor treatment, a procoagulant state exists, which then remains to be present but tends to decrease during the following months of anti-tumor treatment. Patients who once reach complete remission have then lower levels of the hemostasis biomarkers. This correlates with the fact that the highest risk for VTE occurrence is found in patients early after tumor diagnosis and with an active tumor, respectively. Although it would be highly desirable to be able to predict an individual’s risk of VTE by longitudinal investigation of hemostasis biomarkers, this seems to be hindered due to the highly variable course of these biomarkers.
4 Methods

4.1 Study design

The current longitudinal study was conducted within the framework of the Vienna Cancer and Thrombosis Study, which is an on-going, prospective observational single center study.

The study was approved by the ethics committee of the Medical University of Vienna. Patients were recruited at different departments of the General Hospital of Vienna/Medical University of Vienna between January 2011 and December 2012. The study population consists of newly-diagnosed cancer patients suffering from brain or lung cancer, as well as of patients with a colorectal or pancreatic carcinoma. None of the patients received any anti-tumor treatment except surgery before study inclusion. Eligible patients had to be at least 18 years of age and had to give their written informed consent prior to study entry. Patients with an overt bacterial or viral infection and those with a venous or arterial thromboembolic event within the last three months were not allowed to participate. Apart from a portacath implantation, surgery within the last two weeks was an additional exclusion criterion. Further, we excluded patients, who had a continuous anticoagulation treatment with vitamin-K-antagonists or low-molecular weight heparin (LMWH). Patients receiving aspirin, ticlopidine, clopidogrel or LMWH only at high risk situations and with a dosage of thromboprophylaxis, could be included.

At inclusion time, data on the patients’ medical history was collected using a questionnaire (see Appendix 1) and a first blood sample was taken. Demographic and clinical details on the malignant disease (ie. tumor site, histology and stage) were documented. Further, possible existing predisposing factors for VTE, were recorded. Each patient received detailed written information on VTE symptoms. During the following observation period of a maximum of 250 days, up to seven further blood samples during anti-cancer treatment were drawn on a monthly basis and information regarding VTE occurrence and anti-cancer treatment was documented during the observation time using a questionnaire (see Appendix 2). If a patient did not return for the next blood sample, the patient, his relatives or the family doctor were contacted to obtain further information. In addition, patients were advised to report immediately in case of developing any symptoms, which might be caused by a possible VTE.
As various anti-tumor treatments are suggested to alter levels of certain parameters, we analyzed the effect of different anti-tumor treatment modalities on VTE occurrence during the time course. Unfortunately, due to the small number of patients, who had anti-angiogenic treatment or surgery within the study period, we were not able to do a separate analysis on these two factors. Since all patients received chemotherapeutic treatment during the study period and due to the higher VTE risk in patients with platin analogues, two groups; namely, first, the group of patients who had never received platin analogues and second, patients receiving platin analogues within the study period were distinguished. This excludes all patients with brain cancer, as none of them ever received platin analogues. A patient was regarded as receiving RT at a blood sampling time point, when RT was done within the last two weeks.

4.2 Diagnosis of VTE

There was no routine screening for VTE in our patients. When a patient developed symptoms of VTE, objective imaging methods were performed to confirm or exclude a VTE. Deep vein thrombosis of the extremity was diagnosed with duplex ultrasound and/or venography. For the diagnosis of PE, a ventilation/perfusion lung scan, computed tomography or pulmonary angiography was used. In case of death within the observation period, death certificates and autopsy findings, respectively, helped us, to find out if an eventually underlying fatal PE existed. Every venous thromboembolic event was then presented to an independent, “adjudication committee”, which consisted of specialists in the fields of angiology, radiology, and nuclear medicine in order to confirm or rule out the event and decide, whether the event was symptomatic or incidental, respectively. When an event was treated with anticoagulants, it was regarded as clinically significant and counted as an event. Figure 20 shows the schematic of VTE diagnosis.
4.3 Blood sampling

At study entry and at each follow-up time point, venous blood sampling was performed by sterile and atraumatic antecubital venipuncture. All blood sampling took place between 8 and 12 a.m. We collected the blood samples into plasma vacuum tubes (Vacuette®, Greiner-Bio One, Kremsmuenster, Austria) which contained 1:10 volume sodium citrate stock solution at 0.129 mmol/L and into a Vacutainer K3-EDTA tube (Vacuette®, Greiner-Bio One), respectively. Plasma samples were then centrifuged at 3000 g for 10 minutes in order to obtain platelet-poor plasma. The aliquots were then stored at -80 °C until we performed the testing of sP-selectin as well as the thrombin generation assay in series. For the determination of D-dimer and prothrombin fragment 1+2, plasma samples were stored at -20 °C until serial testing was performed. Further, levels of FGEN, FVIII, ATIII, leucocyte, platelet count and hemoglobin, respectively, were measured in the laboratory immediately after the blood samples have been taken.
4.4 Laboratory analysis

The determination of FVIII activity was performed on a Sysmex CA 7000 analyzer by using FVIII deficient plasma (Technoclone, Vienna, Austria) and APTT Actin-FS (Dade-Behring, Marburg, Germany) with a coefficient of variation of 5 % to 8 %. Soluble P-selectin levels were measured by the use of a human sP-selectin Immunoassay (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions, which have been described previously. [98]

The measurement of thrombin generation over time was done with an assay kit (Technothrombin® TGA kit, Technoclone, Vienna, Austria) on an automated computer-controlled microplate reader (FLx800; BioTek, Winooski, VT, USA) and specially adapted software (Technothrombin® TGA, Vienna, Austria). The fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) was used and the reaction was triggered by the TGA RC low reagent. This reagent contains 71.6 pM recombinant human tissue factor lipidated in 3.2 µmol/L phospholipid micelles (phosphatidylcholine 2.56 µmol/L) and phosphatidylserine (0.64 µmol/L). Peak thrombin generation, which illustrates the maximum concentration of thrombin generation, was used for the analysis of correlations between thrombin generation and VTE in our cancer patients.

Further, by the use of a quantitative ELISA (Enzygnost ® F1+2- monoclonal; Dade-Behring, Marburg, Germany), Fibrinogen levels were measured according to Clauss [99] (STA Fibrinogen; Diagnostica Stago, Asnières, France) with a normal range between 180 and 390 mg/dl. F1+2 levels were determined.

For determination of D-dimer, we used a quantitative latex assay (STA-LIAtest® D-DI; Diagnostica-Stago, Asnières, France) on a STA-R analyzer (Diagnostica-Stago), and to measure the quantitative ATIII activity (range: 0 – 140 %), STA Antithrombin III was used.

Thrombocytes, leucocytes and hemoglobin were analyzed with the automated hematology analyzer XE-5000 (Sysmex, Kobe, Japan).

The analytical inter assay coefficients of variation were 4.91 for FVIII, 2.25 for sP-selectin, 15.3 for peak height thrombin, 3.98 for FGEN, 7.15 for F1+2 and 3.09 for D-dimer.
4.5 Statistical analysis

All patients with at least one follow-up blood sample were included for the longitudinal analyses of the primary outcome variables FVIII, peak height thrombin, D-dimer, prothrombin fragment 1+2 (F1+2), FGEN, sP-selectin, ATIII and the routine blood parameters – hemoglobin, thrombocytes and leucocytes. Due to their skew distributions log2-transformed values of the variables FVIII, D-dimer, F1+2 and sP-selectin were used for all statistical analyses. The variables peak height thrombin and FGEN were not transformed before statistical evaluations. Repeated Measures Analysis of Variance (ANOVA) models were performed to test for statistically significant time effects on the parameter levels and to evaluate differences between groups of patients. Within these models differences due to the tumor type, the disease stage, the therapy response and the type of treatment were tested. P-values resulting from multiple comparisons were adjusted using the Tukey method. In order to evaluate whether time effects are different between the tumor types an interaction term was considered within the ANOVA models and subgroup analyses were performed for each tumor type in case of a statistically significant interaction. Statistically significant time effects are described by the estimated monthly relative changes (Δ rel) with 95 % CI in case of log-transformed outcome variables. Presenting time effects in non-transformed outcome variables the estimated monthly absolute changes (Δ abs) with 95 % CI are given. Differences between tumor types were analogously described by the relative differences of means in case of log transformed outcome variables (Δ rel) and by absolute differences (Δ abs of means) in case of non-transformed outcome variables, respectively. Separate ANOVA models were performed for each of the six outcome variables.

Analysing the outcome VTE and survival of patients, all patients were included for statistical analyses independent of their number of follow-up blood samples. Univariate Cox-regression models were used to evaluate the effect of the investigated parameters on the development of VTE and on survival. The observation period started at the study inclusion. With respect to the outcome VTE, the observation endpoint was fatal or non-fatal VTE. Data were censored at death, end of observational period after 250 days or loss to follow-up. Considering the endpoint death, data were censored at the end of the observational period after 250 days or loss to follow-up. Every investigated parameter was considered as a time-
dependent factor updated at the respective time point of the blood sampling. Statistically significant effects are described by the hazard ratios with 95 % CI. All p-values are results of two-sided tests and p-values < 0.05 were considered as indicating statistical significance.


Appendix 1 Patient questionnaire at study inclusion

BASIS-PATIENTENFRAGEBOGEN

Patienten ID Nummer..........................
Datum: .....................................

1. Persönlichen Daten

Wohnadresse: ............................................................
Hausarzt: .............................................................
Körpergröße: ........ cm Körpergewicht: ....... kg
Blutgruppe:.... Rhesusfaktor: ........... ...
Telefonnummer: ................................. Email: .......................

2. Tumorerkrankung

Tumorart:.................................................................
Tumorstadium: .........................................................
Histologie des Tumors:...................................................
Neu diagnostiziert: JA □ Nein □
Zeitpunkt der Erstdiagnose: ...........................................
Frühere Tumorerkrankung: ...........................................
Metastasierung? JA □ Nein □
Bereits durchgeführte Therapie .....................................................
Geplante Therapie .............................................................

3. Familienanamnese

Thrombose/Embolie? Krebserkrankung?
JA □ Nein □ JA □ Nein □

3. Medikamente / Rauchen/ Alkohol

Welche Medikamente nehmen Sie derzeit regelmäßig ein und seit wann?
(auch Kontrazeptiva, Hormonersatztherapie, Thrombo-Ass, Marcoumar oder Sintrom)

<table>
<thead>
<tr>
<th>Medikament</th>
<th>Dosis</th>
<th>Seit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Erhalten Sie derzeit gerinnungshemmende Injektionen (Heparin?)
Nein □ JA □ wenn ja, welche: ............................................
4. Grundkrankheiten

Leiden oder litten Sie jemals unter einer chronischen Erkrankung?
(Herzinsuffizienz ab NYHA III, Diabetes mellitus?)

...................................................................................................................
...................................................................................................................
....................................................................................................................

5. Thromboseanamnese

-Sind Sie schon einmal an einer tiefliegenden Thrombose oder Lungenembolie erkrankt?

<table>
<thead>
<tr>
<th>Lokalisation</th>
<th>Datum</th>
<th>Nachweisverfahren</th>
<th>Ursache</th>
<th>Symptome</th>
</tr>
</thead>
</table>

-Haben Sie schon jemals eine Venenentzündung gehabt?

<table>
<thead>
<tr>
<th>Lokalisation</th>
<th>Datum</th>
</tr>
</thead>
</table>

Hatten Sie schon einmal eine arterielle Thrombose?

<table>
<thead>
<tr>
<th>Datum</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Datum</th>
</tr>
</thead>
</table>

Haben Sie derzeit eine zentrale venöse Verweilkatheter?

<table>
<thead>
<tr>
<th>Ja/Nein</th>
<th>Seit wann</th>
</tr>
</thead>
<tbody>
<tr>
<td>Port-a-Cath</td>
<td></td>
</tr>
<tr>
<td>Hickman</td>
<td></td>
</tr>
<tr>
<td>Sonstige</td>
<td></td>
</tr>
</tbody>
</table>

6. Nur für Frauen

Hatten Sie in den letzten 6 Wochen eine Schwangerschaft oder Geburt?

JA □ Nein □

Nehmen Sie orale Kontrazeptiva (Pille)?

JA □ Nein □

Erhalten Sie eine Östrogentherapie?

JA □ Nein □

Sind Sie schwanger?

JA □ Nein □

7. Zur Zeit vor der Blutabnahme

Hatten Sie in den letzten 6 Wochen eine schwere Verletzung, Fraktur, Infektion, Operation oder andere akute Krankheit?

...................................................................................................................

8. Kamofsky index

100 % Normalzustand, keine Beschwerden, keine manifeste Erkrankung
90 % minimale Krankheitssymptome
80 % normale Leistungsfähigkeit mit Anstrengung
70 % eingeschr. Leistungsfähigkeit, arbeitsunfähig, kann sich alleine versorgen
60 % gelegentliche fremde Hilfe
50 % Krankenpflegerische und ärztliche Hilfe, nicht dauernd bettlägerig
40 % bettlägerig, spezielle Pflege erforderlich
30 % schwerkrank, Krankenhauspflege notwendig
20 % Krankenhauspflege und supportive Maßnahmen erforderlich
10 % moribund, Krankheit schreitet schnell fort
### Appendix 2 Patient questionnaire for follow-up

**Follow-up Patientenfragebogen**

### FRAGEBOGEN FÜR DEN ZEITRAUM VON «last_co_event» BIS ZUM HEUTIGEN TAG

Datum: .........................

Körpergewicht: ....... kg

Geänderte Telefonnummer, Adresse: ... .................................................................

#### Ist eine tiefe Venenthrombose aufgetreten?

<table>
<thead>
<tr>
<th>Nein ☐</th>
<th>JA ☐</th>
</tr>
</thead>
<tbody>
<tr>
<td>An welcher Stelle: .................................................................</td>
<td></td>
</tr>
<tr>
<td>Datum: .......................................</td>
<td></td>
</tr>
<tr>
<td>Beschwerden? : .................................................................</td>
<td></td>
</tr>
<tr>
<td>Wie wurde die Diagnose gestellt: .................................................................</td>
<td></td>
</tr>
<tr>
<td>Auslöser: .................................................................</td>
<td></td>
</tr>
</tbody>
</table>

#### Ist eine Lungenembolie (oder Lungeninfarkt) aufgetreten?

<table>
<thead>
<tr>
<th>Nein ☐</th>
<th>JA ☐</th>
</tr>
</thead>
<tbody>
<tr>
<td>An welcher Stelle: .................................................................</td>
<td></td>
</tr>
<tr>
<td>Datum: .......................................</td>
<td></td>
</tr>
<tr>
<td>Beschwerden? : .................................................................</td>
<td></td>
</tr>
<tr>
<td>Wie wurde die Diagnose gestellt: .................................................................</td>
<td></td>
</tr>
<tr>
<td>Auslöser: .................................................................</td>
<td></td>
</tr>
</tbody>
</table>

#### Ist eine Venenentzündung aufgetreten?

| Nein ☐ | JA ☐, Stelle: ................. |

#### Was sind Ihre derzeitigen Medikamente?

| Was sind Ihre derzeitigen Medikamente? |

#### Haben Sie Thrombosevorbeugungsspritzen bekommen?

<table>
<thead>
<tr>
<th>Nein ☐</th>
<th>JA ☐</th>
</tr>
</thead>
</table>

«ID» «title» «prename» «name»
Haben Sie Blutkonserven bekommen?
Nein ☐ JA ☐ Datum, Krankenhaus?): .................................................................

Haben Sie einen zentralen Venenkatheter bekommen?
Nein ☐ JA ☐ (Datum, Krankenhaus?): .................................................................

<table>
<thead>
<tr>
<th>Nur für Frauen:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nehmen Sie die Pille? Nein ☐ JA ☐</td>
</tr>
<tr>
<td>Bekommen Sie Östrogene (Hormon gegen Wechselbeschwerden)? Nein ☐ JA ☐</td>
</tr>
<tr>
<td>Sind Sie schwanger? Nein ☐ JA ☐</td>
</tr>
</tbody>
</table>

Hatten Sie seit «last_co_event» eine Operation?
Nein ☐ JA ☐
Krankenhaus: .................................................................
Aufnahmedatum: .................................................................
Entlassungsdatum: .................................................................
Welche Operation?: .................................................................
Thrombosevorbeugung? (Welches Medikament?, Wie lange?): .................................................................

Haben Sie seit «last_co_event» eine Chemotherapie bekommen?
Nein ☐ JA ☐
Krankenhaus, Abteilung?: .................................................................
Behandlungsbeginn?: .................................................................
Medikament?: .................................................................
Behandlungsende?: .................................................................

Haben Sie seit «last_co_event» eine Strahlentherapie bekommen?
Nein ☐ JA ☐
Krankenhaus, Abteilung?: .................................................................
Behandlungsbeginn?: .................................................................
Behandlungsende?: .................................................................

Wurden bei Ihnen seit «last_co_event» neue Erkrankungen erkannt?
(Infektionen, Herzinfarkt, Schlaganfall, Verschlusskrankheit der Beine, etc.?) .................................................................

Danke!

«ID» «title» «prename» «name»
Longitudinal analysis of hemostasis biomarkers in cancer patients during antitumor treatment

E. - M. Reitter,* A. Kaider,† C. Ay,‡ P. Quehenberger,‡ C. Marosi,§ C. Zielinski and I. Pabinger*‡

*Clinical Division of Hematology and Hemostaseology, Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna; †Section for Clinical Biometrics, Center for Medical Statistics, Informatics and Intelligent Systems, Medical University of Vienna; ‡Department of Laboratory Medicine, Medical University of Vienna; and §Clinical Division of Oncology, Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria


Essentials

- Hemostasis biomarkers impact thrombosis occurrence and survival in cancer patients.
- We performed a longitudinal analysis of hemostasis parameters in 112 cancer patients.
- Hemostatic parameters are associated with disease state, patients' prognosis, and the risk of VTE.
- The procoagulant state exists not only at diagnosis, but also during the course of disease.

Summary. Background: Hemostasis biomarkers are known to have an impact on venous thromboembolism (VTE) occurrence and survival in cancer patients. Objectives: As there are almost no data on longitudinal changes, we aimed to evaluate those in the present prospective observational study during chemotherapy and the course of disease. Patients/Methods: Patients with cancer of the brain (n = 39), lung (n = 41), colon (n = 15) or pancreas (n = 17) were included before initiation of antitumor therapy. Blood samples for determination of factor VIII, thrombin peak height, D-dimer, F(1+2), fibrinogen and soluble P-selectin (s-selectin) were drawn on a monthly basis. The study endpoints were death, VTE occurrence, or completion of the study period. Results: Overall, 546 blood samples of 112 patients were analyzed. D-dimer and s-selectin levels were significantly higher in patients with distant metastasis than in those without. Patients with complete remission had significantly lower levels of F(1+2), D-dimer and fibrinogen. Peak height thrombin levels showed a decrease over time in all tumor types. Levels of biomarkers behaved differently in the various tumor types. Patients who developed VTE (n = 14) showed increasing levels of FVIII, s-selectin, and D-dimer. At the last blood sampling time-point before VTE occurrence, in 13 patients the D-dimer level was above the median, and in seven of these patients it was even above the 75th percentile; however, the individual course was highly variable. Regarding survival, steadily increased FVIII, s-selectin and D-dimer levels were associated with higher mortality. Conclusion: Hemostatic parameters show an association with disease state, prognosis, and the risk of VTE, not only at diagnosis, but also during the course of antineoplastic treatment.

Keywords: biomarkers, cancer, D-dimer; hemostasis; survival; venous thromboembolism.

Introduction

Tumor-associated venous thromboembolism (VTE) aggravates the clinical course of disease, worsens the survival prognosis [1], and is a common cause of death in cancer patients [2-4]. Malignancies increase the VTE risk fourfold to seven-fold. A peak within the first year after diagnosis can be observed [5], although VTE may occur at any time [6-7]. Cancer patients show systemic activation of coagulation and fibrinolytic, and elevated clotting factor consumption [8], which reflects a hypercoagulable state. Various hemostatic parameters are known to be predictive of VTE and survival in cancer patients [9-13].

In the Vienna Cancer and Thrombosis Study, significantly higher levels of soluble P-selectin (sP-selectin), D-dimer, prothrombin fragment 1 + 2 (F(1+2)) and fac-
Hemostasis biomarkers during treatment

Surgery within the last 2 weeks (except percutaneous implantation), an overt bacterial/viral infection or venous/arterial thrombomalous events within the last 3 months were exclusion criteria. Furthermore, patients receiving continuous anticoagulation with vitamin K antagonists or low molecular weight heparin (LMWH) were excluded. Patients receiving aspirin, ticlopidine, dipyridamole or LMWH at a thromboprophylactic dosage for a certain time (e.g. perioperative) were included.

At inclusion, data on the medical history were collected, and a first blood sample was drawn. Demographic and clinical details of tumor site, histology and stage were documented, and possible predisposing factors for VTE were recorded. Each patient received detailed written information on VTE symptoms. During the following observation period of a maximum of 280 days, up to seven further blood samples were taken monthly, and information regarding VTE occurrence and antithrombotic treatment was documented. If a patient did not return for the next blood sample, the patient, the relatives or the family doctor were contacted to obtain further information. Patients were also advised to report any VTE symptoms immediately.

Furthermore, we analyzed the effects of different treatment modalities on VTE occurrence, and investigated potential differences in the associations between various hemostatic parameters and survival over time.

Diagnosis of VTE

There was no routine screening for VTE. Whenever a patient developed VTE symptoms, objective imaging methods (e.g. duplex ultrasound and computed tomography) were performed to confirm or exclude VTE. In case of death within the observation period, death certificates and autopsy findings helped to show a possibly underlying fatal pulmonary embolism (PE). Every VTE was presented to an independent adjudication committee of specialists in the fields of angiology, radiology, and nuclear medicine, to assess and confirm the event and evaluate its clinical significance.

Blood samples

At inclusion and at each follow-up, venous blood samples were drawn by sterile and atraumatic venipuncture, and collected in plastic vacuum tubes (Vacuette; Greiner-Bio One, Kremsmünster, Austria), which contained 1 : 10 volume sodium citrate stock solution at 0.129 mmol L⁻¹ and in a Vacutainer K3-EDTA tube (Vacutette; Greiner-Bio One).

Blood samples were scheduled to be drawn at the following time points: (i) baseline, i.e. before any antithrombotic treatment (TP0); (ii) between day 14 and day 40 (TP1); (iii) between day 41 and day 70 (TP2); (iv) between day 71 and day 101 (TP3); (v) between day 102 and

Materials and methods

Study design

The study was conducted within the framework of the Vienna Cancer and Thrombosis Study, an ongoing prospective observational single-center study, and was approved by the Ethics Committee of the Medical University of Vienna.

Patients with newly diagnosed brain, lung, colorectal or pancreatic cancer were recruited at different departments of the Vienna General Hospital of the Medical University of Vienna between January 2011 and December 2012. None of the patients received chemotherapy or radiotherapy before study inclusion. Eligible patients were aged at least 18 years, and gave their written informed consent.

© 2013 International Society on Thrombosis and Haemostasis
day 131 (TP4); (v) between day 132 and day 162 (TP5); (vi) between day 163 and day 200 (TP6); and (vii) between day 201 and day 250 (TP7).

For further analyses, plasma samples were centrifuged at 3000 × g for 10 min to obtain platelet-poor plasma. The aliquots were stored at −80 °C until testing for sP-selectin and a thrombin generation assay (TGA) were performed in series. For determination of D-dimer and F$_{1+2}$ levels, plasma samples were stored at −20 °C until serial testing was performed. FGEN and FVIII levels were immediately measured at the Department of Laboratory Medicine.

**Laboratory analysis**

For D-dimer determination, a quantitative latex assay (STA-Liat est D-Di; Diagnostica Stago, Asnieres, France) was used on an STA-R analyzer (Diagnostica Stago). F$_{1+2}$ levels were determined with a quantitative ELISA (Enzymost F$_{1+2}$ monoclonal; Dade-Behring, Marburg, Germany). Thrombin generation was measured with an assay kit (Technothrombin TGA kit; Technoclone, Vienna, Austria), as previously described [15]. sP-selectin levels were measured with a human sP-selectin immunocassay (R&D Systems, Minneapolis, MN, USA), as previously described [27]. FVIII activity was determined on a Sysmex CA 7000 analyzer by using FVIII-deficient plasma (Technoclone) and activated partial thromboplastin time Actin FS (Dade-Behring). FGEN levels were measured according to Claus [28] (STA Fibri nogen; Diagnostica Stago).

The analytical interassay coefficients of variation were 4.9% for FVIII, 2.25% for sP-selectin, 15.3% for peak height thrombin, 3.98% for FGEN, 7.15% for F$_{1+2}$, and 3.09% for D-dimer.

**Statistical analysis**

The study was conducted as a hypothesis-generating study. Patients were recruited over a period of ~18 months, and it was assumed that strong effects should also be seen in a rather small cohort. Patients with at least one follow-up blood sample were included for the longitudinal analyses of the primary outcome variables FVIII, peak height thrombin, D-dimer, F$_{1+2}$, FGEN, and sP-selectin. As VTE treatment influences hemostatic parameters, values measured after VTE occurrence were not considered in our analyses. Because of the skew distribution, log-transformed values of FVIII, D-dimer, F$_{1+2}$, and sP-selectin were used for all statistical analyses. The variables peak height thrombin and FGEN were not transformed prior to statistical evaluations. Repeated measures ANOVA models were performed to test for statistically significant time effects on the parameter levels, and to evaluate differences between the various patient groups. Within these models, differences attributable to tumor type, stage, therapy response and treatment type were assessed. P-values resulting from multiple comparisons were adjusted with the Tukey method. To evaluate whether time effects were different for the various tumor types, an interaction term was considered within the ANOVA models, and subgroup analyses were performed for each tumor type in case of a statistically significant interaction. Statistically significant time effects were described by the estimated monthly relative changes (Δt) with 95% confidence intervals (CIs) in case of log-transformed outcome variables. For time effects on non-transformed outcome variables, the estimated monthly absolute changes (Δt) with 95% CIs were analyzed. Separate ANOVA models were performed for each of the outcome variables.

For analysis of the two key outcomes VTE and survival, all patients were included, independently of their number of follow-up blood samples. Univariate Cox regression models were used to evaluate the effects of the investigated parameters on VTE development and survival. The observation period started at study inclusion. Regarding VTE, the observation endpoint was fatal or non-fatal VTE; data were censored at death, end of the observation period after 250 days, or loss to follow-up. Regarding the endpoint death, data were censored at the end of the observation period or loss to follow-up. Every investigated parameter was considered as a time-dependent factor to be updated at the next time of blood sampling. Statistically significant effects are described by hazard ratios (HRs) with 95% CIs.

All P-values are results of two-sided tests, and P-values of <0.05 were considered to indicate statistical significance.

**Results**

**Patient characteristics**

Between January 2011 and December 2012, 112 patients with newly diagnosed brain (n = 39), lung (n = 41), colorectal (n = 15) or pancreatic (n = 17) cancer were prospectively enrolled. In total, 546 blood samples were drawn and analyzed. Three patients died before their first follow-up, and four patients did not return for a follow-up, owing to their poor overall condition. These seven patients (three with brain cancer, two with pancreatic cancer, and one each with colorectal and lung cancer) were only included in the analyses for VTE and survival. Whereas 105 patients were eventually included in the follow-up analyses.

The median age of all patients was 62.4 years (range: 21.3–80.3 years); 64 (57.1%) were males and 48 (42.9%) were females.

During the median follow-up period of 250 days, which refers to time to VTE and survival, respectively (minimum, 15 days; maximum, 250 days), a median of six blood sam-
samples (minimum, 1; maximum, 8) were collected. Because of restricted mobility, some patients did not attend consecutively. For these, some blood samples are missing. Table 1 shows the number of patients for each blood sampling time-point according to tumor type.

During the entire study period, all patients received chemotherapy treatment according to the tumor-specific regimens. As patients treated with platinum-based drugs are at relatively high risk of VTE, we distinguished two groups of patients: who had never received platinum-based drugs (n = 32), and patients receiving platinum-based drugs (n = 53).

Overall, 30 patients (47.6%) also received radiation and five (4.3%) received angiogenesis inhibitors. The baseline patient characteristics are summarized in Table 2.

**Differences in the longitudinally investigated parameters according to disease stage and therapy response**

For analysis of the influence of disease stage and therapy response, brain cancer patients were excluded. Among the 40 patients with lung, stomach or pancreatic cancer, 22 were diagnosed at an advanced stage (i.e., presence of distant metastasis), 24 had lymph node metastasis, and 23 had local disease only. sP-selectin levels were significantly higher in patients with distant metastasis than in those without (P = 0.005; median of means = 0.74 [95% CI 0.59–0.93]), and ΔΔΔ of means = 1.25 [95% CI 1.08–1.69], respectively). D-dimer levels also differed significantly during the follow-up period among the various disease stages: patients with distant metastasis had higher levels than those with lymph node metastasis (P = 0.002; median of means = 2.22 [95% CI 1.27–4.00]), or those without metastatic spread (P = 0.023; median of means = 1.81 [95% CI 1.01–3.23]). Concerning the other investigated parameters (FVIII, FGEN, F1 + 2, and peak height thrombin), no significant correlation with disease stage was found. Possible associations between complete remission and the investigated parameter levels were evaluated. Patients who experienced complete remission after the observation period (n = 36: eight brain, 16 lung, eight colorectal, and four pancreatic cancer) had significantly lower levels of F1 + 2 (ΔΔΔ of means = 0.82 [95% CI 0.59–0.96], P = 0.017), D-dimer (ΔΔΔ of means = 0.54 [95% CI 0.38–0.71], P ≤ 0.001) and FGEN (ΔΔΔ of means = -4.88 [95% CI -88.61 to -9.1], P = 0.014) over the time course than those without complete remission (Fig. 1). Levels of FVIII, sP-selectin and peak height thrombin showed no correlation regarding remission of disease (data not shown).

**Table 1** Number of patients at each time-point (TP) of blood sampling according to tumor type

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Brain (n)</th>
<th>Lung (n)</th>
<th>Colorectal (n)</th>
<th>Pancreas (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP0, day 0</td>
<td>39</td>
<td>41</td>
<td>15</td>
<td>17</td>
<td>112</td>
</tr>
<tr>
<td>TP1, days 1-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-40</td>
<td>30</td>
<td>34</td>
<td>19</td>
<td>9</td>
<td>82</td>
</tr>
<tr>
<td>TP2, days 41-70</td>
<td>22</td>
<td>32</td>
<td>11</td>
<td>18</td>
<td>78</td>
</tr>
<tr>
<td>TP3, days 71-100</td>
<td>25</td>
<td>16</td>
<td>11</td>
<td>9</td>
<td>61</td>
</tr>
<tr>
<td>TP4, days 101-150</td>
<td>23</td>
<td>24</td>
<td>5</td>
<td>9</td>
<td>61</td>
</tr>
<tr>
<td>TP5, days 151-200</td>
<td>30</td>
<td>22</td>
<td>7</td>
<td>10</td>
<td>58</td>
</tr>
<tr>
<td>TP6, days 201-250</td>
<td>22</td>
<td>19</td>
<td>6</td>
<td>9</td>
<td>56</td>
</tr>
</tbody>
</table>

Table 2 Baseline characteristics of the total study population (n = 112) and the patients who were followed during chemotherapy (n = 105)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.4</td>
</tr>
<tr>
<td>Sex (%)</td>
<td>21.3-0.3</td>
</tr>
<tr>
<td>Male (%)</td>
<td>64 (37.1)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>48 (42.9)</td>
</tr>
<tr>
<td>Follow-up period (days)</td>
<td>Median 250</td>
</tr>
<tr>
<td>Range</td>
<td>15-250</td>
</tr>
<tr>
<td>Tumor type, n (%)</td>
<td>Brain 39 (34.6)</td>
</tr>
<tr>
<td>Lung</td>
<td>41 (36.8)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>12 (10.9)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>17 (15.3)</td>
</tr>
<tr>
<td>Distant metastasis, n (%)</td>
<td>46 (41.3)</td>
</tr>
<tr>
<td>Previous VTE, n (%)</td>
<td>8 (7.3)</td>
</tr>
<tr>
<td>BMI ≥ 35 kg m⁻², n (%)</td>
<td>2 (1.9)</td>
</tr>
</tbody>
</table>

BMI, body mass index; CHT, chemotherapy; ESA, erythropoiesis-stimulating agent; sP-selectin, soluble P-selectin; VTE, venous thromboembolism.
sP-selectin levels also significantly decreased over time in brain cancer patients (Δrel per month = -0.56 [95% CI 0.94–0.58], P < 0.001). For the other investigated tumor entities, no significant change in sP-selectin levels over time was observed (Table 3a).

Peak height thrombin levels decreased over time in all tumor types (Δabs per month = -12.9 [95% CI -17.0 to -8.8], P < 0.001). Lung cancer patients showed significantly higher peak height thrombin levels than brain cancer patients (Δabs of means = 57.3 [95% CI 92-106.9]) and pancreatic cancer patients (Δabs of means = 75.7 [95% CI 107.7-149.8]) (P = 0.008 for both) (Table 3a).

FGFR levels differed significantly among various tumor types (P < 0.001); namely, between brain and lung cancer patients (Δabs of means = -111.2 [95% CI -161.5 to -61.0], P < 0.001), between brain and colorectal cancer patients (Δabs of means = -100.3 [95% CI -170.8 to -29.8], P = 0.002), and between lung and pancreatic cancer patients (Δabs of means = 91.6 [95% CI 22.5-160.2], P = 0.009). FGFR levels were highest in lung cancer patients, followed by pancreatic cancer patients, with the lowest levels being found in brain cancer patients (Table 3b).

Over the time course, F1+2 levels were significantly different between brain and pancreatic cancer patients (Δrel of means = 0.68 [95% CI 0.49-0.95], P = 0.016), with brain cancer patients showing significantly lower values (Table 3b).

We also analyzed D-dimer levels, and found significant differences over time between the various tumor types (P = 0.001): in lung cancer patients, D-dimer levels increased during follow-up (Δrel per month = 1.06 [95% CI 1.02-1.10], P < 0.001), whereas in pancreatic cancer patients, D-dimer levels significantly decreased over time (Δrel per month = 0.91 [95% CI 0.84-0.98], P = 0.049) (Table 3b).

Boxplots of the various parameter levels for the different time-points in all patients and depending upon tumor site are shown in Figs S1 and S2.

Also, the least square means over the total time period according to tumor type are shown in Table S1.

Differences in the investigated longitudinal parameters with respect to chemotherapy and radiotherapy

Brain cancer patients who did not receive platinum-based drugs were excluded before analysis of possible interactions between platinum-based drugs and the investigated parameters. As patients with colorectal or pancreatic cancer did not receive radiotherapy, they were excluded from this analysis.

Regarding different antitumor treatments, significantly higher FVIII levels were found in patients receiving platinum-based drugs than in patients receiving other chemotherapeutic agents (Δrel of means = 1.13 [95% CI 1.06-1.20], P < 0.001). Moreover, patients treated with platinum-based drugs showed increased F1+2 levels (Δrel of means = 1.10 [95% CI 1.00-1.21], P = 0.050). During radiotherapy, higher F1+2 levels were observed (Δrel of means = 1.12 [95% CI 1.01-1.24], P = 0.021). No further correlations could be found between treatment modalities (chemotherapy and radiotherapy) and the investigated parameters.

Outcome VTE events

Overall, 14 patients (12.5%) developed a symptomatic VTE during the observation period of 250 days. The cumulative incidence after 3 months was 6% (95% CI 3-13%), and that after 6 months was 11% (95% CI 7-20%). Seven of these patients had a deep vein thrombosis of the lower extremity, four had PE, and three had thrombosis at other venous sites (one each deep vein
<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (Q1-Q3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII total</td>
<td>210.0 (170.0-280.0)</td>
</tr>
<tr>
<td>Brain</td>
<td>270.0 (174.3-305.5)</td>
</tr>
<tr>
<td>Lung</td>
<td>184.5 (164.5-236.5)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>21.5 (171.0-221.0)</td>
</tr>
<tr>
<td>Colon</td>
<td>21.0 (171.0-210.1)</td>
</tr>
<tr>
<td>IP-10 total</td>
<td>3.5 (2.5-4.1)</td>
</tr>
<tr>
<td>Brain</td>
<td>2.9 (2.3-4.1)</td>
</tr>
<tr>
<td>Lung</td>
<td>3.4 (2.5-4.2)</td>
</tr>
<tr>
<td>Colon</td>
<td>3.6 (2.6-4.1)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3.5 (2.5-4.5)</td>
</tr>
<tr>
<td>TGF-beta total</td>
<td>208.5 (161.5-357.5)</td>
</tr>
<tr>
<td>Brain</td>
<td>330.0 (164.9-317.5)</td>
</tr>
<tr>
<td>Lung</td>
<td>300.0 (197.0-317.0)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>319.0 (226.5-317.0)</td>
</tr>
<tr>
<td>Colon</td>
<td>228.0 (155.4-312.0)</td>
</tr>
<tr>
<td>IP-10 total</td>
<td>4.0 (2.2-3.0)</td>
</tr>
<tr>
<td>Brain</td>
<td>3.7 (2.0-4.8)</td>
</tr>
<tr>
<td>Lung</td>
<td>4.7 (3.8-5.1)</td>
</tr>
<tr>
<td>Colon</td>
<td>4.8 (3.1-5.4)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3.8 (3.0-4.8)</td>
</tr>
<tr>
<td>TGF-beta total</td>
<td>352.0 (230.0-338.0)</td>
</tr>
<tr>
<td>Brain</td>
<td>130.0 (130.0-220.0)</td>
</tr>
<tr>
<td>Lung</td>
<td>231.0 (163.5-254.0)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>234.0 (192.0-246.0)</td>
</tr>
<tr>
<td>Colon</td>
<td>252.0 (195.0-328.0)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.6 (0.3-1.0)</td>
</tr>
<tr>
<td>Median (Q1-Q3)</td>
<td>1.0 (0.3-2.1)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.7 (0.3-1.0)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.9 (0.6-2.0)</td>
</tr>
<tr>
<td>Colon</td>
<td>1.5 (0.7-2.2)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.6 (1.0-1.9)</td>
</tr>
</tbody>
</table>

**Table 3:** Median and quartiles (lower [Q1] and upper [Q3] quartile) of the various parameter levels for the different time-points (TP) in all patients (total) versus according to tumor type.

- **TP0:** Median (Q1-Q3)
- **TP1:** Median (Q1-Q3)
- **TP2:** Median (Q1-Q3)
- **TP3:** Median (Q1-Q3)
- **TP4:** Median (Q1-Q3)
- **TP5:** Median (Q1-Q3)
- **TP7:** Median (Q1-Q3)

**FGEN, fibronogen; IP-10, soluble IP-10; TGF, thrombin generation assay.**
thrombosis of the upper extremity, thrombosis of the iliac vein, and thrombosis of the mesenteric vein). Regarding tumor size, 10.3% (n = 4) of all brain cancer patients, 9.8% (n = 4) of all lung cancer patients, 29.4% (n = 5) of all pancreatic cancer patients and 6.7% (n = 1) of all colorectal cancer patients developed VTE.

In Cox regression analyses (Table 4), an increase in FVIII level was significantly associated with VTE occurrence during the follow-up time period (HR per two-fold increase = 3.49 [95% CI 1.36–8.99], P < 0.01).

Increased sP-selectin levels also correlated with VTE occurrence (HR per two-fold increase = 2.44 [95% CI 1.21–4.51], P = 0.005), and so did increased D-dimer (HR per two-fold increase = 1.76 [95% CI 1.32–2.35], P < 0.001) and F1 + 2 (HR per two-fold increase = 2.11 [95% CI 1.47–3.03], P < 0.001) levels. No association of peak height thrombin and FGEN with VTE occurrence was found.

Boxplots of the various parameters in patients with and without VTE within 250 days are shown in Fig. 2. Figure 3 compares individual values of patients developing VTE within 250 days with the interquartile range of patients without VTE. Levels of the various biomarkers over the course of disease were associated with VTE occurrence. In those patients who developed VTE, the D-dimer level at the last blood sampling time-point before VTE was above the median in 13, and above the 75th percentile in seven of these 13. However, the individual course was highly variable, and there was no continuous increase in a specific parameter before VTE occurrence.

Outcome survival

Within the follow-up period of 250 days, 22 patients (15.6%) died: seven (31.8%) each with brain, pancreatic and lung cancer, and one (4.5%) with colorectal cancer.

In Cox regression analyses (Table 4), higher mortality was found to be associated with increased levels of FVIII (HR per two-fold increase = 4.43 [95% CI 1.85–10.60], P < 0.001), sP-selectin (HR per two-fold increase = 3.09 [95% CI 1.68–5.68], P < 0.001), and D-dimer (HR per two-fold increase = 1.60 [95% CI 1.23–2.07], P < 0.001).

No statistically significant associations with survival were found for the other investigated parameters. Figure 4 compares individual values of prognostic factors in patients who died within 250 days with the interquartile range of survivors.

Discussion

In the present longitudinal study, we investigated whether alterations over time of various hemostatic parameters in patients with brain, lung, colorectal or pancreatic cancer can be detected during anti-tumor treatment (chemotherapy and radiotherapy). Furthermore, we were interested in whether these parameters were associated with disease stage, prognosis and VTE occurrence during the first 250 days.

We found that sP-selectin and D-dimer levels were higher in patients with more advanced disease than in those with local disease. As we demonstrated, this was not only the case at diagnosis, as previously shown [29], but was also the case for the whole course of disease. Previously, D-dimer levels were reported to be significantly higher in lung cancer patients with metastatic disease, and in non-responders [30]. Over the course of disease, no associations with disease state of the other investigated parameters could be found. Von Tempelhoff et al. [31] also found no correlation between the International Federation of Gynecology and Obstetrics stage of ovarian cancer patients and FGEN levels over time.

In our study, patients who had complete remission had significantly decreased levels of F1 + 2, D-dimer, and FGEN. Previous retrospective studies have indicated that some hemostatic parameters are associated with remission at the time of diagnosis [32–34]. However, thus far, no longitudinal studies on this topic exist.

Our data indicate that, over time, brain cancer patients showed a decrease in FVIII and sP-selectin levels, whereas levels of peak height thrombin declined in all four tumor entities. Interestingly, we found significantly different levels of FGEN, F1 + 2 and D-dimer between the four tumor entities over the whole observation time. No data on the course of hemostatic parameters in

Table 4. Results of univariate Cox regression analyses

<table>
<thead>
<tr>
<th>Outcome VTE HR 95% CI P-value</th>
<th>Outcome survival HR 95% CI P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII* 1.40* 1.16–3.29 0.0006</td>
<td>FVIII 4.43* 1.15–10.60 0.0008</td>
</tr>
<tr>
<td>sP-selectin* 2.44* 1.31–4.53</td>
<td></td>
</tr>
<tr>
<td>TGA peak height 1.00 0.99–1.00 0.50</td>
<td>TGA 1.00 1.00–1.01 0.83</td>
</tr>
<tr>
<td>Fibrinogen 1.00 1.00–1.002 0.50</td>
<td>Fibrinogen 1.00 1.23–2.07 0.0004</td>
</tr>
<tr>
<td>F1 + 2* 2.41* 1.47–3.03 0.0001</td>
<td>F1 + 2 1.00 1.00–1.01 0.98</td>
</tr>
<tr>
<td>D-dimer* 1.76* 1.32–2.35</td>
<td>D-dimer 0.0008 0.08–2.26 0.17</td>
</tr>
</tbody>
</table>

CI, confidence interval; HR, hazard ratio; TGA, thrombin generation assay; VTE, venous thromboembolism. *Log_{10} transformed, which implies that the corresponding HRs refer to a two-fold increase in these variables.

© 2015 International Society on Thrombosis and Haemostasis
patients with brain cancer or pancreatic cancer were found in the literature. We surmise that these changes are attributable to an at least temporary response to antitumor treatment. Our data do not support the assumption that antitumor treatment itself fuels procoagulatory mechanisms, with the exception of platinum-based drugs, which seemed to increase FVIII levels and, moderately, also $F_1 + 2$ levels, as discussed in more detail below.

Certainly, for other tumor entities it has already been shown that hemostatic parameters change over the course of disease [31,35,36]. Nevertheless, because each cancer patient receives specific antitumor treatment, these changes could also be caused by the treatment itself.

We found a decrease rather than an increase in hemo-
static biomarkers, correlating with data from a prospective study conducted by Oerhoff et al., in which breast cancer patients had adjuvant treatment with tamoxifen. During the observed 6-month treatment period, decreases in D-dimer, fibrinogen and $F_1 + 2$ levels were found as compared with the baseline levels [37]. Moreover, Giudicini et al. observed alterations of hemostatic parameters during treatment in
children with acute lymphoblastic leukemia. Patients showed increased thrombin generation and higher D-dimer levels at diagnosis, before any chemotherapy had been started. Under treatment, D-dimer and F1.2 levels decreased, whereas D-D-dimer levels were observed to increase [35]. Another study in patients with ovarian cancer found a continuous decrease in F1.2 levels postoperatively after six cycles of chemotherapy with cisplatin, epirubicin, and cyclophosphamide [31]. Concerning anti-neoplastic treatment, we analyzed whether patients receiving platinum-based drugs showed significant differences in the investigated parameters longitudinally as compared with patients who had never received platinum-based drugs. We found that patients treated with platinum-based drugs showed higher F1.2 and FVIII levels. To the best of our knowledge, there are no longitudinal studies of parameters of hemostasis treated with cisplatin or carboplatin.

During radiotherapy, patients had increased F1.2 levels. Our results are also in line with the findings of a study conducted by Sender et al. in 45 rectal cancer patients. In that study, significant increases in F1.2 levels were observed when the levels at completion of long-term radiotherapy were compared with the baseline levels. Furthermore, a significant fluctuation of F1.2 levels was seen in patients treated with long-term low-intensity radiotherapy [38].

Furthermore, we found that patients who developed VTE had elevated FVIII, D-dimer, D-D-dimer and F1.2 levels over the entire observation period. Although, within the study period, VTE occurrence was associated...
with varying levels of the investigated biomarkers, individual courses were highly variable, and in our opinion it is rather questionable that monitoring of one of the hemostatic parameters would allow individual and practical risk prediction, either for VTE occurrence in general, or for the timepoint when VTE would be most likely to occur. One study investigated patients with multiple myeloma, and the authors found no association between alterations in FVIII:C and FGEN levels and VTE occurrence before and during treatment [39].

Our analyses also showed that significantly higher levels of FVIII:C, P-selectin and D-dimer were associated with shorter survival. This is in accordance with another study by Stender et al. [40], in which a positive preoperative D-dimer level correlated with survival in colorectal cancer patients without VTE, matching the data from our own group [41]. Moreover, van Marion et al. [39] performed a longitudinal study on multiple myeloma patients, and found that, before the start of chemotherapy, high levels of both FVIII:C and FGEN were significantly associated with mortality.

The main limitations of our study are the rather small number of patients included and the overall heterogeneous patient population. Furthermore, the loss to follow-up rate was rather high, which was caused by the poor prognosis and performance status of patients. Therefore, we were unable to perform separate analyses on antiangiogenic treatment or surgery, as the respective

© 2015 International Society on Thrombosis and Haemostasis
subpopulations were too small. However, performance of such a study is rather cumbersome for patients and laborious for the investigators. The difficulties and challenges connected with consecutive blood sampling in cancer patients with accurate documentation of all treatment modalities and events might be the reason why only few longitudinal studies are available in the literature. Our own study being by far the most extensive with regard to patient numbers and numbers of investigated time-points. To our knowledge, the present study is the first in which six different hematologic biomarkers, namely, FVIII, D-dimer, PGG2, sP-selectin, and peak height thrombus, were measured longitudinally in cancer patients, which is a real strength of our study.

In conclusion, the present longitudinal study provides novel data suggesting that, in newly diagnosed cancer patients, before the start of antithrombotic treatment, a procoagulant state exists. During the following months of antithrombotic treatment, the procoagulant state remains, but then tends to decrease in patients who have complete remission. Although levels of certain parameters (D-dimer, sP-selectin, and FVIII) were considerably increased before VTE occurred, it remains questionable whether continuous monitoring, rather than a single measurement, would be practical and improve individual VTE prediction and patient care.

Addendum

E.-M. Reiter was responsible for the acquisition of data, study concept and design, analysis and interpretation of data, drafting of the manuscript, statistical analysis, patient inclusion and management, and critical revision of the manuscript for important intellectual content. A. Kaider was responsible for the acquisition and interpretation of data, statistical analysis, and critical revision of the manuscript for important intellectual content. C. Ay was responsible for study concept and design, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. P. Quenlenberger was responsible for the acquisition of data and critical revision of the manuscript for important intellectual content. C. Marosi was responsible for patient inclusion and management, and critical revision of the manuscript for important intellectual content. C. Zielinski was responsible for critical revision of the manuscript for important intellectual content. I. Pabinger was responsible for the acquisition of data, study concept and design, analysis and interpretation of data, drafting of the manuscript, statistical analysis, patient inclusion and management, critical revision of the manuscript for important intellectual content, and supervision of the study.

Acknowledgements

We would like to thank T. Altreiter for proofreading the manuscript, and S. Koder for her assistance with laboratory analysis. The Vienna Cancer and Thrombosis Study was supported by a grant from the Jubiläumsfonds of the Austrian National Bank (project numbers 10935 and 12739).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Boxplots of (I) FVIII, (II) sP-selectin and (III) peak height thrombus at different time points (a) in all patients and (b) according to tumor entity.

Fig. S2. Boxplots of (I) fibrinogen, (II) F1 + 2 and (III) D-dimer at different time points (a) in all patients and (b) according to tumor entity.

Table S1. Least squares (LS) means of the various parameter levels (with 95% confidence intervals) over the total time period according to tumor type (ignoring possible interactions with time).

References

Curriculum vitae

Persönliche Daten

Staatsbürgerschaft: Österreich
Familienstand: ledig
Religionsbekenntnis: röm.-kath.

Ausbildung

September 1996 bis Juni 2004:
Neusprachliches Gymnasium Mater Salvatoris, 1070 Wien, dabei mehrjähriger Besuch der Physik- bzw. Chemieolympiade
Matura in Physik mit Fachbereichsarbeits- Thema: Optik in der Augenheilkunde

Oktober 2004 bis August 2010:
Studium der Humanmedizin an der Medizinischen Universität Wien
Diplomarbeit: New risk factors for retinal vein occlusion (Betreuer: Univ.-Prof. Dr. Christoph Prünte / Assoc. Prof. Priv.-Doz. Dr. Wolf Bühl, in Kooperation mit Univ.-Prof. Dr. Ingrid Pabinger-Fasching)

Seit Oktober 2010:
PhD-Studium an der Medizinischen Universität Wien, Universitätsklinik für Innere Medizin I

Seit April 2014:
Assistenzärztin an der Medizinischen Universität Wien, Universitätsklinik für Innere Medizin I
<table>
<thead>
<tr>
<th>Publikationen</th>
<th>IF</th>
</tr>
</thead>
</table>