Risk factors of thrombosis in cancer : the role of microparticles

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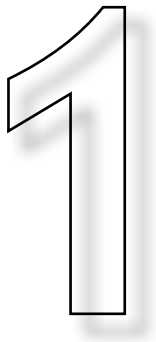


General introduction

General background

Cancer is known to be associated with venous thrombosis with a spectrum of clinical manifestations varying from deep vein thrombosis of the leg and pulmonary embolism, recurrent thrombophlebitits saltans et migrans (also called Trousseau's syndrome) to disseminated intravascular coagulation and arterial embolism. The link between activation of the blood coagulation system and malignancy dates back to 1823. Bouillaud described the first cancer patients with deep venous thrombosis (1) recently unearthed by Buller *et al*. (2). Later in 1865 it was Armand Trousseau, often considered to be the first, who more extensively described the high incidence of venous thrombosis in a group of patients with gastrointestinal carcinoma (3). Thereafter it has been extensively recognized that venous thrombosis occurs frequently in patients with malignancy (4-7).

The causes of venous thrombosis can be divided in environmental risk factors such as bed rest, surgery, plaster cast, trauma, long-distance travel, oral contraceptives or pregnancy and puerperium and genetic risk factors such as factor V Leiden and prothrombin 20210A mutation (8). Various mechanisms may contribute to the development of venous thrombosis in cancer patients, including inflammation due to necrosis or release of acute phase reactants and haemodynamic disorders such as stasis. Tumour-specific mechanisms include the ability of tumour cells to activate the coagulation cascade by several pathways. The tumour cells are able to interact with host blood cells, such as platelets, leukocytes and endothelial cells, by releasing inflammatory cytokines (IL-1, TNF and VEGF) or by direct cell-to-cell interactions. This may lead to down-regulation of anticoagulant and up-regulation of procoagulant proteins which may contribute to the general hypercoagulable condition of these subjects (9). Cancer cells themselves are able to produce a number of procoagulant substances including tissue factor, the initiator of the clotting cascade (10;11), and 'cancer procoagulant' (12). This way, tumour tissue can directly activate the clotting cascade, leading to thrombin generation and fibrin formation. Tissue factor and other proteins, such as thrombin, may favour clotting but also seem to be involved in the process of metastasis of the tumour cells (11).

The incidence of venous thrombosis in solid tumours varies in most clinical studies presented in current literature and is the highest in patients with pancreatic cancer (cumulative incidence up to 57%). Various studies have been initiated in Leiden in the context of collaboration between the departments of Clinical Epidemiology, Clinical oncology, Thrombosis and Haemostasis and other departments to further investigate the

relationship between cancer and thrombosis. Blom *et al*. (13) analyzed 3220 unselected patients with deep vein thrombosis or pulmonary embolism and 2131 controls of the MEGA study, and showed that cancer patients had a seven-fold increased risk of venous thrombosis, with a particularly high risk in patients with adenocarcinomas such as ovarian, lung, prostate and gastro-intestinal carcinoma. It has frequently been observed among clinicians that adenocarcinomas confer a higher risk of thrombosis than other types of solid tumours. However there are few data to support this notion. Estimations of the incidence of different histological types of cancer arising in the same organ have rarely been made. Up till recently only one study dealt with this issue: Blom *et al*. (14) demonstrated an increased risk of venous thrombosis in patients with adenocarcinoma of the lung versus squamous carcinomas of the lung.

Another risk factor of venous thrombosis frequently encountered in cancer patients, the use of indwelling central venous catheters, was further investigated in a prospective study in patients with haematological malignancies. Indwelling central venous catheters in patients with a malignancy were shown to be associated with an overall cumulative incidence of upper extremity thrombosis of 28.6% (15). Subsequently, other cohort studies were performed in Leiden in patients with solid tumours to unravel the relationship between cancer and thrombosis and further investigate the role of cellular fragments released in the bloodstream of cancer patients.

Hypercoagulability seems to contribute to the two most frequent causes of death in cancer patients, namely metastasis and venous thrombosis. Venous thrombosis often precedes the diagnosis of cancer (16), whereas thrombotic disease in cancer patients is associated with a detrimental course of disease. Local growth and metastatic behaviour of malignancies are influenced by clotting (17;18). Tissue factor, thrombin, fibrin, platelets and other haemostatic components can all play a role in tumour progression. Chemotherapy with or without the use of central venous catheters and hormone therapy may further impair the haemostatic balance by causing alterations of the blood vessel wall or by affecting levels of proteins involved in the coagulation cascade (19).

The poor prognosis of cancer patients with thrombosis as compared to those without supports the hypothesis that the local or systemic hypercoagulability state confers a growth advantage to tumour cells or more aggressive tumours are more likely to have a hypercoagulability state. Although abnormal coagulation profiles, *e.g.* elevated clotting factors and thrombocytosis, are frequently found in cancer patients, not all patients with such abnormalities develop venous thrombosis. The mechanism by which tumour

predispose to thrombosis has not been elucidated yet, although recently new risk factors have been identified and hypothesis-generating findings have been reported which give more insight in the relationship between cancer and thrombosis.

Epidemiology and risk factors for venous thrombosis in patients with cancer

Two large population-based studies demonstrated that the incidence of a diagnosis of cancer is increased particularly in the first year following the diagnosis of thrombosis (20;21). In these studies by Baron *et al.* (21) and Sörensen *et al.* (20) the authors examined data from both cancer and thrombotic disease registries and calculated standardized incidence ratios (SIR) for cancer, separately for patients with either deep vein thrombosis or pulmonary embolism. In these studies, patients with a venous thrombotic event were more likely to be diagnosed with cancer, especially of lung, pancreas and brain. Others examined the incidence of thrombosis in cancer patients (7;13;22). In the 1980s, the frequency of venous thrombosis in cancer patients was estimated to be approximately 15%, depending on the type of primary tumour (23). However, more recently, cumulative incidence of 8% have been reported which may reflect the more widespread use of thrombosis prophylaxis in modern practice (7). In these studies solid tumours, such as stomach, kidney, pancreatic, brain and ovarian cancer were most strongly associated with thrombosis.

Patients with adenocarcinomas, especially arising from the gastrointestinal tract such as pancreatic carcinoma, are believed to have the highest risk of developing venous thrombosis (13). Although patients with mucin-producing adenocarcinomas are most likely to develop thrombosis, the most frequent types of cancers found in patients with thrombosis are those most prevalent in the population as reported by Levitan et al (22) and Blom *et al.* (13), in which particular patients with lung cancer, gastrointestinal and urological malignancies were frequently found to develop thrombosis

Immobilisation

As stasis is the major cause of thrombosis, the risk is of venous thrombosis is increased in all circumstances associated with immobilisation, such as bed rest, plaster cast and long-distance travel (24;25) due to the interference with the function of the calf musculature in pumping the blood upstream through the veins. Cancer patients may be less mobile

due to their illness or admission to the hospital. Shen and Pollack (26) reported death caused by pulmonary embolism and confirmed by autopsy in 14% of patients with cancer as compared to 8% of patients without cancer who all died in the hospital.

Surgery

Surgical interventions carry a high risk of venous thrombosis, depending on the type of surgery. The highest risks are orthopaedic surgery and neurosurgery. In generally, the larger the intervention, the greater the risk, but in orthopaedic surgery even minor interventions such as arthroscopy affect the risk of venous thrombosis. Due to routine use of anticoagulant prophylaxis, symptomatic venous thrombosis have declined from up to 50% to around 3% (27;28). The incidence of postoperative deep-vein thrombosis is about two times higher in patients with cancer than in patients without cancer (29;30). The risk of developing a fatal pulmonary embolism postoperatively is 3-fold increased in cancer patients as compare to those without cancer. Factors contributing to this high incidence are advanced age, long and complicated surgical procedures and delayed mobilisation plus prolonged postoperative hospitalisation due to the patient's poor condition. If thrombosis prophylaxis is not extended for four weeks after surgery for cancer, patients with cancer remain at risk of developing late venous thrombosis (31;32).

Anti-cancer agents

Systemic treatment for a number of malignancies has changed dramatically during the last years resulting in reduced morbidity and mortality in cancer patients with the introduction of new classes of anti-cancer agents. Examples of these are anti-hormonal agents, chemotherapeutics and angiogenic inhibitors. Despite the high cytotoxicity of these agents on tumour cells, vascular damage, such as venous and arterial thrombosis, stroke and pulmonary embolism are frequently reported in patients using these agents.

The vascular endothelium plays an important role in the regulation of vascular tone, haemostasis, immune and inflammatory responses by secretion of regulatory factors. The three most important endothelial-derived substances are nitric oxide (NO), endothelin (ET-1) and prostacyclin (PGI2). NO and PGI2 act as vasodilators, whereas ET-1 serves as a vasoconstrictor. Vascular damage causes an alteration in the formation and release of these endothelial factors, resulting in less production of NO and PGI2. This causes a constant adrenergic vasoconstrictor tone, which can lead to increased vascular tone and vasospasm. Furthermore, decreased production of the two endothelial factors NO and

PGI2, can lead to increased platelet adhesion and aggregation and therefore enhance vascular disorders including thrombosis.

Estimates of the incidence of thrombotic complications in patients undergoing chemotherapy originate from controlled clinical trials of systemic therapy in women with breast cancer (33;34). The frequency of chemotherapy-induced thrombotic complications in women with stage II breast cancer undergoing chemotherapy was on average 7% in studies assessing this risk, compared to 0.8% in those with no adjuvant treatment (35). Among patients with stage IV breast cancer the risk was even higher (23). Hormone therapy combined with chemotherapy further increased the risk of thrombotic complications in women with breast cancer (33). When tamoxifen is given as chemoprevention in women at high risk, or as monotherapy in breast cancer patients after removal of the primary tumour to prevent recurrence of breast cancer, the risk of venous thrombosis is slightly increased (36;37). By comparison hormone therapy with third-generation aromatase inhibitors is associated with a lower rate of thrombotic events (38).

With the development of new drugs such as vascular endothelial growth factor inhibitors and other targeting specific molecules, the incidence of thrombosis in patients with solid tumours seems even to have increased. Thrombosis frequencies of 30-40% have been reported in patients with multiple myeloma and renal cell carcinoma when thalidomide was given in combination with chemotherapy (39;40). In early-phase clinical trials, new antiangiogenic agents have been associated with an unexpectedly high risk of thrombotic complications (41). Confirmation of the association between venous thrombosis and this new generation drugs awaits analysis of larger cohorts of patients.

Central venous catheters

Central venous catheters (CVCs) are frequently used in patients for a variety of indications such as cancer treatment, diagnostic monitoring, and parenteral nutrition, and are of great comfort in the management of patients with cancer (42). The benefit derived from CVCs may be offset by venous thrombosis and associated complications, such as pulmonary embolism, CVC dysfunction, infection or loss of central venous access. In the long term, patients with venous thrombosis may suffer from a post-thrombotic syndrome. The occurrence of catheter-related thrombosis differs but is especially high in cancer patients undergoing chemotherapy (43;44). The incidence of deep-vein thrombosis in patients with a central venous catheter varies considerably in the literature. Bern and colleagues (45) found that in the absence of thromboprophylaxis, the rate of catheter-related thrombosis as demonstrated by phlebography was 37%, whereas Monreal et al.

(46) found an even higher rate. In studies in which ultrasonography was used to detect catheter-related thrombosis (47) in symptomatic patients, but also in a study by Verso et al (43) who used venography at fixed time points, a much lower rate of thrombosis has been reported. Van Rooden et al (15) performed a prospectively controlled clinical study and demonstrated that Doppler-ultrasound screening may be useful to identify those patients that are at high or low risk for clinically manifest CVC-related thrombosis. The lower sensitivity of radiological non-invasive methods to detect venous thrombosis compared to phlebography, as well as the differences in material and coating of catheters used nowadays, plus the introduction of new procedures to reduce vessel damage during placement of the catheter, are likely to account for the discrepancies in studies on the incidence of catheter-related thrombosis.

Platelets and thrombocytosis

Under normal physiological conditions, endothelial cells prevent platelets from binding to the vascular wall and thereby preserve blood flow. In pathological circumstances and perhaps during chemotherapy-induced vascular damage, the resistance of the vascular wall to platelet binding is disturbed. Once several platelets adhere to the vascular wall, increased platelet adherence and production of a fibrin clot begins. Furthermore, Brock and colleagues (48) reported that VEGF can induce endothelial cells to release Von Willebrand factor, which is involved in adhesion of platelets. The increased platelet-binding capacity of the tumour vasculature and the subsequent activation of platelets are regulated by stimuli of the tumour cells and may differ for each tumour type Platelets may adhere to tumour vessels, form microthrombi, and release granules that contain VEGF, platelet-derived endothelial cell growth factor, and platelet-derived growth factor, together with inhibitors, such as thrombospondin and platelet factor 4. Activated platelets as well as stimulated endothelial cells express P-selectin (CD62P), a member of the selectin family of cell adhesion molecules. P-selectin-mediated cell adhesive interactions seem critically important in the inflammatory processes but also in the pathogenesis of thrombosis and the growth and metastasis of cancers. P-selectin is involved in the interaction with P-selectin glycoprotein ligand-1 (PSGL-1, CD162), and is responsible for leukocyte rolling on stimulated endothelial cells and heterotypic aggregation of activated platelets onto leukocytes. Cross-linking of PSGL-1 by P-selectin also induces production of cytokine and chemoattractant-induced beta2-integrin in leukocytes required for activation and adhesion of leukocytes. Furthermore, P-selectin mediates aggregation of activated platelets to cancer cells and adhesion of cancer cells to stimulated endothelial cells.

Tissue factor and other prothrombotic factors

Tumour cells can express several procoagulant factors, including tissue factor(TF). The activation of coagulation leads not only to the development of venous thrombosis, but might also be related to enhanced tumour growth and angiogenesis (52). TF plays a central role in the hypothesis that clotting and tumour growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. TF is a transmembrane receptor compromised of a 219-amino-acid extracellular domain, a 29-amino-acid hydrophobic transmembrane region and a 21-amino-acid intracellular tail. Binding of factor VIIa to the extracellular domain of TF initiates the extrinsic pathway of coagulation on the cell surface membrane and activates signalling through the MAPK pathway. Under physiological condition, TF expression is tightly controlled; the factor is normally not expressed but inflammatory cytokines or endotoxin can induce the expression of TF on monocytes, macrophages and endothelial cells. In malignant cells, however, TF has been shown to be expressed. In several types of cancers, including breast cancer, colorectal cancer and pancreatic cancer, elevated TF expression on tumour cells correlates with tumour grade and tumour progression (10;11;53). Wojtukiewicz and colleagues (54) showed marked expression of TF, prothrombin and fibrinogen in situ in pancreatic cancer, while expression of the anticoagulant and antiangiogenic protein tissue factor pathway inhibitor and plasminogen activators as assessed by immunohistochemical staining was minimal suggesting that local conditions favour the process of coagulation and angiogenesis.

Tumour-specific prothrombotic properties contribute to tumour growth and dissemination. The formation of thrombin and production of fibrin, the final product of the activation of blood coagulation, are coagulation-dependent mechanisms of tumour progression. In addition, tumour prothrombotic properties can interfere with the malignant process by coagulation-independent mechanisms. TF also has other functions, including the ability to modulate vascular endothelial growth factor (VEGF) expression by malignant cells and normal vascular cells. Tissue factor thus seems to play an important role in various processes and is important with respect to tumour neovascularisation. Tissue factor may provide a link between coagulation, inflammation, and thrombosis and cancer growth and paraprotein metastasis (55).

By contrast, ‘cancer procoagulant’, a cysteine proteinase that directly activates factor X independently of factor VII, has been found in tumour cells and in tissues of the amnion and chorion but not in normal, differentiated cells. The finding that the classical

severe coagulopathy in acute promyelocytic leukaemia patients which seems mediated by leukaemia blast-cell procoagulant activities resolves in parallel with disappearance of the malignant of leukaemia cells following treatment supports the role of tumour procoagulants in promoting clotting complications in malignant disorders (56).

Tumour cells can express proteins that regulate the fibrinolytic system, including the urokinase-type and tissue-type plasminogen activators, plasminogen-activator inhibitors 1 and 2, and plasminogen-activator receptor (57). The increase in plasma concentrations of plasminogen-activator inhibitors and impairment in plasma fibrinolytic activity in patients with solid tumours indicate a new tumour-associated prothrombotic mechanism.

Tumour cells induce platelet activation and aggregation by direct cell–cell contact or by release of soluble factors, such as ADP, thrombin, and other proteases (58). Upon activation circulating platelets expose on their surface P-selectin, whereas they release their granule contents upon aggregation. Activation of platelets thus enhances their capacity to interact by specific adhesive mechanisms with endothelial cells, leucocytes, and tumour cells.

Tumour cytokines

Tumour cells produce and secrete a number of different cytokines, including TNFα, interleukin 1ß, and VEGF, which may be involved in the development of thrombotic disorders in patients with cancer (59). The major targets of these cytokines are the vascular endothelium and leucocytes. TNFα and interleukin 1ß induce the expression of endothelial procoagulant activity and simultaneously down-regulate the expression of thrombomodulin, the endothelial surface high-affinity receptor for thrombin, which complexes thrombin to activate the potent anticoagulant protein-C system. Together, up-regulation of TF and down-regulation of thrombomodulin lead to a prothrombotic condition in the vascular wall. The same cytokines stimulate the production of the fibrinolysis inhibitor plasminogen-activator inhibitor-1 (PAI-1), thus impairing the endothelial antithrombotic response. The release of VEGF by tumour cells may account for the increased microvascular permeability found in a variety of tumours. VEGF is a chemotactic for macrophages and also induces expression of tissue factor by endothelial cells and monocytes, which implies involvement of tissue factor in tumour neovascularisation (60). Finally, cytokines induce changes in expression of endothelial-cell adhesion molecules, thus increasing the capacity of the vessel wall to attract leucocytes and platelets and promoting local activation of clotting and formation of fibrin.

Tumour cells also have the ability to interact with the monocyte-macrophage system to express TF on their surfaces (61;62). Mononuclear phagocytes do not express TF under resting condition, but can generate this in response to various stimuli, including bacterial endotoxines, immune complexes and lymphokines (61). Tumour-associated macrophages obtained from experimental and human tumours express substantially more TF than control cells. Tumour cytokines also attract and activate polymorphonuclear leucocytes, which release reactive oxygen species and intracellular proteases that have several activities on endothelial cells and platelets, tipping the haemostatic balance towards a prothrombotic state (63).

Tumour Cell-Host cell interactions

Tumour cells can interact with the vascular endothelium by both direct and indirect mechanisms. The presence of cell-adhesion molecules on the surface of tumour cells allows them to interact with normal cells and during the process of haematogenous spread interaction may occur with endothelial cells, platelets, and leucocytes. The capacity of tumour cells to adhere to both resting and stimulated endothelium is well known (49) and adhesion-molecule pathways specific to different tumour-cell types have been identified. Malignant cells attached to the vessel wall promote localized clotting activation and thrombus formation and promote the adhesion and arrest of leucocytes and platelets by releasing cytokines. Cancer cells also directly activate platelets, adhere and migrate through the vessel wall, and are assisted by polymorphonuclear leucocytes in their interaction with endothelial cells.

Mucins and adenocarcinomas

Mucins are large glycoproteins with clustered O-linked glycans (64). Cancer cells frequently upregulate the expression of a variety of mucin polypeptides. These are often carriers of sialylated fucosylated sulfated glycans and may act as ligands for the selectin family of adhesion molecules (65). Such selectin-mucin interactions are implicated in the haematogenous spread of tumour cells (65-67). Trousseau syndrome is most commonly observed in patients with mucin-producing adenocarcinomas, in which mixtures of aberrant mucins shed in significant amounts by cancer cells circulate in the bloodstream (68;69). Such circulating tumour-derived mucins are used as prognostic markers in the clinic. It has been hypothesised that circulating mucins are directly involved in the pathogenesis of Trousseau syndrome. Indeed, some early studies suggested a procoagulant

role for mucins (70;71). L-, P-, and E-selectins comprise a family of carbohydrate-binding adhesion molecules expressed by leukocytes, platelets, and vascular endothelium (72). L-selectin is constitutively expressed on neutrophils, monocytes, and naïve lymphocytes. P-selectin is stored in secretory granules of resting platelets and endothelium and is rapidly translocated to the cell surface upon activation. E-selectin is newly synthesized in endothelial cells via transcriptional activation initiated by various proinflammatory agonists. While all three selectins recognise structurally related ligands containing sialic acid and fucose residues, optimal ligand formation for L- and P-selectin also requires sulfate esters (72). Many experiments have shown that heparin can inhibit P- and L-selectin recognition of ligands (73;74) and that heparin blockade of tumour metastasis is at least partly explained by selectin inhibition, rather than by its anticoagulant activity (66;67). P-selectin interactions with circulating carcinoma mucins may be involved in Trousseau syndrome as cancer mucins may act as templates to aggregate activated platelets via P-selectin. In the study performed by Wahrenbrock *et al.* (64) in which TF-free cancer mucins were administered intravenously to mice, platelet-rich microthrombus formation was observed which was dependent not only on P-selectin but also on leukocyte-derived L-selectin. Furthermore, microthrombus formation could occur independently of thrombin generation. Similar findings were obtained using in vitro studies with whole blood. These authors are the first to explain the classical microangiopathy of Trousseau's syndrome in patients with mucin-producing adenocarcinomas and indicate to the superiority of therapy with heparins over vitamin K antagonists in such patients.

Microparticles

Already in the 1940s it was known that plasma containing platelets clotted faster than platelet-poor plasma. High-speed centrifugation of platelet-poor plasma prolonged the clotting time, implying the presence of a subcellular fraction in platelet-poor plasma (75). In 1967, Wolf *et al.* (76) demonstrated the presence of a factor which he though to originate from platelets and called "platelet dust". Subsequently it became apparent that platelets release small vesicles, now called microparticles (MP) upon stimulation. MP are small membrane vesicles reported to range in size between approximately 100 and 1000 nm, which are released from cells upon activation or during the process of apoptosis. Circulating blood cells as well as endothelial cells are able of releasing these small membrane vesicles, which express on their surface some of the antigenic markers distinctive of the cell of origin. For a long time, MP were considered to be cellular debris

reflecting cellular activation or damage, but there is now increasing evidence that these MP interact with other cells and may acquire a pathophysiologic potential. There are several lines of evidence supporting the procoagulant activity of MP. Platelet-derived microparticles generated in vitro in response to stimuli have demonstrable haemostatic properties which include the binding of factors Va (77), VIII or IXa (78) as well as the prothrombinase complex (79) and tissue factor (80;81). Although the precise role is still unknown, MP isolated from various patient populations support in vitro coagulation (82-85) and several small studies have demonstrated that MP levels are elevated in individuals suffering from thrombotic events (86;87). Lastly, human MP injected into rats were highly thrombogenic, and this effect was abolished by pre-incubation of MP with anti-tissue factor antibodies (81). On the other hand in patients with a hereditary bleeding disorder, known as Scott syndrome, impaired membrane vesiculation leading to decreased numbers of MP has been found (88).

Methods widely used to detect MP

Flow cytometry is widely used to characterize cell-derived MP although the accuracy to assess the size of MP less than 488 nm is a matter of discussion. MP can be isolated from cell-free plasma before labelling with different antibodies. By fluorescently-labelled antibodies or annexin V, antigens and phosphatidylserine exposed on the MP surface can be detected and quantified by flow cytometry. However, a wide variety of methodologies are used by different laboratories, precluding direct comparison in some cases and which may result in inconsistent or conflicting data (89). Major differences exist in the preparation of the MP samples, for instance the mode of centrifugation and cell lineage-specific antigenic markers. These differences probably account for some of the different findings among groups using different methodologies. More recently, various groups are exploring the use of other techniques to detect and quantify number and characteristics of MP isolated from blood of different individuals.

Source of tissue factor expressed by MP

Platelets are known to be able to endocytose numerous plasma proteins. TF has been demonstrated on platelet-derived MP by flow cytometry, and it has been debated whether leukocytes or platelets are the source of this TF, as platelets are considered not to express TF. Rauch *et al*. (90) had demonstrated that a monocytoid cell line can transfer TF to activated platelets via MP, whereby platelets-derived MP became TF-positive. Recently,

platelet-derived MP isolated from human blood stimulated in vitro have been found to express TF procoagulant activity, suggesting that in vivo these MP may carry TF to different sites and initiate coagulation locally or at distant site (91). In vitro activated platelets and platelet products may also induce TF-activity in other cells such as monocytes (92). Platelets as well as platelet-derived MP have also been shown to transfer to monocytes, and this may well be one of the most efficient and important mechanisms involved in decrypting TF activity *in vivo*. The negatively charged phosphatidylserines expressed on MP are thought to provide the catalytic surface facilitating thrombin formation. The assumption that platelets themselves do not produce tissue factor has been a matter of intense debate, but the discussion was recently fuelled by the discovery that quiescent platelets can express TF pre-mRNA and -following their activation- splice TF pre-mRNA into mature mRNA. This is associated with increased TF protein expression procoagulant activity and accelerated formation of cloths (93).

Other experimental animal studies of laser-induced vessel wall injury (94), revealed a critical role for P-selectin in the recruitment of TF to the thrombi and the subsequent generation of fibrin. Interestingly the TF present in these thrombi did not come only from the blood vessel wall, but also from blood-borne TF associated with cell-derived MP in the circulation.

Almost 25 years ago it was observed that the cell free supernatants taken from tumour cell lines contained procoagulant activity. Dvorak *et al*. (95) demonstrated that this procoagulant activity was lost following ultra-centrifugation and recovered following resuspension of the pellet. By using electron microscopy, these pellets were noted to be composed of membrane-derived vesicles. The procoagulant behaviour of these MP was consistent with the presence of TF (96). Since then, it has been demonstrated that numerous other tumour cell lines are able to form MP which apparently carry procoagulant activity (97), as did blood samples taken from patients with leukaemia but not healthy controls (98). In 1987, Bona *et al*. observed the transfer of cytoplasmic TF to plasma membrane and ultimately to membrane vesicles shed from promyelocytic leukaemia cells (99). More recently, it was demonstrated that human cancer cell lines shed vesicles containing intact tissue factor. The quantity of MP-associated TF correlated directly with production of tissue factor by cancer cells (100). Although the initial observation of an association between MP and malignancy were made in the early 1980s, in 2007 we could demonstrate the association between cancer-associated thrombosis and TF-bearing MP in cancer patients (100).

Thrombosis profylaxis studies: anticoagulants and metastasis

In current clinical practice, the initial therapy of venous thrombosis in cancer patients consists of low-molecular-weight heparin (LMWH) followed by oral anticoagulation with coumarin derivatives (vitamin K antagonists). The safety and efficacy of oral anticoagulants are critically dependent on maintaining the international normalized ratio (INR) within the target range during treatment. The narrow therapeutic window requires that the anticoagulant effect be carefully monitored with regular laboratory testing.

Insufficient data are available from cancer patients to determine the optimal duration of secondary prophylaxis. In the absence of data from clinical trials, the general view is that following an initial thrombotic event thrombosis prophylactic therapy should be continued indefinitely in patients with cancer, or certainly for as long as the cancer is active and patients are treated with anti-tumour therapy (101). Cancer patients with thrombosis are at an increased risk of recurrence compared to patients with thrombosis without cancer. The post-hoc analyses of data from two multi- centre, randomized clinical trials has shown an increased risk of recurrent venous thrombosis among cancer patients (102). In that analysis, the incidence of recurrent thrombosis among patients receiving oral anticoagulant therapy for 3 months was 27 per 100 patient-years for cancer patients, compared with 9 per 100 patient-years for patients without cancer, while the cancer patients were also at a greater risk of anticoagulant-associated bleeding than patients without cancer.

Several trials have compared LMWHs with oral anticoagulants as long-term prevention of secondary venous thrombosis, but the use of anticoagulants were generally of short duration and did not focus on patients with cancer only. The studies consistently found no difference in the rate of thrombosis between LMWH and oral anticoagulant therapy. The CLOT trial was the first large-scale study to investigate whether in patients with cancer secondary prophylaxis with a LMWH would be a useful alternative to long-term oral anticoagulant therapy (103). In this study 676 patients with cancer and symptomatic venous thrombosis were randomised to receive either once-daily LMWH, followed by oral anticoagulant therapy for 6 months, or LMWH alone for 6 months. During the 6-month study period, the incidence of recurrent thrombosis in patients treated with LMWH alone was about half that observed in patients allocated to long-term oral anticoagulant therapy (8.1% *versus* 16.0%, respectively). Importantly, the incidence of major bleeding in the two groups was not different. Furthermore, the risk of recurrent VT at 6 months was only 9%

in the LMWH only group, compared with 17% in the oral anticoagulant group. Although the long-term self-injection of dalteparin in this study was acceptable it is likely that this diminishes the patients' quality of life as compared to the use of oral anticoagulants.

Numerous animal studies have demonstrated that heparins may exert an anticancer effect by interaction with the process of coagulation and other mechanism. Such preclinical studies demonstrating anti-tumour effect of heparins including LMWHs formed the basis of various clinical studies. The potential antitumour effect of LMWH may be of particular importance in less advanced disease. In view of this hypothesis, a post-hoc analysis of the CLOT results was performed to determine whether a treatment-related difference in mortality existed between patients with metastatic or non-metastatic solid tumours (104). At 12-month follow-up, 70% of the patients with metastatic disease had died and there was no difference in mortality between the two treatment groups. In contrast, among those with non-metastatic disease at entry to the study, the 12-month cumulative mortality was 20% for those in the LMWH group compared with 35% in the oral anticoagulant group. This difference in mortality among patients with non-metastatic disease at randomization could not be attributed to a difference in fatal PE between treatment groups and is consistent with the theory that LMWHs may exert clinically relevant antineoplastic effects in non-metastatic cancer. The findings of the post-hoc analysis of the CLOT data are consistent with the results of a sub analysis of the FAMOUS trial. In this randomized placebo-controlled trial of LMWH therapy in patients with advanced solid tumours without evidence of underlying thrombosis, with the aim of determining the effect of LMWH on survival at 1 year. With regard to the primary endpoint of the study LMWH administration did not improve 1-year survival rates in patients with advanced malignancy. The authors subsequently calculated the survival in a group of patients (not defined a priori) with a better prognosis and who survived more than 17 months. Based on this estimation there was a survival advantage for the LMWH group, with survival estimates at 2 and 3 years after randomization of 78% respectively 60% for the LMWH group and 55% and 36% for the placebo group. The median survival time in the LMWH group was 43.5 months (95% CI, 33 to 52.3 months) compared with 24.3 months (95% CI, 22.4 to 41.5 months) in the placebo group. There was no difference in bleeding rates between the two groups. Although in general this type of statistical analysis based on selection of study outcome should be avoided, the data seem to support that LMWH indeed has a long-term favourable effect on tumour cell biology that results in improved survival of patients having a good prognosis (105). Another study of Klerk *et al.* (106),

showed that a 6-week course of LMWH favourably influenced the survival and prolonged median survival from 6.6 to 8.0 months. Altinbas et al reported improved tumour response rates and survival in patients with small cell lung cancer randomized to receive LMWH and combination chemotherapy compared with chemotherapy alone (107).

In conclusion

Venous thrombosis is a common complication of cancer and is causally associated with the generation of a hypercoagulable state. Clinical manifestations of venous thrombosis in cancer include deep venous thrombosis, pulmonary embolism, disseminated intravascular coagulation, and Trousseau's syndrome. Cancer cells activate platelets and express several procoagulant factors, including tissue factor and thrombin. The activation of coagulation might also be related to enhanced tumour growth and angiogenesis. Various factors may contribute to the development of venous thrombosis in cancer patients, and circulating mucins as well as circulating microparticles which express active TF on their surface may provide a missing link between cancer and thrombosis in (adeno) carcinoma patients.

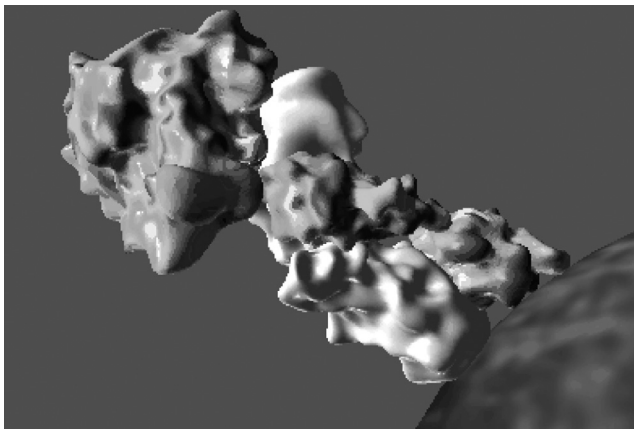
Treatment options include vitamin K antagonists and low-molecular-weight heparins, and the long-term use of these heparins in prevention of venous thrombosis may improve the outcome in comparison with oral anticoagulants. Further research is needed to better understand the morbidity and mortality associated with thrombosis in cancer patients and to optimise strategies of prevention and treatment.

Outline of the thesis

This thesis elaborates the occurrence of venous thrombosis in cancer patients. In **Chapter 1** a general introduction of the relation between cancer, thrombosis and metastasising behaviour of tumour cells is given. In **Chapter 2**, the use of thrombosis prophylaxis in cancer patients with a long-dwelling central venous catheter is discussed based on the experience in a single centre. **Chapter 3** is a review of the problems of catheter-related thrombosis related to the use of long-dwelling central venous catheter in the general practice of the medical oncologist and the different ways how doctors deal with these issues. The aim of this chapter is to describe incidence and risk factors, complications, prevention and treatment of catheter-related thrombosis for clinicians.

Although the belief that patients with mucin-producing adenocarcinomas are more likely to develop thrombosis is widespread, strikingly few papers have been published on the incidence of venous thrombosis in patients with different histological types of cancer. In **Chapter 4** a review of venous thrombosis in lung cancer, the tumour type with the highest incidence worldwide, is presented. The results of an analysis of the incidence of thrombosis in patients with gastrointestinal tract carcinomas are described in **Chapter 5**. In this analysis the concept that adenocarcinomas compared to other histological types are associated with a higher risk for venous thrombosis is investigated, in a cohort of 1000 patients with carcinomas (adenocarcinoma and squamous cell carcinoma) originating from the upper gastro-intestinal tract .

Chapter 6, **7** and **8** deal with the results of preclinical work investigating the pathogenesis of thrombosis in cancer patients. In Chapter 6 we discuss the role of tumour-derived microparticles and microparticle-associated TF-activity in the development of cancer-related thrombosis in a cohort of advanced and early stage disease breast and advanced pancreatic cancer patients in comparison to MP and MP-associated TF activity in healthy subjects and individuals who present with thrombosis but who are not known to have cancer. In **chapter 7** differences in microparticle-associated TF-activity in patients with different cancer types and who did or did not develop thrombosis are presented. In **Chapter 8** circulating MP with TF activity in relation with venous thrombosis and expression of TF on the tumour tissue in a cohort of 55 pancreatic cancer patients are presented. The results of this thesis are summarized and discussed in **Chapter 9**, whereas **Chapter 10** concerns the Dutch summary.



Tissue factor

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2 Risk factors for catheter-related thrombosis in cancer patients

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Abstract

We investigated the risk factors for venous thrombosis in cancer patients with implantable ports undergoing chemotherapy. One hundred and seventy one ports were placed in a central ("chest ports") and 84 in a peripheral vein ("arm ports"), 181 received prophylactic nadroparin and 10 coumarin. Clinically overt thrombosis was confirmed by ultrasound or angiography. Catheter-related thrombosis incidence without anticoagulants was 28% in arm and 33% in chest ports, but with anticoagulants this was 32% in arm and only 1% in chest ports (odds ratio (OR) 34.8 95% confidence interval (CI) 7.3–165). Left-sided placement compared with right-sided and catheter tip position in the superior *vena cava* compared with right atrium were associated with a 3.5 respectively 2.6-fold increased risk. Thrombosis was associated with elevated homocysteine levels (OR = 3.8, 95% CI 1.3–11.3), but not with factor V Leiden or prothrombin 20210A gene mutations, or high concentration of factor VIII, IX or XI. Prophylaxis with anticoagulants is recommended for chest, but not for arm ports. Determination of plasma homocysteine levels may identify patients at an increased risk for thrombosis.

Introduction

The use of centrally or peripherally inserted venous catheters with implantable ports has become common in cancer patients receiving chemotherapy. Catheters are implanted for the long-term administration of chemotherapy courses with sclerosing agents, in patients with toxic chemotherapy regimens and anticipated haematological toxicity requiring frequent blood sampling and, more recently, in patients who require continuous administration of drug infusions. Many different types of implantable devices consisting of a small-volume subcutaneous injection (s.c.) port (commonly called ports) have been introduced with different types of catheters and devices. Port-associated complications such as infections, thrombosis or even pulmonary embolism, are the cause of significant morbidity and occasionally mortality and remain a significant problem in current daily practice.

Catheter-related thrombosis is probably frequently under-diagnosed as most patients with catheter-related thrombosis are asymptomatic or have non-specific symptoms. The reported incidence of catheter-related venous thrombosis varies considerably, in part due to the method of detecting thrombi, with incidences of greater than 60% being reported (1;2). De Cicco and colleagues (1) reported a very high incidence of 66%, but only 6% of patients with catheter-related thrombosis, screened by venography, were symptomatic. Lokich and colleagues (3) reported an incidence of 42%, of which 28.3% were symptomatic. Van Roode and colleagues (4) showed that in patients with haematological malignancies, 26 of 105 patients (25%) developed subclinical thrombosis, of whom nine became clinically manifest. Clinical presentation of catheter-related thrombosis may include arm or head swelling, erythema, pain and congestion of collateral veins, whereas catheter malfunctioning may be the first clinical manifestation of an otherwise asymptomatic catheter-related thrombosis. Thrombosis may lead to prompt catheter removal and anticoagulant treatment.

A hypercoagulable state associated with malignancy, co-morbidity of cancer patients, the use of certain anti-cancer drugs and the presence of a foreign body may contribute to the higher venous thrombosis incidence observed in cancer patients (5). Thrombogenicity of different central venous catheters has been reported to vary depending on the catheter material and size of the catheter used. Polyethylene catheters are associated with a higher incidence than silastic catheters (6;7), whereas there is no difference in the incidence of venous thrombosis following the use of silastic or hydromer-coated polyurethane catheters (6;7).

While several factors may contribute to the development of venous thrombosis, few of these factors have been examined in well-controlled studies (8;9). We therefore investigated various risk factors and the incidence of catheter-related thrombosis in a cohort of cancer patients undergoing chemotherapy and determined the risk of catheter-related thrombosis associated with anticoagulant treatment and prothrombotic risk factors.

Patients and methods

Patients

Between April 1994 and January 2003, 243 consecutive cancer patients in the Leiden University Medical Centre Department of Clinical Oncology received a central venous catheter for either repeated administration of chemotherapy with sclerosing agents known to irritate the veins resulting in frequent phlebitis or for continuously infused chemotherapy. Patients were treated with various combination chemotherapy regimens, most of which contained cisplatin, doxorubicin or both cisplatin and (epi)-doxorubicin. Of these patients, 132 were men and 111 were women, with a mean age of 44 years (range 14–78 years). The most frequently treated types of cancer were bone tumours, *i.e.*, osteosarcoma or Ewing's sarcoma (124 [51%] patients) and distal oesophagus or stomach cancer (54 [22%] patients) (Table 1). One hundred and thirty-nine (57%) patients had distant metastases at the time of insertion of the catheter.

Implantable ports

All catheters implanted were composed of two parts, namely a single lumen radio-opaque catheter connected to an injection reservoir, the port, containing a silicone diaphragm. For the chest ports, we used the Port-A-Cath (Sims Deltec, St-Paul, MN) and for the arm ports, either a Port-a-Cath (Smiths Medical Deltec) or a Vital port (Cook Pacemaker Corporation, Leechburg, PA). The injection port reservoir of the chest ports was made of Titanium and the catheter of polyurethane, with an outer diameter of 2.6 mm and an inner diameter of 1.6 mm. Arm ports also had a Titanium reservoir and the material of the catheter was made of polyurethane in the Deltec and of silastic in the Vital Cook catheters. The outer diameter of the Deltec catheter was 1.9 mm; the internal diameter was 1.0 mm. The outer diameter of the Vital Cook catheter was 1.7 mm; the inner diameter was 0.9 mm.

All catheters were introduced into the veins by experienced interventional radiologists in the angiography interventional radiology suite. Under local anaesthesia, the catheter was tunnelled and a surgeon made the connection between the reservoir and catheter during the same session. After placement, correct positioning of the tip of the catheter was confirmed by chest X-ray and shown to be localised in either the *vena cava* superior or the right atrium. In general, ports were not immediately removed after cessation of chemotherapy in order to be able to use the ports for second- or third-line chemotherapy, if needed. Experienced nurses flushed all ports every 4–6 weeks with 5 ml of a heparin-sodium solution 100 IE/ml and at the end of each cycle of chemotherapy infusions to maintain patency. In patients in whom the port was replaced, the time period of insertion of both catheters was analysed. Chest ports were placed in the subclavian vein, but preferably in the jugular vein. Arm ports were placed in an arm vein.

Table 1

	Patients with thrombosis *n* (%)	Patients without thrombosis *n* (%)	OR (95% CI)
Gender			
Female	19 (57.6)	92 (43.8)	1
Male	14 (42.4)	118 (56.2)	0.6 (0.3-1.2)
Median age in years (range)	42 (16-67)	44 (14-78)	
Type of tumour			
osteo-or Ewing's sarcoma	19 (57.6)	105 (50.0)	1.4 (0.7-2.9)
oesophagus/stomach	7 (21.2)	47 (22.4)	2.1 (0.9-5.3)
ovarian	5 (15.2)	9 (4.3)	5.6 (1.5-20.4)
mamma	0 (0.0)	12 (5.7)	
miscellaneous	2 (6.1)	37 (17.6)	0.9 (0.4-1.7)
Metastatic disease	15 (45.5)	124 (59.0)	1.8 (0.7-4.2)
Chemotherapeutic agents			
anthracyclines	26 (78.8)	171 (81.4)	0.7 (0.2-3.0)
cisplatin	28 (84.8)	156 (74.3)	0.5 (0.2-1.4)
taxane	5 (15.2)	13 (6.2)	0.3 (0.1-1.8)
5-FU civ	8 (24.2)	72 (34.3)	1.6 (0.4-3.5)
Platelet count (*10^9 cells/L)			
≤ 400	4 (12.1)	28 (13.3)	1
> 400	29 (87.9)	182 (86.7)	1.1 (0.4-3.5)

Thrombosis and prophylaxis

The diagnosis of symptomatic thrombosis (*n* = 28), suspected by either symptoms such as arm swelling, pain or bluish discoloration, or suspected because of device malfunctioning (*n* = 5) was confirmed by duplex ultrasonography or phlebography of the upper extremity venous system, while the investigator was unaware of any antithrombotic medication.

Before 1998 (*n* = 66), no prophylaxis with anticoagulants was given, but two patients already received anticoagulant treatment with coumarins for various unrelated reasons, e.g., myocardial infarction, recent surgery, and remained on coumarin treatment. Since 1998, all patients (*n* = 177) received thrombosis prophylaxis with low molecular weight heparin (LMWH) (nadroparin 2850 IE s.c. daily), except for the three patients who had already received coumarin treatment for other reasons did not receive additional prophylaxis with LMWH. Five patients received a second catheter due to catheter-related thrombosis and received coumarin.

Blood sampling

Since 1999, citrated (room temperature) and acidic citrated blood (on melting ice) samples were obtained from 101 patients, after informed consent was obtained. For factor VIII, IX, XI, G1691A (FV) mutation and G20210A (FII) mutation analysis, blood was collected in tubes containing 0.106 mol/l trisodium citrate. Plasma was prepared by immediate centrifugation for 10 min at 3200 rotations per minute (rpm) and stored at −70°C. DNA was extracted from white cells and the G1691A mutation and G20210A mutation determined by the polymerase chain reaction (PCR). The fibrinogen concentration was determined according to Clauss (10). Factor VIII: C (11), factor IX: C and factor XI: C levels were measured by a one-stage clotting assay. Elevated levels of factor VIII were defined as >200 IU/dl and of factor IX and factor XI as >150 IU/dl. For homocysteine concentration assays, blood was collected in Stabilyte tubes containing 0.5 mol/l trisodium citrate and plasma was prepared by immediate centrifugation and stored at −70°C. Total homocysteine concentration was determined with the high performance liquid chromatography (HPLC) sodium borohydride / monobromobimane method ($NaBH_4$/mBrB method used $NaBH_4$ for reduction and mBrB derivation) (12). Elevated levels of homocysteine were defined based on the distribution of plasma levels in cohorts with different age and gender. For women, elevated levels of homocysteine were defined as greater than 13.4 mmol/l for those aged 19–59 years, greater than 16.4 mmol/l for those aged 60–70 years and greater than 17.4 mmol/l for patients over 70 years of age. For men, this was defined as greater than

15.2 mmol/l for 19–59 year old, greater than 18.3 mmol/l for those aged 60–70 years and greater than 19.1 mmol/l for patients over 70 years of age.

Statistical analysis

We compared catheters in patients who experienced catheter-related thrombosis with catheters in patients who did not experience such events.

We investigated putative risk factors by calculating exposure odd ratios (ORs) as an estimate of the relative risk (RR). The ORs show how much higher the risk of disease, *e.g.*, thrombosis, is in the presence of a risk factor than in its absence. An OR ratio of 1 indicates the absence of an association.

Results

Of 243 patients who received 255 devices, in 171 (67%) a catheter was placed in a central vein, *i.e.*, jugular internal or subclavian vein ("chest ports") and in 84 (33%) instances in a peripheral vein, i.e., cubital or basilical vein ("arm ports"). The mean time *in situ* for the chest ports was 207 days (median 178 days; range 9–1092 days) and for arm ports 352 days (median 321 days; range 7–1795 days).

Thirty-three (14%) of the 243 cancer patients developed a catheter-related thrombosis during chemotherapy; 28 (85%) were associated with patient symptoms and five detected because of device malfunctioning. The mean time until detection of thrombosis was 22 days (median 51 days; range 6–309 days), and eighty-five percent occurred within 2 months. In four of the 28 (14%) thromboses that occurred within 2 months, the port was still functioning.

Except for ovarian cancer, there was no association between tumour type, presence or absence of metastatic disease, platelet count, number of chemotherapeutic cycles or type of chemotherapy and thrombosis incidence (Table 1). Both arm and chest posts were implanted on the right (n = 152) and left (n = 103) side; the risk of venous thrombosis was 3.5-fold higher for left-sided placement compared with right-sided placement (OR = 3.5, 95% CI 1.6–7.5) (Table 2).

Table 2. Risk of thrombosis in relation to site of placement of catheter

	Venous thrombosis		OR (95% CI)
	Left-sided placement	Right-sided placement	
All catheters (*n* = 255)	22/103	11/152	3.5 (1.6–7.5)
Chest ports (*n* = 171)	4/38	4/133	3.8 (0.9–15.9)
Arm ports (*n* = 84)	18/65	7/19	1.5 (0.5–4.5)

The position of the tip of the catheter (atrium versus *cava superior* vein) was associated with the risk of venous thrombosis: the risk was almost 3-fold higher when the catheter tip was located in the *superior vena cava* compared with the atrium (OR = 2.7, 95% CI 1.1–6.6). We did not find an association with the type of catheter, or the manufacturer. In 8 (5%) of 171 chest ports and 25 (30%) of 84 arm ports, venous thrombosis occurred, *i.e.*, the risk of venous thrombosis was 8-fold higher for arm ports than for chest ports (OR = 8.1 95% CI 3.5–19.1, Table 3).

Table 3. Risk of thrombosis in relation to the type of catheter and use of anticoagulants

	Venous thrombosis		OR (95% CI)
	Arm ports	Chest ports	
All catheters (*n* = 255)	25/84	8/171	8.1 (3.5–19.1)
Without anticoagulants (*n* = 64)	13/46	6/18	0.8 (0.2–2.5)
With anticoagulants[a] (*n* = 191)	12/38	2/153	34.8 (7.3–165)

[a] Nadroparin (*n* = 181) or coumarin (*n* = 10).

In 64 patients who did not receive anticoagulants, the risk to develop venous thrombosis was similar in patients with arm ports and patients with chest ports; in 13 (28%) of the 46 arm ports, and 6 (33%) of the 18 chest ports, venous thrombosis occurred. In patients who received anticoagulants (in 95% of the catheters nadroparin s.c. was given) catheter-related venous thrombosis occurred more often in those with arm ports (12 [32%] of 38 ports) than in those with chest ports (2 [1%] of 153 ports), OR = 34.8 95% CI 7.3–165 (Table 3). None of the 10 patients on coumarin therapy developed venous thrombosis.

From 101 patients in whom prothrombotic factors were determined, eighteen (18%) had developed venous thrombosis. The prevalence of factor V Leiden and prothrombin 20210A did not differ between the group of patients with venous thrombosis and the group

of patients without evidence of catheter-related thrombosis, nor did we find an association between elevated levels of FVIII, FIX and FXI and the development of thrombosis (Table 4). However, elevated plasma homocysteine levels were more frequently found in the group of patients with venous thrombosis (median 12.7 mmol/l; range 5.4–31.8), i.e., 8 (44.4%) of 18 patients with venous thrombosis, compared with 14 (16.9%) of 83 patients without venous thrombosis (median 12.3 mmol/l; range 8.3–20.1). Elevated plasma homocysteine concentration was associated with a 3.8-fold increased risk of development of thrombosis (OR = 3.8 95% CI 1.3–11.3, Table 4), there was no linear correlation between the actual plasma homocysteine level and thrombosis.

Table 4. Number of patients with factor V Leiden or factor II 20210A gene mutations, elevated plasma levels of clotting factors or homocysteine

	Patients with thrombosis (*n* = 18) *n* (%)	**Patients without thrombosis (*n* = 83) *n* (%)**	**OR (95% CI)**
Factor V Leiden/ factor II 20210A mutation	1 (5.6)	7 (8.4)	0.6 (0.1–5.5)
↑ FVIII	3 (16.7)	6 (7.2)	2.2 (0.5–9.6)
↑ FIX	4 (22.2)	25 (30.1)	0.9 (0.3–3.2)
↑ FXI	2 (11.1)	5 (6)	2.0 (0.4–11.0)
↑ Homocysteine	8 (44.4)	14 (16.9)	3.8 (1.3–11.3)

Discussion

We found an incidence of 14% of catheter-related thrombosis in cancer patients receiving anthracycline- and cisplatin-containing combination chemotherapy via implantable central or peripheral venous ports. Most cases of venous thrombosis occurred within 2 months after insertion of the catheter. The administration of anticoagulants, mainly prophylaxis with s.c. administered nadroparin, was associated with a markedly reduced incidence of thrombosis for chest, but not for arm ports. We identified arm ports, left-sided placement, catheter tip location in the *superior cava* vein and elevated levels of homocysteine as important risk factors for the development of thrombosis.

Platelet counts, the presence of stage IV metastatic disease, individual cytotoxic drug and the cumulative dose of the cytotoxic drugs administered, were not associated with the development of catheter-related thrombosis. Ovarian carcinoma seemed to be an additional risk factor for the development of catheter-related thrombosis, probably reflecting the intrinsically high risk of developing thrombosis in patients with ovarian carcinoma (13), compared with other tumour types.

Most studies on peripherally inserted catheters, although performed in patients with diseases other than cancer, showed a much lower incidence of thrombosis of up to 5% (14) and (15). In agreement with our findings, Kuriakose and colleauges (16) also observed a higher incidence in peripheral ports of 11% compared with 3% in chest ports in patients with mainly cancer or myeloproliferative disorders undergoing chemotherapy.

Left-sided placement as well as location of the catheter tip in the *superior vena cava* instead of the right atrium was found to be associated with a more than 3-fold respectively 2.6-fold increase in risk for the development of thrombosis. The high incidence of thrombosis, despite the prophylactic use of anticoagulants, found in patients with arm ports compared with chest ports may have resulted from arm movements, kinking of the catheter, mechanical displacement of the catheter, or a nod in the catheter at the level of the armpit. Such factors may have contributed to changes in blood flow or injury of the vascular endothelium and release of clotting activators, especially in cancer patients with peripherally inserted arm ports.

Conflicting data exist in the literature with regard to the association between gene abnormalities and the risk of catheter-related thrombosis in cancer patients. We found no association between the risk for thrombosis and known risk factors for venous thrombosis, nor with elevated levels of factor VIII, IX or XI. This is consistent with our previous data (17) and data from Ramacciotti and colleagues (18), who also did not find an association between gene polymorphisms tested, i.e., Factor V Leiden, factor II G20210A, factor XIII val 34leu and Methylene tetrahydrofolate reductase (MTHFR) C677T, and the risk of venous thrombosis in cancer patients. In agreement with these findings, Riordan and colleagues (19) found a low prevalence of factor V Leiden gene mutation in 28 cancer patients with catheter-related venous thrombosis. In contrast, other groups did find an association between factor V Leiden and prothrombin gene mutations and thrombosis in paediatric (20) and in adult patients with haematological malignancies (21). One explanation could be that gene mutations only carry a low additional risk that does not have a substantial impact on thrombosis incidence when other factors already result in a high incidence of venous thrombosis.

Elevated plasma homocysteine levels have been identified as a risk factor for venous thrombosis. Damage of the endothelial cells by homocysteine has been proposed as a cause of homocysteine-associated venous thrombosis, but the exact mechanism is unknown (22). Elevated levels of homocysteine, as observed in our cancer patients, may thus play a causative role in the development of catheter-related thrombosis, in particular during the administration of endothelial cell-damaging chemotherapy. In cancer patients, plasma homocysteine levels may originate from proliferating cancer cells (23). The role of elevated homocysteine levels, MTHFR polymorphism, possibly associated with cancer dietary deficiency, and the protective effect of dietary supplementation in cancer patients (24) and (25) clearly deserves further investigation in larger cohorts of patients. Reduced dietary folate intake in cancer patients, may well contribute to increased homocysteine levels and the development of venous thrombosis in these patients.

The high incidence of thrombosis and the associated complications rate compel further investigation into the exact role of elevated homocysteine levels.

Prophylaxis with anticoagulants is a controversial issue at present and policies differ in different countries. The question of whether prophylaxis with either LMWH or coumarin could protect against catheter-related venous thrombosis in cancer patients, either solid tumour or haematological patients, has been addressed in five randomised studies, of which three have appeared as full papers (2,26-29). Monreal and colleagues (2) and Bern and colleagues (26) both performed a randomised placebo-controlled study in solid tumour patients. Both studies demonstrated that prophylaxis, with LMWH and warfarin, respectively, could protect against catheter-related venous thrombosis. In both studies, a venography was performed as the end-point in each patient after 90 days of insertion of the catheter. On the basis of these two studies, the American College of Chest Physicians recommended prophylaxis with LMWH or low-dose warfarin in cancer patients with central venous catheters (30). More recently, Heaton and colleagues (27) randomly assigned 88 patients with haematological malignancies to low-dose warfarin or no treatment. The end-point of this study was clinically suspected venous thrombosis, confirmed by venography. No significant difference in venous thrombosis incidence was found between the warfarin and control groups. More recently, two randomised studies have been performed (28,29). One in mostly haematological malignancy patients (28) with or without the use of warfarin, and the other (29) in mostly solid tumour patients, employing LMWH. In both studies, no major differences were observed, but the studies have not yet been reported as peer-reviewed papers.

In conclusion, this is the first report identifying elevated plasma homocysteine levels as a major risk factor for catheter-related thrombosis. As our study was based on small numbers, further investigation of homocysteine levels in a larger group of cancer patients is warranted to unravel the relationship between factors influencing plasma homocysteine levels in cancer patients undergoing chemotherapy and the occurrence of venous thrombosis. Based on the high incidence of thrombosis in our patient group, despite the use of prophylactic anticoagulants, we strongly advise against the use of arm ports in cancer patients undergoing anthracycline- and/or cisplatin-based combination chemotherapy. In contrast, the use of a chest port to facilitate administration of such chemotherapy is associated with a low risk of thrombosis, provided that thrombotic prophylaxis is given. Our data underscore the findings of Monreal and colleagues (2) and Bern and colleagues (26), with respect to the need for thrombosis prophylaxis in cancer patients with central venous catheters. Conflicting data in the literature with regard to this question may be explained by the absence of stratification for identified prothrombotic risk factors (*e.g.*, elevated levels of homocysteine, arm ports and side of catheter placement) resulting in such factors not being well balanced between the two randomised groups of patients.

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3 Deep vein thrombosis associated with central venous catheters- a review

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Introduction

Central venous catheters (CVCs) are frequently used in patients for a variety of indications such as cancer treatment, diagnostic monitoring, parenteral nutrition, hemodialysis, cardiac pacing, and administration of fluids, blood products or medication (1). The benefit derived from a CVC may be offset by thrombosis and associated complications, such as pulmonary embolism (PE), CVC dysfunction, infection or loss of central venous access. In the long term patients with thrombosis may suffer from a post-thrombotic syndrome (1;2).

The CVC-related thrombosis is an issue of importance to many clinicians, and insight into the different aspects is crucial to guide decisions in treatment in often vulnerable patients in daily practice. In medical literature, there is a lack of uniformity and uncertainty about several entities of CVC-related thrombosis. First, two types of CVC-related thrombosis must be clearly distinguished; i.e. clinically manifest and subclinical thrombosis. Furthermore, the type of thrombosis and the incidence is defined by the diagnostic strategy in patients with a CVC.

Anticipation of the risk of CVC-related thrombosis and the identification of certain 'high-risk' patients who are prone to develop thrombosis and secondary complications, is essential to initiate early preventive measurements such as prophylactic anticoagulation. The need for anticoagulant prophylaxis is however still a subject of discussion (3;4). Finally, for the treatment of established CVC-related thrombosis, several therapeutic options were evaluated in literature. General recommendations of anticoagulant treatment, and whether CVC removal is necessary or not, is warranted.

The primary aim of this review is to describe the diagnostic methods and their performance, the incidence and risk factors, complications, prevention and treatment of CVC-related thrombosis from a practical clinical point of view. English medical literature studies were retrieved by an extensive Medline search (Pubmed®) and bibliographies of the obtained studies were crosschecked where necessary. For each subject, only those studies with the strongest level of evidence, as defined and discussed in the subsequent paragraphs, were selected and reviewed.

Diagnosis of CVC-related thrombosis

In view of diagnosis of CVC-related thrombosis, two types of thrombosis can be distinguished; clinically manifest thrombosis and subclinical thrombosis. Clinically manifest thrombosis is defined as thrombosis objectified by diagnostic imaging (ultrasound, venography) upon overt symptoms and signs, such as pain or tenderness, warmth, swelling or edema, bluish discoloration or visible collateral circulation. Subclinical thrombosis, defined as thrombosis in the absence of signs and symptoms, is demonstrated by screening diagnostic imaging. Most thrombotic events associated with CVCs remain subclinical, or complications such as PE are the first presenting symptom (5-7).

Radiologically, thrombosis can have a typical appearance of enveloping sleeve surrounding the CVC (Fig 1) or be characterized by mural thrombosis adherent to the venous vessel wall (8). Mural thrombosis, present in approximately 30% of patients with CVCs, may cause subtotal stenosis (Figure 2) or occlusion of the venous lumen and lead to clinically manifest thrombosis or associated complications (6). Mural thrombosis is often found near the entry site of the CVC into the vessel or at the junction of large veins, although it may be extended or located into adjacent venous segments or the right atrium.

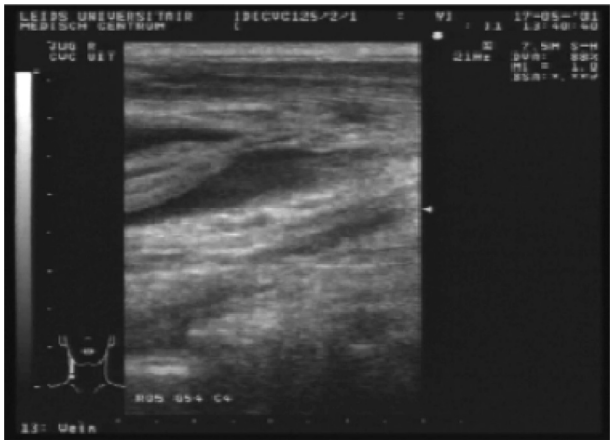

Figure 1. Ultrasonic appearance of a typical enveloping 'fibrin sheath' demonstrated immediately after central venous catheter removal (Jugular vein).

In the diagnostic work-up of CVC-related thrombosis, diagnostic imaging upon a clinical suspicion of thrombosis is mandatory. A diagnosis based solely on clinical symptoms and

signs of thrombosis is non-specific, as in deep vein thrombosis (DVT) of the leg. In only about a third to a half of all patients in whom thrombosis is clinically suspected, the diagnosis is confirmed (9-11).

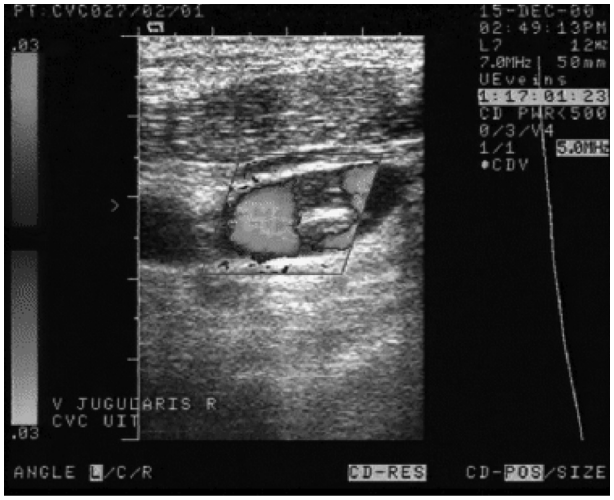

Figure 2. Nearly occlusive mural thrombosis visualized by a flow defect, detected by Doppler Flow Imaging, just after central venous catheter removal.

Contrast venography is widely recognized as the reference standard in the diagnosis of thrombosis (12). However, ultrasound is most often used clinically, because it is non-invasive, does not expose to ionizing radiation, can easily be performed at the bedside and is well accepted by patients. In modern ultrasonography, real time gray-scale images (B-mode) are obtained and the criteria of non-compressibility (compression ultrasound) and direct visualization of thrombotic material in the venous lumen can be used to establish the presence or absence of thrombosis. Besides, real time changes in vessel diameter due to respiration may detect occlusive thrombosis more centrally located. In addition, Doppler techniques can add the advantage of evaluation of blood-flow. With pulsed Doppler signals added to gray scale imaging (Duplex ultrasound) qualitative and quantitative information of blood flow can be obtained. Color Doppler Flow Imaging (CDFI) displays blood flow in color in addition to gray scale imaging. A combination of all three modalities is called color duplex ultrasound.

In symptomatic lower extremity DVT, compression ultrasonography has been validated in clinical practice (13), but specifically for thrombosis associated with femorally inserted

CVCs, no studies are available in which ultrasound was compared with venography. With regard to the upper-extremity DVT, venography has high to moderate inter-observer agreement rates (71%-83%) and can be used as a reference test in clinical practice (14). In several studies the diagnostic accuracy of ultrasound in upper extremity thrombosis compared with venography was evaluated.

For the purpose of this review, we selected those studies in which ultrasound was compared with routine contrast venography in the diagnosis of upper-extremity DVT in the entire cohort of reported patients, and which results were independently interpreted by blinded observers. Overall, six studies were retrieved (Table 1) in which patients with CVCs were included. The reported sensitivity of ultrasound in the diagnosis of upper extremity DVT among these studies ranged from 56% to 100%, whereas the specificity ranged from 77% to 100% (10;11;15-18).

Table 1. Diagnostic accuracy of Doppler-ultrasound in the diagnosis of upper extremity thrombosis with routine contrast venography as the reference standard

Study (reference)	Patients (*n*)	CVC (%)*	Technique	Sensitivity (%)	Specificity (%)	Manifest/ subclinical†
Prandoni *et al.* (10)	58	14	CUS	96	94	Manifest
Prandoni *et al.* (10)	47	NI	Duplex	81	77	Manifest
Prandoni *et al.* (10)	34	NI	CDFI	100	93	Manifest
Baarslag *et al.* (11)	99	NI	CDFI	82	82	Manifest
Baxter *et al.* (15)	19	74	CDFI	100	100	Manifest
Köksoy *et al.* (16)	44	100	CDFI	94	96	Mixed
Haire *et al.* (17)	43	100	Duplex	56	100	Mixed
Bonnet *et al.* (18)	40	100	Doppler	93	93	Mixed

CUS, compression ultrasound; CDFI, color Doppler flow imaging; NI, not indicated.

*Percentage of patients with a central venous catheter (CVC).

†For definition manifest/subclinical, see text.

Reports specifically aimed at patients with CVCs are limited to three studies only (16–18), Importantly, in patients with CVC-related thrombosis, thrombosis tends to be located more centrally than in patients with thrombosis not related to CVCs (4). As a consequence, the diagnostic technique of ultrasound, and therefore the accuracy, in patients with suspected thrombosis because of CVCs is different than those without (history of) CVC. In one study continuous wave Doppler without gray scale imaging only was used, a technique hardly applied nowadays (18). Applying modern techniques, Duplex ultrasound was reported to have an excellent specificity (100%), however the sensitivity was substantially lower (56%) (17). In another study, CDFI was found to be more sensitive (sensitivity 94% specificity 96%) (16).

Summary

In summary, reliable data on the accuracy of ultrasound in CVC-related thrombosis are limited. In lower extremity CVC-related thrombosis no studies are available. In upper extremity CVC-related thrombosis specifically, only three studies are available, of which CDFI had the best performance (sensitivity 94%, specificity 96%). In view of the advantages of ultrasound mentioned, and the high specificity, patients with clinically suspected CVC-related thrombosis, should undergo ultrasound initially. However, the safety of withholding treatment in case of a negative ultrasound in patients suspected for thrombosis is uncertain (19). As a consequence, in patients with normal ultrasound additional venography could be performed. Alternative strategies such as serially performed ultrasound, spiral CT or MRI may be useful and of potential interest, but are not validated yet.

Incidence and risk factors of CVC-related thrombosis

Incidence

In numerous studies the incidence of CVC-related thrombosis has been evaluated. In most studies, clinically manifest thrombosis was used as the primary endpoint. Among these studies incidences ranging from 0% to 28% were reported (20;21). However, the decision to refer for diagnostic imaging upon clinical signs and symptoms for thrombosis lacks uniformity and may be subjective. A more reliable estimate is given by studies in which

routine diagnostic screening (ultrasound or venography) was used in consecutive patients with CVCs to determine to assess a diagnosis of thrombosis. For the purpose of this review these studies are selected and summarized in Table 2, according to the indication for the CVC, i.e. the underlying disease and the type of thrombosis (subclinical, clinically manifest and overall) (5;6;8;22–44).

Overall, the reported incidences of CVC-related thrombosis in these studies ranged widely from 2% to 67% (Table 2). The wide range in observed incidence may be partly caused by different diagnostic modalities (venography, ultrasound), the used criteria, and patient- and CVC characteristics. On average, a 30% cumulative incidence can be found in hospitalized patients and the overall majority of thrombotic events remained subclinical (6). The percentage of clinically manifest thrombosis in these studies ranged from 0% to 12% (Table 2).

In some specific populations, such as patients with hemophilia, prospective (screening) studies are not available. In cohort-studies with merely clinical manifest thrombosis as an endpoint incidences ranged from 0% to 3% (45). Whether in patients with inherited bleeding disorders the risk of thrombosis is reduced as compared with other patients, is not known because of the lack of large studies in which all patients were screened systematically for thrombosis.

Risk factors

The individual risk of CVC-related thrombosis in a patient is the result of the interaction between patient characteristics, i.e. inherited and acquired risk factors; and the CVC (Figure 3). There are numerous studies in which risk-factor analysis of CVC-related thrombosis was performed. For inherited and common acquired risk factors cohort studies were considered to represent the highest level of evidence (level 1); case control studies as level 2. For CVC characteristics, randomized trials were considered to represent level 1 of evidence; cohort studies as level 2.

Table 2. Incidence of CVC-related thrombosis amongst studies with routine diagnostic imaging performed in consecutive patients (Doppler-Ultrasound or venography)

Study (reference)	Population	N	Technique	DVT % (manifest %)	Location entry site CVC
Chastre *et al.* (22)	ICU	33	V	67 (0)	Jugular vein
Durbec *et al.* (23)	ICU	70	V	36 (0)	Femoral vein
Timsit *et al.* (24)	ICU	208	D	33 (0)	Subclavian & jugular vein
Wu *et al.* (25)	ICU	81	D	56 (0)	Jugular vein
Joynt *et al.* (26)	ICU	124	D	10 (2)	Femoral vein
Martin *et al.* (27)	ICU	60	D	58 (2)	Axillary vein
Stoney *et al.* (28)	Cardiology	203	V	34 (3)	Cephalic & jugular vein
Goto *et al.* (30)	Cardiology	100	V	23 (0)	Cephalic & subclavian vein
Lin *et al.* (29)	Cardiology	109	D	6 (0)	Cephalic & subclavian vein
Antonelli *et al.* (31)	Cardiology	40	V	28 (5)	Cephalic & subclavian vein
Van Rooden *et al.* (32)	Cardiology	145	D	23 (2)	Cephalic & subclavian vein
Valerio *et al.* (33)	Oncology	18	V	33 (6)	Subclavian vein
Brismar *et al.* (34)	Oncology	53	V	36	Subclavian vein
Bozetti *et al.* (35)	Oncology	52	V	28 (0)	Subclavian vein
Haire *et al.* (5)	Haematology	35	V	63 (9)	Subclavian vein
Balesteri *et al.* (8)	Oncology	57	V	56 (0)	Subclavian vein
De Cicco *et al.* (37)	Oncology	95	V	66 (6)	Subclavian vein
Biffi *et al.* (38)	Oncology	302	D	4 (2)	Subclavian & cephalic vein
Luciani *et al.* (39)	Oncology	145	D	12 (3)	Subclavian vein
Harter *et al.* (40)	Oncology	233	D	2 (0)	Jugular vein
Lordick *et al.* (41)	Haematology	43	D	30 (0)	Jugular vein
Van Rooden *et al.* (42)	Haematology	105	D	28 (12)	Jugular & subclavian vein
Nowak-Gottl *et al.* (43)	Pediatrics	163	D	11 (11)	Subclavian vein
Beck *et al.* (44)	Pediatrics	93	D	18 (8)	Jugular & subclavian & femoral vein
Van Rooden *et al.* (6)	Mixed	252	D	30 (7)	Jugular & subclavian vein

V, venography; D, Doppler-ultrasound; DVT, deep venous thrombosis.
For definition of manifest, see text.

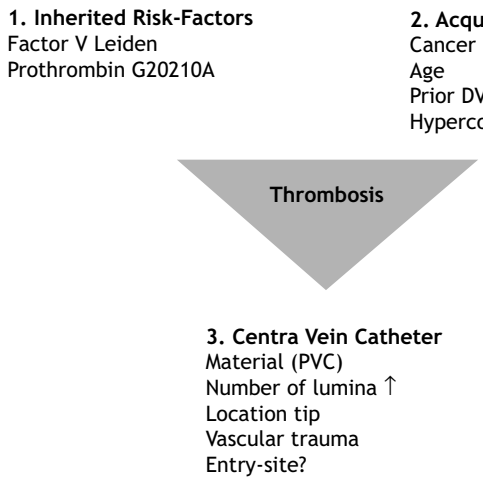

Figure 3. Interaction of inherited, acquired risk-factors of thrombosis with catheter characteristics play an important role the development of central venous catheter-related thrombosis.

Inherited coagulations disorders have been reported to contribute substantially to CVC-related thrombosis in large cohort studies (level 1). Factor V Leiden (FVL) was strongly associated with clinically manifest thrombosis in patients who underwent bone marrow transplantation (*n* = 277); *i.e.* 54% of patients with FVL developed thrombosis, in comparison with 10% of patients without (Cox proportional hazard ratio 7.7) (46). In a large hospital population of 252 patients, the presence of FVL and prothombin G20210A mutation increased the overall risk of CVC-related thrombosis almost threefold (6). Two other recent performed studies also suggested a contribution of these commonly inherited coagulations disorders (47;48). In contrast to these studies, a case–control study (level 2) reported no increased prevalence of FVL in patients with CVC-related thrombosis as compared with the general western population (49). In children, similar risk estimates as in adults have been reported. In cohort studies, the risk of thrombosis in FVL carriers in pediatric patients was substantial in patients with acute lymphoid leukemia, as well in mixed populations (43;50;51). With regard to common acquired risk factors of venous thrombosis there are numerous studies of different level of evidence. In cohort studies, the presence of cancer or active cancer treatment in both, adults and children (6;44), prior thrombo-embolism (32), acquired (temporary) hypercoaguable state (43;52) and a

high platelet count at CVC insertion (53) were associated with thrombosis. Age was also associated with CVC-related thrombosis; the risk was higher with increasing age, and in very young children (24;44).

Table 3. Studies in which the benefit from anticoagulant prophylaxis for CVC-related thrombosis was evaluated. Studies were classified into three categories: (i) randomized-controlled trials with routine mandatory diagnostic imaging; (ii) randomized-controlled trials with clinically manifest thrombosis or associated complications; and (iii) observational studies

Study (reference)	Population	*n*	Intervention	Thrombosis (%)	Thrombosis (controls) (%)	Endpoint
Randomized-controlled trials - mandatory diagnostic imaging						
Bern *et al.* (74)	Oncology	82	Warfarin 1 mg	9.5	42	Mandatory venogram
Monreal *et al.* (75)	Oncology	29	Dalteparin 2500 IU	6	62	Mandatory venogram
Abdelkefi *et al.* (76)	Hematology	128	UFH (100 IU kg^{-1})	1.5	12.6	Mandatory ultrasound
Brismar *et al.* (34)	Nutrition	49	UFH (5000 IU q 6 h)	21.7	53.8	Mandatory venogram
Ruggiero and Aisenstein (80)	Nutrition	34	UHF (1000 IU L^{-1})	53	65	Mandatory venogram
Fabri *et al.* (81)	Nutrition	46	UFH (3000 IU L^{-1})	8.3	31.8	Mandatory venogram
Fabri *et al.* (82)	Nutrition	40	UFH (3000 IU L^{-1})	0	0	Mandatory venogram
Macoviak *et al.* (79)	Nutrition	37	UHF (1 U ml^{-1})	17.6	15.6	Mandatory venogram
Pierce *et al.* (78)	Pediart. Crit. Ill	209	UFH bonded CVC	8	0	Mandatory ultrasound
Massicotte *et al.* (77)	Pediatr. Oncology	158	Reviparin 30-50 IU kg^{-1}	14.1	12.5	Mandatory venogram
Randomized-controlled trials - Clinical endpoints						
Heaton *et al.* (84)	Hemato-oncology	88	Warfarin 1 mg	17.7	11.6	Including PE & malfunction
Anderson *et al.* (85)	Oncology	255	Warfarin 1 mg	4.6	4	No PE or malfunction
Reichardt *et al.* (83)	Oncology	425	Dalteparin 5000 IU	3.4	3.7	No PE, malfunction

Study (reference)	Population	*n*	Intervention	Thrombosis (%)	Thrombosis (controls) (%)	Endpoint
Cohort studies (consecutive patients *vs*. controls)						
Boraks *et al*. (86)	Hemato-Oncology	223	Warfarin 1 mg	5	13	CMT
Lagro *et al*. (87)	Hemato-Oncology	323	Nadroparin 2850 IU	7	6	CMT
Lagro *et al*. (87)	Hemato-Oncology	323	Nadroparin 5600 IU	8	6	CMT

UHF, unfractionated heparin; RR, risk reduction; CMT, clinically manifest thrombosis; PE, pulmonary embolism.

Many CVC characteristics have been associated with an increased risk of CVC-related thrombosis. The type of CVC may be an important factor in the development of CVC-related thrombosis. CVCs composed of silicon or polyurethane are less often associated with local thrombosis than CVCs made of polyethylene (35;54;36). In addition, the risk of thrombosis tends to increase with the number of CVC lumina (5;55). The role of the puncture-site of CVC insertion is still much debated. In two randomized trails (level 1) in intensive care unit patients insertion via the subclavian route had a low risk of thrombosis as compared to a femoral route (0% vs. 25%, respectively 6%) (56;57). A similar observation was found in a cohort (level 2) study in patients with subclavian vein CVC as compared with jugular CVCs (11% *vs*. 42%) (24). In both studies patients were routinely screened by ultrasound for CVC-related thrombosis. However, the methodology of comparing femoral with subclavian vein thrombosis associated with CVCs can be debated as the technique and accuracy of ultrasound in asymptomatic upper and lower DVT differ. In a recent cohort study (level 2) in children, the subclavian route had an increased risk of thrombosis as compared with the jugular route as assessed by a combination of routine venography and routine ultrasound (58). In cohort studies, a left insertion side has been reported to increase the risk of thrombosis (37;53;58) and with a CVC tip position into the subclavian or innominate vein, thrombosis was more often observed in comparison to a superior caval vein or right atrial tip location (39). Additional factors in cohort studies that have been reported to increase the risk of thrombosis are a percutaneous insertion procedure, prior CVC at the same puncture site and a prolonged stay of the CVC for over 2 weeks (58;59).

Summary

In summary, CVC-related thrombosis is a multicausal disease. Prothrombotic factors (e.g. FVL) and the underlying disease (cancer) may play an important role in the development of CVC-related thrombosis. Some important CVC characteristics increase the risk of thrombosis, such as the type and material of the CVC, vascular trauma and the duration of stay of the CVC.

Complications

Catheter related thrombosis may be associated with several complications including PE, infection of the thrombus, CVC dysfunction and subsequent loss of intravenous access and post-thrombotic syndrome or recurrent thrombosis.

Pulmonary embolism

The reported incidence of PE as a complication of catheter-related thrombosis varies. In only one study, all patients with proven thrombosis systematically underwent screening for PE (ventilation-perfusion scan) and a 15% cumulative incidence was reported (60). In other studies incidences of PE, using merely clinical endpoints, varied greatly. Whereas incidences of symptomatic PE up to 17% have been reported, others did not observe any PE (61;62). PE associated with CVC-related thrombosis has been reported to be the cause of death (7;60).

Screening for PE if a diagnosis of CVC-related thrombosis is established is usually not mandatory, as in most patients anticoagulant treatment is initiated, eventually with a removal of the CVC. A firm evidence regarding clinical outcome needs however to be established prospectively.

Infection

The CVC-related thrombosis and CVC-related infection have been reported to be associated (24;41;63;64). The pathogenesis of catheter-related infection seems to depend on the development of thrombosis of the catheter. Several thrombo-proteins were shown to increase the risk of subsequent infection (65;66). Results from a postmortem study in 72 patients with a CVC at death revealed that in all patients with catheter-related sepsis

(*n* = 7) mural thrombosis after a CVC was present, out of a total number of 31 patients with thrombosis (63). In a study in 265 critically ill patients the risk of infection and sepsis was 2.6-fold increased in patients with catheter-related thrombosis (24). In 43 patients undergoing intensive chemotherapy, 13 patients had objectified subclinical thrombosis of whom 12 developed infection (41).

In addition, CVC-related infection may also increase the risk of subsequent clinically manifest thrombosis. In one study CVC-related infection increased the risk of thrombosis (24%) markedly in comparison with those without infection (3%) (relative risk 17.6) (64).

In the presence of CVC-related infection, it may be useful to screen patients for thrombosis with ultrasound, even in the absence of other clinical overt signs and symptoms. Whether such a strategy is clinically beneficial, improves clinical outcome, and is cost-effective should be further investigated.

Early CVC removal and dysfunction

The CVC dysfunction because of clot formation may occur due to obstruction within the CVC lumina, or occlusion due to an enveloping sheath obstructing the CVC luminal tip. Clot formation of the CVC has been identified as the principal cause of catheter dysfunction in prospective follow-up studies. In a study in 85 CVCs placed for hemodialysis, 16 (19%) clot formation occurred leading to catheter malfunctioning requiring removal of the catheter in all cases (67). In another study in 92 CVCs inserted for hemodialysis, 11 CVCs had to be removed because of catheter complications (68). In six (55%) of these cases, occlusion because of clot was the major reason for removal of the catheter. In a study of 949 CVC placed for ambulatory chemotherapy in cancer patients, 152 (18%) of the catheters had to be removed because of complications (69). In this study infection of the CVC was the leading cause of removal of the CVC, 47 (31%) out of 152 CVCs, but also 38 (25%), had to be removed due to catheter-related thrombosis or dysfunction due to clot. In a large study based on the Strategic HealthCare Programs National Database, catheter complications that occurred in 45 333 CVCs used in an outpatient setting in a 17-month period between 1999 and 2000 were evaluated (70). In 1871 catheters, dysfunction occurred and in 511 (27%) cases dysfunction occurred as a consequence of clot formation. In this study different types of central catheters were shown to carry a different complication rate but thrombosis was the most commonly reported cause of catheter dysfunction for peripherally and centrally inserted CVC with implantable ports.

Post-thrombotic syndrome and recurrent DVT

The incidence of the post-thrombotic syndrome, characterized by venous hypertension, swelling of the extremity and pain (10), has been studied in patients without a CVC who experienced an episode of DVT. In such patients, an incidence of up to 80% of the post-thrombotic syndrome has been reported (71). However, data on post-thrombotic syndrome occurring as a sequela of CVC-related thrombosis are scarce and show contradictory results. Hingorani *et al*. reported a cumulative incidence of 4%, whereas Hicken found a much higher cumulative incidence of 50% (62;72). In a prospective study of a large group of 405 children with various diseases who all developed thrombosis of the upper or lower extremity, 244 (60%) had a CVC (73). Of these 405 children, 40% had thrombosis of the lower and 60% had thrombosis of the upper extremity. Post-thrombotic syndrome was found to occur in 50 (12%) of the 405 children. Of the 50 children who developed a post-thrombotic syndrome, 23 had a CVC. In this study a CVC was not an indicator for post-thrombotic syndrome (OR 0.59; 95% CI 0.28–0.94).

There are no reliable data concerning recurrent DVT after an episode of proven CVC-related thrombosis.

Summary

In summary PE is an understudied and probably underdiagnosed complication of catheter-related thrombosis and together with infection of the thrombus a serious life-threatening complication. In clinical practice, an established diagnosis of infection may render it worthwhile to screen for thrombosis with ultrasound. Besides, luminal clot is the most commonly reported cause of catheter malfunctioning and removal of the catheter. The post-thrombotic syndrome causes severe morbidity, however, whether a CVC is an important risk factor is unclear.

Prevention

In several studies among different patient populations the effectiveness of anticoagulant prophylaxis was evaluated. Basically, three groups of patients were distinguished: (i) patients with hematological or solid tumor malignancies; (ii) non-cancer patients (usually

patients with parenteral nutrition); and (iii) critically ill patients. For the purpose of this review three types of studies, according to level of evidence, are discussed subsequently (Table 3): (i) Randomized-controlled studies with routine diagnostic imaging (venography or ultrasound) to define CVC-related thrombosis as an endpoint. Interpretation of data was blindly assessed. (Level 1); (ii) Randomized-controlled studies (double-blind) with clinically manifest thrombosis (or associated complications) as the primary endpoint (Level 2); and (iii) Observational studies which evaluated routine implementation of anticoagulant prophylaxis in a cohort of consecutive patients compared with historical controls without (Level 3).

Adult and pediatric populations are discussed separately.

RCT with routine diagnostic imaging

Three randomized-controlled trials (RCTs) in which routine diagnostic imaging was used were performed in adult cancer patients, and two in pediatric populations (74–78) and five RCT in patients receiving parenteral nutrition (34;79–82).

Cancer patients

In cancer patients with subclavian CVCs, Bern *et al*. (74) studied the benefit of a randomly allocated fixed low dose warfarin (1 mg once daily orally) compared with controls without. Among patients on warfarin a substantially lower frequency of CVC-related thrombosis, as demonstrated by venogram, was observed (9.5% *vs*. 42% in controls). Monreal *et al*. (75) observed a similar benefit from a low molecular weight heparin (Dalteparin 2500 IU subcutaneously) in cancer patients with subclavian inserted Port-a-Caths. In patients on Dalteparin a 6% rate in thrombosis was observed by routine venogram, as compared with 62% in patient without. In a recent study in 128 hemato-oncology patients a benefit from continuously administered unfractionated heparin (UFH) (100 IU kg^{-1} day^{-1}) was observed (76). In the heparin group a 1.5% of patients were diagnosed with thrombosis by routine ultrasound, in the control group 12.6%. There were three events of severe bleeding in the heparin group, as compared with two in the control group (P = NS). Combining the results of Monreal *et al*. and Abdelkefi *et al*. revealed a clear benefit from heparin as compared with placebo in adult cancer patients (RR 0.11; 95% CI 0.03–0.45).

In a study of 158 children with hematological malignancies no substantial benefit was obtained with a LMWH as prophylaxis (77). A total of 14% (11 of 78) of patients on LMWH and 13% (10 of 80) in control patients got thrombosis. In critically ill children, the effect of a heparin bonded catheter has been evaluated to reduce the risk of thrombosis (78). A significant reduction in thrombosis from 8 of 103 (8%) to 0 of 97 was observed (78).

Non-cancer patients/parenteral nutrition

In patients who received parenteral nutrition, only the benefit of UFH in various dosages added to the infusate has been assessed (Table 3). The statistically power of these studies was however limited, because of the small number of patients of each study. Combining the results of these studies, a trend in risk reduction of thrombosis by adding UFH to the infusate was calculated (RR 0.6; 95% CI 0.34–1.06).

RCT with clinical endpoints

Cancer patients

In RCTs with clinically manifest thrombosis as a primary endpoint no clear benefit from anticoagulant prophylaxis was noticed in all three available studies (83–85) (Table 3). Remarkably, the absolute risk of clinically manifest thrombosis in the control group without anticoagulant prophylaxis was low in all these studies (4%), which might explain the lack of statistical power of these studies. The reason for the discrepancy with observational studies with incidences of up to 13% (Table 3) is unclear, but may be caused by selection of patients or referral criteria for diagnostic imaging.

There have been no studies in non-cancer patients or critically ill patients or pediatric patients in this category of studies.

Observational studies

Cancer patients

In cancer patients two cohort studies were performed which evaluated the effect of LMWH (two regimens) or a fixed low dose warfarin on CVC-related thrombosis (Table 3) (86;87). In a study among hematology patients a fixed low dose warfarin (1 mg orally) revealed

a 5% clinically manifest thrombosis, as compared with 13% in historical controls without (86). In another study with retrospective controls, a 7- (2850 IU) and 10-day (5700 IU) course of a LMWH in hematology patients was analyzed. Overall, there was no difference in the cumulative incidence of clinically manifest thrombosis between the groups who received nadroparin (7% and 8% respectively) and those without (6%) (87). However, in this study most thrombotic events occurred after stopping prophylaxis while the CVC remained in place. It is unknown whether a prolongened course would have been effective.

Combining the results of RCT and cohorts-studies, neither an effect of warfarin or heparin was calculated, with regard to the risk of clinically CVC-related thrombosis (warfarin: RR 0.72, 95% CI 0.27–1.9; heparin 0.92, 95% CI 0.57–1.49).

In order to reduce CVC the risk of intraluminal clot formation or dysfunction flushing or locking CVCs with UFH is performed routinely in many clinics. Whether such strategy is more beneficial as compared with saline is unsure. Currently there are no reliable data addressing this theme with clearly defined endpoints including routine assessment by contrast linogram, ultrasound/venography, response-rate to subsequent thrombolysis and safety.

Summary

In summary, the risk of thrombosis may be reduced by applying routine anticoagulant prophylaxis in patients with CVCs in cancer patients. However, a clear benefit was only demonstrated in cancer patients who underwent mandatory diagnostic imaging, including risk reduction of subclinical events. It is therefore debatable whether routine implementation of prophylaxis for CVCs is warranted. Besides, the safety of anticoagulant prophylaxis, a matter of serious concern especially with regard to patients with cancer, has not been studied well. In a recent survey, it was reported that a major reason for clinicians not to comply with consensus guidelines was the risk of bleeding due to thrombocytopenia, which presumably outweighed the risk of thrombosis, particularly in patients with cancer (88–90). In this view, individualized strategies upon allocation of risk assessment in certain vulnerable patients with CVCs and a high risk of thrombosis – such as those with (chemotherapy induced) thrombocyptopenia – might be potentially useful to guide decisions on anticoagulant prophylaxis.

In non-cancer patients or critically ill patients no clear benefit from anticoagulant prophylaxis was observed. Available data consisted of small studies. With the improvement of CVC material no definite recommendations in these groups of patients can be made, until a large interventions study becomes available.

In critically ill children one study showed a risk reduction of CVC thrombosis using heparin bonded CVCs. These CVCs might be a safe alternative to systemic prophylactic anticoagulation, and this needs to be evaluated in other populations in need for short term catheterization.

Treatment

For the treatment of CVC-related thrombosis, various options are available. Anticoagulant treatment, removal or replacement of the CVC, or thrombolytic therapy may be used after a diagnosis of thrombosis is established. In this review randomized-controlled intervention-trials evaluating the recurrence rate of thrombosis and complications, and safety of therapy are considered most convincingly (level 1), cohort studies as level 2, case series as level 3.

Currently, no randomized trails have appeared in the literature. In one cohort study, 112 cancer patients with catheter-related thrombosis, a diversity of therapeutic interventions (several anticoagulation strategies with or without CVC removal) were shown not to result in major differences in clinical outcome (61). Treatment consisted of anticoagulation (n = 39), anticoagulation with CVC removal or replacement (n = 22), CVC removal or replacement (n = 32), other therapy (n = 7) or no therapy (n = 8). In no patients recurrent DVT or secondary complications or death of unknown cause occurred within 2 weeks of diagnosis, while in four patients with CVC replacement only symptoms of edema were persistent. In a prospective case-series of 46 outpatients with upper extremity DVT, in whom 16 (35%) had a central-vein catheter, showed that LMWH (Dalteparin 200 aXa IU kg^{-1}) for a minimum of 5 days together with oral anticoagulants was shown to be safe and effective (91) Evaluation after 12 weeks showed one recurrent DVT (2%), no secondary complications of DVT and one major bleeding event (2%). However, seven patients died, all presumably to underlying disease. Another study evaluated 36 patients with proven DVT of the upper extremity, mostly related to CVCs, up to 1 year after the diagnosis. With LMWH followed by oral anticoagulants (6 months), no recurrent DVT or secondary complications were noted. Nine patients died, presumably due to underlying disease (25%) (92).

A number of non-randomized studies of thrombolytic therapy in catheter related thrombosis have been carried out (93–96). In a retrospective analysis of 95 patients with an upper-extremity thrombosis of whom 62 patients were treated with anticoagulants and 33 with systemic thrombolysis, it was shown that in 21% of the patients, bleeding complications were observed after thrombolysis compared with no complications in the group of anticoagulants only (97). Besides, in the long term no clinical differences with regard to recurrent DVT and post-thrombotic syndrome were observed between thrombolysis and anticoagulation.

For the treatment of fibrin sheaths or luminal occlusion which can lead to CVC dysfunction, the first choice of therapy is local thrombolytic therapy with low dose tissue plasminogen activator (98;99) or urokinase (100,101). After 2-h treatment with 2 mg per 2 mL recombinant tissue plasminogen activator (Alteplase), function was restored to 74% in the alteplase arm and 17% in the placebo arm ($P < 0.0001$ compared with placebo) (98). After another dose (2 mg per 2 mL), function was restored in 90% of patients. There were no serious study-drug-related adverse events, no intracranial hemorrhage, no major hemorrhage, and no embolic events (98). Similar results were confirmed in a large randomized trial in over 1000 patients (99).

Summary

In summary, the treatment of catheter-related thrombosis is controversial. There are no randomized designed studies on the best treatment of catheter-related thrombosis, but in most cohort studies anticoagulant therapy is given. The necessity to remove the catheter depends on the underlying diagnosis and need for vascular access. There is a definite need for well designed studies evaluating the optimal treatment in CVC-related thrombosis. Because of the high rate of complications during systemic thrombolysis, this therapy should be reserved to life-threatening or extremity-threatening venous thrombosis.

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4 Risk of venous thromboembolism in lung cancer

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Abstract

Purpose of review

To evaluate risk factors of venous thromboembolism (VTE) in lung cancer patients.

Recent findings

Calculated incidence rates of VTE in lung carcinoma patients vary from 40 to 100 cases per 1000 person-years, which is much higher than the estimated 1-2 cases per 1000 person-years of the general population. Patients with adenocarcinoma have a higher risk of VTE than patients with squamous cell lung carcinoma. The risk of VTE seems to be 2-fold higher in NSCLC than in SCLC patients.

Other risk factors of VTE are pneumonectomy, metastatic disease, the use of specific chemotherapeutic drugs in combination with novel targeted drugs, *e.g.* anti-angiogeneic agents (*e.g.* anti-vascular endothelial growth factor, VEGF) agents, and elevated pre-chemotherapy platelet counts.

Interestingly, tissue factor (TF), the initiator of the clotting cascade, may be (over) expressed in lung carcinoma cells. Active TF bearing microparticles (MP), which may originate from the tumour cells themselves, have been found in the circulation of cancer patients. MP-associated TF activity may provide a causative link between cancer and thrombosis and play a decisive role in the pathogenesis of the prothrombotic state in cancer patients.

Summary

Risk factors of VTE in lung cancer patients are adenocarcinoma, metastatic disease, pneumonectomy and anti-cancer therapy including chemotherapy and anti-VEGF targeted drugs. Other risk factors identified include pretreatment platelet counts and active TF-expressing circulating microparticles.

Introduction

Incidence and mortality risk of VTE in the general population are rare. The risk ratio of VTE in the general population is not precisely known and was estimated to be between 1–3 in 1000 per year (1;2). Recently, incidence and mortality of a first venous thrombosis was estimated in a general population of Norway (3). The incidence rate for all first venous thrombosis was 1.43 per 1000 person-years (95% CI: 1.33-1.54), for deep vein thrombosis it was 0.93 per 1000 person-years (95% CI: 0.85-1.02) and for pulmonary embolism it was 0.50 per 1000 person-years (95% CI: 0.44-0.56).The incidence rates increased markedly with age and were slightly higher in women than in men. The 30-day case-fatality was higher in patients with pulmonary embolism compared to those with deep vein thrombosis (9.7% *vs.* 4.6%, risk ratio 2.1 (95% CI: 1.2-3.7). It was much higher in patients with cancer compared to patients without (19.1% vs. 3.6%, risk ratio 3.8 (95% CI 1.6-9.2)). The risk of dying was highest in the first months subsequent to the venous thrombosis.

Known risk factors of thromboembolism are surgery, immobilization, the use of oral anticonceptives, and the presence of specific gene mutations in factor V Leiden and in the prothombine gene. More recently also lipid abnormalities have been suggested to contribute to the development of VTE Elevated triglyceride levels were associated with a doubling of risk of venous thrombosis in postmenopausal women, whereas elevated HDL cholesterol levels were associated with a decreased risk (4).

VTE and cancer

Thromboembolism is a well-recognized complication of malignant disease with a spectrum of clinical manifestations varying from venous thromboembolism (VTE) and Trousseau's syndrome to disseminated intravascular coagulation (5). The link between activation of the blood coagulation system and malignancy dates back to 1865 (6). Thereafter venous thrombosis has been reported to be a common complication in patients with malignancy (7;8), but although lung cancer is the second most common cancer in western countries and the leading cause of cancer death in men and women (9) strikingly few papers on the phenomenon of VTE in lung cancer patients are found and data on mortality due to VTE are limited.

Utilizing a Medicare database, Levitan et al found that the incidence of VTE is high among cancer patients, and lung cancer belonged to the group of malignancies with the highest incidence rates (10). More recently, the overall risk of venous thrombosis was found

to be 7-fold increased in patients with a malignancy versus persons without malignancy (11). Patients with haematological malignancies had the highest risk of venous thrombosis followed by lung cancer. The risk of venous thrombosis was highest in the first few months after the diagnosis of malignancy and in the presence of distant metastases. Cancer patients who were carriers of the factor V Leiden and prothrombin 20210A mutations, known risk factors to develop venous thrombosis, appeared to have an even higher risk (11).

Risk of VTE in different histological types of lung cancer and stages of the disease

Although lung cancer incidence has increased during the last decades and Non Small Cell Lung Cancer (NSCLC) accounts for approximately 80% of all lung tumours, few reports exist on the incidence of VTE in NSCLC. For small cell lung cancer (SCLC), which accounts for up to one-fifth of all lung cancers, even less reports exist on the risk of VTE.

The association between VTE and lung cancer has been reported more than twenty years ago (12;13). Recently, Blom *et al*. (14) investigated the thrombotic risk in 537 NSCLC patients and observed that the risk of VTE was 20-fold higher than in the general population (standardized morbidity ratio (SMR): 20.0 (14.6-27.4). Patients with adenocarcinoma of the lung had a 3-fold higher risk (incidence: 66.7 per 1000 years) than patients squamous cell carcinoma of the lung (incidence: 21.2 per 1000 years). In adeno- and squamous carcinoma together, they observed 39 events of VTE over 879 years of follow-up for an overall incidence of VTE of 44.4 per 1000 person-years. During chemotherapy or radiotherapy and in the presence of metastases the risk of VTE was even higher (14).

In the past, autopsy and retrospective studies had already indicated that various adenocarcinomas are most strongly associated with VTE (15;16) and this has led to the widespread belief that mucin-producing adenocarcinomas are indeed the most often tumours associated with VTE. The findings of Blom *et al*. (14) seem to support the notion that specific properties of adenocarcinomas are indeed responsible the observed increased thrombosis in adenocarcinoma patients.

According to data reported in abstract form at the American Thoracic Society International Conference of 2006, the overall incidence of VTE in lung cancer patients was even higher (17). For their analysis, the investigators determined the occurrence of an objectively defined VTE in 598 consecutive patients with a histologically confirmed diagnosis of lung cancer. Seventy-three (12.2%) patients developed a VTE for a total of

730 person-years, which translates into an incidence rate of 100 cases per 1,000 person years. Multivariate analysis indicated that patients with NSCLC were 2.1 times more likely than patients with SCLCS to develop VTE. Age, sex, stage of the lung cancer, ECOG performance, and recent surgery did not predict VTE. This study has not been reported yet as a peer-reviewed paper (17).

VTE as first sign of occult lung cancer

White *et al*. (18) used the California Cancer Registry to identify diagnosed cases of 19 common malignancies during a 6-year period. Cases were linked to a hospital discharge database to identify incident VTE events within 1 year before the cancer diagnosis date. Among 528,693 cancer cases, the incidence of preceding VTE was increased over that expected in the year preceding the diagnosis of cancer, but in particular only during the 4-month period immediately preceding the cancer diagnosis date ($P < .001$). Almost all of these unexpected VTE cases were associated with a diagnosis of metastatic-stage cancer with a standardized incidence ratio (SIR) of 2.3 (95% confidence interval, 2.0-2.6; $P < .001$). Only 7 cancer types were associated with a significantly elevated SIR: acute myelogenous leukaemia, non-Hodgkin lymphoma; and renal cell, ovarian, pancreatic, stomach, and lung cancer (SIR, range, 1.8-4.2).

Pathogenesis of cancer-related thrombosis

The development of VTE in cancer patients seems to be a multifactorial event involving several mechanisms, including inflammation due to necrosis or release of acute phase reactants and haemodynamic disorder, such as stasis. Tumour-specific mechanisms may result in down-regulation of anticoagulant and an up-regulation of procoagulant proteins, which might contribute to the general hypercoagulable condition of cancer patients (19). Cancer cells themselves may produce a number of procoagulant substances including tissue factor (TF), the initiator of the clotting cascade as for instance reported by Yu *et al*. (20). Few studies have attempted to correlate the haemostatic abnormalities in cancer patients with the clinical event of VTE, but so far -with the exception of a small study by Falanga *et al*. (21), in which preoperative thrombin-antithrombin complex levels correlated with the risk of postoperative VTE in cancer patients- no significant difference in clotting factor profile has been found to distinguish cancer patients who did develop VTE from those who did not. Importantly, to our knowledge extensive studies of hypercoagulability in malignancy have not successfully demonstrated a specific coagulation abnormality in cancer patients which predicts for development of VTE.

Tissue factor, microparticles and VTE

Importantly, lung cancer, in particular NSCLC, has been shown to express tissue factor; Callander *et al*. (22) indeed showed that lung cancer expresses TF, but Ornstein et al (23) reported that lung carcinomas (both squamous cell and adenocarcinoma) rarely and inconsistently expressed TF. More recently Sawada *et al*. (24) showed that NSCLC cells produce various amounts of TF but they did not determine the functional activity of the TF. This information is critically important, as only active and not inactive TF is able to initiate coagulation. The activation state of membrane-bound TF depends on the conformational state of TF which is determined by the presence or absence of a specific disulfide bond.

Sato *et al*. (25) reported Trousseau's syndrome in a patient with an adenocarcinoma of the lung in which TF seemed to play a pivotal role in the pathogenesis of recurrent VTE. In their case a markedly elevated plasma TF level was found. Furthermore, cancer cells were shown to express tissue factor as demonstrated by staining of tumour tissue with an anti-tissue factor monoclonal antibody, suggesting that TF in the circulation was released by the lung carcinoma cells.

Tesselaar *et al*. (26) investigated the association between clinically manifest VTE and procoagulant properties of circulating microparticles (MP) isolated from blood of unselected pancreas and breast adenocarcinoma patients, of whom a number presented with ultrasound or CT-scan confirmed VTE. They showed that elevated MP-associated TF activity significantly correlated with development of VTE in cancer patients with disseminated mucinous carcinomas. They also investigated individuals (without cancer) who presented with VTE, in one of these subjects who presented with idiopathic VTE of both legs, a highly elevated MP-associated TF activity was found. Strikingly, within one month following presentation with bilateral deep venous thrombosis, this patient was being diagnosed with a disseminated mucinous adenocarcinoma of the lung. Their findings suggest that the presence of active TF-bearing MP in the circulation of individuals who present with VTE may indicate the presence of an occult adenocarcinoma. Whether MP-associated TF activity indeed predicts the presence of occult adenocarcinomas will now be investigated by them in a prospective cohort study.

Interestingly, Del Conde *et al*. (27) reported a patient with a giant-cell lung carcinoma with a severe form of Trousseau's syndrome, who -despite receiving potent antithrombotic therapy- suffered eleven major arterial and venous thrombotic events over a 5 month period. This patient had a 41-fold higher concentration of plasma tissue factor

as compared to the mean plasma TF concentration examined in 16 normal individuals. Tumour cells from a lymph node stained intensely for TF. Microvesicles derived from patient plasma were found to express TF, and these cell-derived MP may have been shed by the cancer cells.

VTE and surgery in lung cancer patients

Cancer patients undergoing surgery have at least twice the risk of postoperative deep venous thrombosis and were known to have a more than 3-fold increased risk of fatal pulmonary embolism than non-cancer patients undergoing similar procedures (28). Interestingly, mortality due to VTE has been reported to occur predominantly in patients with squamous cell lung carcinoma following surgery (29). Patients with cancer are also more likely to develop VTE post-operatively despite thrombosis prophylaxis (30;31). After surgery, both pneumonectomy and lobectomy, pulmonary vein thrombosis has been shown to occur (32). Compared to patients who undergo surgery for other reasons than lung malignancies, *e.g.* orthopaedic or gynaecological operations, patients who undergo thoracic surgery seem to have a lower risk of VTE (33) This seemingly contradiction could be explained by the high risk for other severe of even fatal complication following pneumonectomy compared to other types of operations, which may result in an underestimation of the risk of VTE. .

Pulmonary embolism is the second cause of mortality after pneumonectomy for a malignancy and such patients have the highest risk to die from pulmonary embolism (29).

In a more recent published study of Mason *et al.* (34) the incidence of postoperative VTE after pneumectomy for malignancies was higher (7.4 %) as compared to that reported in older literature with a peak incidence within 7 days after the operation. Most patients had already been discharged from the hospital. Higher pack-years of smoking were associated with increased risk, as well as with earlier occurrence of VTE. Patients with VTE had a poor survival compared to patients who did not and this was valid for upper- as well as for lower extremities thrombosis. The difference in survival persisted after censoring for deaths directly attributable to venous thromboembolism. Based on these findings, VTE is a frequent event after pneumonectomy, and improved prophylaxis in high risk patients may prevent morbidity and mortality after pneumonectomy for lung malignancy and improve the poor survival outcome in these patients.

VTE in lung cancer patients undergoing chemotherapy and treatment with novel targeted drugs

Combined chemotherapy (either in combination with radiotherapy) is the current standard treatment for advanced stage NSCLC as well as in the treatment of SCLC patients and VTE is a well known complication of anticancer therapy (35-37). Since chemotherapy increases the risk of thrombosis in cancer patients (38), VTE may well become an important clinical issue in the general practice of physicians who treat lung cancer patients.

In SCLC high response rates are achieved by chemotherapy, but long-term outcome is still poor due to the majority of patients relapsing. This has resulted in the exploration of a number of new agents and novel strategies for the treatment of small-cell lung cancer, but with little benefit for the patient thus far.

Recommended first-line treatment in patients with advanced non-small-cell lung cancer (NSCLC) is chemotherapy with combinations of cisplatin plus gemcitabine, vinorelbine, or taxanes, with or without targeted therapy. Numico *et al*. (39) prospectively assessed the occurrence of VTE in patients with NSCLC who were treated consecutively with cisplatin and gemcitabine. They observed 22 VTE in 19 of 108 stage II-IV NSCLC patients who underwent chemotherapy (17.6%; 95% confidence interval [95% CI], 10.3-24.8%). A second thrombotic event was observed in patients who were given further chemotherapy after resolution of the first event, underscoring that indeed chemotherapy contributed to or even caused the thrombotic event.

Khorana *et al*. (40) analyzed data from a prospective, multicentre study in 3003 patients treated with at least one cycle of chemotherapy. VTE occurred in 58 (1.93%) over a median follow-up of 2.4 months (0.8%/month). The incidence varied significantly by site of cancer (P = 0.01) with highest rates in upper gastrointestinal (2.3%/month) and lung cancer (1.2%/month). Interestingly, an elevated pre-chemotherapy platelet count was associated with a 3-fold increased rate of VTE. In multivariate analysis, elevated pre-chemotherapy platelet count, site of cancer, haemoglobin <10 g/dL or use of erythropoietin, and use of white cell growth factors in high-risk sites of cancer were significantly associated with VTE. Their findings suggest that thrombosis prophylaxis should be considered in lung cancer patients undergoing chemotherapy who have elevated pre-chemotherapy platelet counts. Zecchina G, *et al*. (37) observed that thrombotic risk was further potentiated by chemotherapy in 49 unresectable, locally advanced, or metastatic lung cancer. In accordance with the literature in patients with other types of cancer, no alterations of coagulation inhibitors or activation of disseminated intravascular

coagulopathy and/or fibrinolysis as factors that induce chemotherapy-related thrombosis in lung cancer were found. Their findings also suggested that thrombocytosis played a role in triggering thrombotic complications.

Because combination chemotherapy schedules have induced higher better response rates than single agent chemotherapy, but did not result in major improvement of survival in NSCLC patients, many new drugs and drug combinations are currently under investigation. These include targeted therapies with epidermal growth factor receptor (EGFR) inhibitors, such as gefitinib and erlotinib, are effective in advanced NSCLC patients after first-line chemotherapy. Also VEGF-targeted inhibitory agents (inhibitors of vascular endothelial growth factor [VEGF] and its receptors) are currently being investigated for the treatment of NSCLC. A phase III study proved a significant benefit when bevacizumab was added to first-line chemotherapy in advanced NSCLC (41) and phase III clinical trials are currently in progress to investigate the efficacy of dual VEGF/EGFR inhibition, alone or in combination with chemotherapy, in patients with NSCLC. Clinical trials have shown that the use of anti-angiogenic agents is associated with an increase in incidence of VTE, but also haemorrhages. In the first reported phase I study of combined treatment with SU5416 plus gemcitabine-cisplatin more than 20% of the patients developed VTE (42). Combined modality treatment consisting of chemotherapy, radiotherapy and the antiangiogenic agent thalidomide in advanced NSCLC resulted in excessive toxicities with major thrombotic events (43), similar to what has been reported in other malignancies. Yoon *et al*. (44) reported that chemotherapy with bevacizumab, irinotecan, 5-fluorouracil and leucovorin was found to be associated with a large, embolizing thrombus in the thoracic aorta.

Behrendt *et al*. (45) conducted a retrospective cohort study in 1023 patients with stage IIIB, IV, or recurrent non-small cell lung cancer (NSCLC) who were followed during 2 randomized, double-blind trials of prinomastat versus placebo, plus gemcitabine/cisplatin (GC) or paclitaxel/carboplatin (PC). VTE included deep venous thrombosis (DVT) or pulmonary embolism (PE) confirmed by imaging or autopsy. The use of these novel matrix metalloproteinase inhibitor prinomastat in combination with chemotherapy, but not alone, resulted in approximate doubling of the risk of VTE in advanced NSLC patients.

Also in a large Phase III study in previously untreated stage IIIB/IV NSCLC patients who were treated with chemotherapy, with or without aprinocarsen, a new antisense oligonucleotide directed against protein kinase C-alpha, VTE was significantly increased in the experimental arm (46).

Thus, since the use of anti-angiogenic agents is associated with a marked increase in thromboembolic events, the clinician should be alert to the occurrence of such vascular complications. However, since some of these compounds may be associated with bleeding as well as with VTE, preventive measures are not easy to implement.

Another factor that nowadays may increase the risk of VTE in lung cancer patients is the recommended use of eythropoietin which has been shown to improve anaemia in cancer patients undergoing chemotherapy. Various trials reported the increased risk of thrombotic events following the use of eythropoietin indicating that the widespread use of eythropoietin should be cautioned

In conclusion

Lung cancer patients are at increased risk for VTE, and the life expectancy of a cancer patient with thrombosis is poor compared to a cancer patient without VTE. This supports the hypothesis that a local or systemic hypercoagulability state confers a growth advantage to tumour cells, while characteristics of individual patients' tumour cells may at the same time determine the tendency to develop thrombotic events. Risk factors for VTE are adenocarcinoma, NSCLC in comparison to SCLC, pneumonectomy, distant metastases and chemotherapy either alone or in combination with novel anti-angiogenic targeted drugs.

Abnormal coagulation profiles do not correlate with the development of thrombosis and are thus not useful to determine. The mechanism by which cancer predispose to thrombosis is still a challenging subject for research and a matter of controversies among researchers. One newly recognized mechanism by which cancer leads to thrombosis is microparticle-associated active tissue factor, which may be derived from various cells of the body, including the tumour cells themselves.

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5 Risk of venous thrombosis in patients with adeno-and squamous cell carcinoma of the upper gastro-intestinal tract

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submitted

Abstract

Background

Patients with malignancy are at increased risk for venous thrombosis and it is widely assumed that those with adenocarcinomas are at highest risk.

Patients and methods

To determine the incidence of venous thrombosis in patients with adenocarcinoma or squamous cell carcinoma of the upper-gastrointestinal tract we performed a cohort analysis and reviewed the medical records of 1000 consecutive patients diagnosed with upper-gastrointestinal cancer diagnosed between 1980 and 2000 in one academic centre. These were 535 esophageal carcinoma patients, of whom 216 (40%) had an adenocarcinomas and 319 (60%) a squamous cell carcinoma, and 465 patients with adenocarcinoma originating in the stomach.

Results

The mean age was the same for patients with cancer arising in the esophagus or stomach, those with squamous cell carcinoma and with adenocarcinomas; nor did the numbers of years at risk differ. There were 70 venous thrombotic events (incidence rate 3.9 per 100 person-years). In patients with esophageal cancer the incidence of thrombosis was 3.3 per 100 person-years, and in those with gastric cancer it was 4.5 per 100 person-years. The incidence of venous thrombosis in patients with adenocarcinoma (59/681, 4.8 per 100 person-years) was 2.6-fold increased (HR 2.63, 95% CI: 1.38-5.00) compared to the risk in patients with squamous cell carcinoma (11/319, 1.9 per 100 person-years). The survival was worse for patients who developed venous thrombosis (HR 2.13, 95% CI: 1.64-2.88) than other patients, irrespective of whether the tumor originated in the stomach or the esophagus.

Conclusions

The risk of venous thrombosis in upper-gastrointestinal cancer patients is 10 to 15-fold increased as compared to the general population. Patients with adenocarcinoma have a higher risk to develop thrombosis than patients with squamous cell carcinoma, whereas patients who develop thrombosis have a worse survival compared to those who do not.

Introduction

The association of thrombosis and cancer originates from the observations of Armand Trousseau, who in 1865 noted that patients who present with idiopathic venous thrombosis frequently had an occult malignancy (1). In the past years several investigators have confirmed the strong association between cancer and venous thrombosis. Hospital-based and national registries offer an opportunity to study the risk of venous thrombosis in large cohorts of cancer patients, which is essential in decisions on prophylactic anti-coagulant treatment. Two large population-based studies performed by Sorensen *et al*. (2) and Baron *et al*. (3) have demonstrated that the incidence of cancer is increased in the years following the diagnosis of venous thrombosis. The peak incidence of cancer in the first year after the diagnosis of venous thrombosis strongly suggests that these patients already had cancer during their episode of venous thrombosis.

Blom *et al*. (4) showed that the overall risk of venous thrombosis was 7-fold increased in patients with a malignancy versus persons without malignancy. Patients with hematological malignancies, followed by lung cancer and gastrointestinal cancer had the highest risk of venous thrombosis. Sallah *et al*. and Levitan and *al*. (5;6) showed that of the solid tumors, gastrointestinal tract cancer, kidney, brain and ovarian cancer were most strongly associated with venous thrombosis. Although the overall incidence of venous thrombosis has been shown to be increased in cancer patients, few data exist on the incidence for different histological types of cancer in one organ site. Although squamous cell carcinomas and adenocarcinomas both arise from epithelial cells, adenocarcinomas are presumed to carry a higher risk of thrombosis than squamous cell carcinomas.

To study the incidence of venous thrombosis in adenocarcinomas and squamous carcinomas arising in the same organ site, we performed a follow-up study in patients with upper gastro-intestinal cancer, *i.e*. gastric and esophageal carcinoma.

PAtients and methods

Patients

All patients who were treated between January 1980 and January 2001 in our hospital for upper gastro-intestinal cancer were identified from the Cancer Registry database of the Leiden University Medical Center (LUMC). This database contains information concerning

all patients diagnosed with cancer who are admitted to our hospital for treatment. The database has been set up in 1970 and has since been staffed by a specialized team of oncological data managers.

Data were collected from 1177 consecutive patients who presented with either a malignancy of the esophagus or stomach at the Leiden University Medical Center (Figure 1). In all patients, the malignancy was histologically confirmed. Of these 1177 patients, 568 patients had esophageal carcinoma and 609 patients had a malignancy of the stomach. Of the 568 patients with esophageal carcinoma, 24 cases were excluded because of incomplete records and 11 cases because they had a different malignant tumor type than adeno- or squamous cell carcinoma. Of the remaining 535 esophageal carcinoma patients, 216 (40%) had an adenocarcinoma and 319 (60%) had a squamous cell carcinoma (Figure 1). Of the 609 patients with gastric carcinoma, 4 were excluded because of incomplete records and 140 patients because of a different malignant tumor type (mainly lymphoma or sarcoma). The 465 remaining patients with gastric carcinomas all had an adenocarcinoma. Thus, a total of 1000 patients were included in the study.

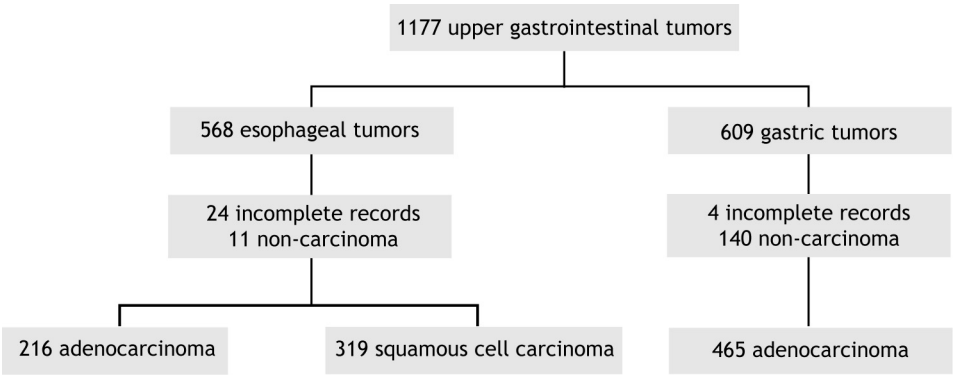

Figure 1

Patient and tumor characteristics were recorded from the medical records and information about the occurrence of venous thrombosis was collected from medical records or through the Leiden Anticoagulation Clinic. In all cases the diagnosis of venous thrombosis was confirmed by duplex ultrasonography or venography in case of deep vein leg thrombosis, high-probability ventilation-perfusion scans in case of pulmonary embolism and in some cases confirmation was also obtained by autopsy. We counted all venous thrombosis which occurred at the time of, or after the diagnosis of cancer was made.

Statistical analysis

We counted person-years of follow-up for each subject from the date of diagnosis of cancer until the date of a thrombotic event, the date of death, or the end of the study period (31 December 2006), whichever occurred first. A total of 1790 person-years accrued, with 4 patient lost to follow-up 3 or more years after their initial diagnosis of cancer. We computed the incidence rates by dividing the number of cases of venous thrombosis by the number of person-years, and cumulative incidences by dividing the number of thrombosis cases by the number of patients at start of follow-up. A Standardized Morbidity Ratio was calculated using the incidence rate of deep venous thrombosis of the leg and pulmonary embolism in the Dutch population of 1994 (7). We compared thrombosis incidence between various groups of patients with Cox modeling.

For the survival analysis of patients who developed a venous thrombosis after diagnosis of esophageal cancer we used a Cox proportional hazards model with the thrombotic event as a time-dependent covariate.

Results

Characteristics of the 1000 patients with upper-gastrointestinal tract carcinoma are shown in Table 1. Most of the patients were men and the mean age did not differ between squamous and adenocarcinoma patients, nor between patients divided into those with a tumor located in the esophagus versus stomach. The median survival of patients with a tumor arising in the esophagus respectively stomach was 9.7 respectively 9.0 months, with 143 (14.3%) patients alive after 3 years of follow-up.

We observed 70 cases of venous thrombosis with 1790 person-years of follow-up in all upper gastro-intestinal tract carcinoma patients, for an incidence rate of 3.9 per 100 person-years. Of the 70 patients who developed venous thrombosis, 26 patients had a pulmonary embolism and 11 patients died as a consequence of the thrombotic event. Five patients with a venous thrombosis developed their venous thrombosis within one month after surgery for their primary tumor. The median time from first admittance for upper-gastrointestinal tract carcinoma until development of the venous thrombosis was 2.0 months (range 0.0–150 months). The cumulative incidence of venous thrombosis was 7%: 5.6% after the first and 6.3% after the second year of follow-up.

When analysis was performed according to localization, we observed 28 venous thromboses, including 13 cases with pulmonary embolism in esophageal cancer patients (Table 2) in 857 person-years with an incidence rate of venous thrombosis of 3.3 per 100 person-years. The incidence of venous thrombosis in the first 6 months after the diagnosis of esophageal carcinoma was 9.3 per 100 person-years and 0 per 100 person-years in the next six months. In patients with gastric carcinoma, 42 events of venous thrombosis including 13 cases with pulmonary embolism were observed in 933 person-years with an incidence rate of venous thrombosis of 4.5 per 100 person-years. The incidence of venous thrombosis in the first 6 months after the diagnosis of gastric carcinoma was 17.2/100 person-years, which is 25-fold higher than in the subsequent time period.

Table 1. Patient's characteristics in 535 esophageal and 465 gastric carcinoma patients at the time of diagnosis.

Histology	**Patients with esophageal cancer**				**Patients with gastric cancer**	
	Squamous cell carcinoma *n* = 319		Adenocarcinoma *n* = 216		Adenocarcinoma *n* = 465	
	No	(%)	No	(%)	No	(%)
Sex						
Men	192	(60.2)	168	(77.8)	340	(73.1)
Female	127	(39.8)	48	(22.2)	125	(26.9)
Age (years)						
mean	65.6		68.2		65.4	
range	22-89		39-88		21-92	
Survival (months)						
median	9.6		9.5		8.8	
range	0.2-300		0.2-255		0.1-300	
Initial Treatment						
urgery	93	(29.2)	94	(43.5)	338	(72.7)
radiotherapy	214	(67.1)	120	(55.6)	31	(6.7)
chemotherapy‡	3	(0.9)	11	(5.1)	20	(4.3)
Distant Metastases						
yes	60	(18.8)	73	(33.8)	205	(44.1)
no	256	(80.3)	141	(65.3)	256	(55.0)
unknown	3	(0.9)	2	(0.9)	4	(0.9)

‡ Another 76 patients received chemotherapy later during the course of the disease; 39 patients with esophageal cancer (21 squamous cell and 18 with adenocarcinoma) and 37 patients with gastric adenocarcinoma.

The thrombotic risk in esophageal carcinoma was 10 times higher than in the Dutch population [SMR: 10.6 (6.8-14.4)], as 2.73 cases instead of the observed 28 were expected based on age- and sex-specific incidence rates from the general Dutch population. The thrombotic risk in gastric cancer patients was 15 times higher than expected (42 observed and 2.73 expected) in the Dutch population [SMR 15.2 (13.7-22.4)].

Table 2. Risk of thrombosis in patients with squamous and adenocarcinoma (esophagus and stomach) of the upper gastrointestinal tract and in patients with either sqamous or adenocarcinoma of the esophagus.

	VT‡ Yes	VT No	Total	HR	95% CI
All Patients					
Squamous cell carcinoma	11	308	319		
Adenocarcinoma	59*	622	681	2.63	1.38-5.00
Esophageal cancer patients					
Squamous cell carcinoma	11	308	319		
Adenocarcinoma	17‡	199	216	2.47	1.16-5.29

‡ Of the patients with a venous thromboembolism, 26 had a pulmonary embolism; 13 patients with esophageal cancer (7 squamous cell and 6 adenocarcinoma) and 13 patients with an adenocarcinoma of the stomach.
* Three patients with an adenocarcinoma had a second venous thrombosis (VT) during the course of their disease, 1 with esophageal cancer (‡) and 2 with gastric carcinoma.

Among the 319 patients with a squamous cell carcinoma we observed 11 (3.4%) cases with venous thrombosis, whereas in 681 patients with an adenocarcinoma we found 59 (8.7%) cases with venous thrombosis (Table 2). The incidence rate among the patients with a squamous cell carcinoma was 1.9 per 100 person-years. Incidence rates among patients with an adenocarcinoma were 4.8 per 100 person-years of follow-up. The hazard of venous thromboses was 2.6-fold increased in patients with adenocarcinoma versus patients with squamous cell carcinoma (HR 2.63, 95% CI: 1.38-5.00, Table 2.).

Among 110 patients who received chemotherapy, 15 (13.6%) developed thrombosis, versus 55 (6.2%) out of 890 who did not receive chemotherapy (HR 2.40 95% CI 1.30-4.41). This difference was restricted to those with gastric cancer, amongst whom 11 of 57 with chemotherapy developed venous thrombosis, versus 31 out of 408 who did not. There was no difference in venous thrombosis in patients with or without radiotherapy (data not shown).

We analyzed survival in the 1000 cancer patients using as predictor variables age, sex, metastatic disease, esophageal or gastric carcinoma, squamous or adenocarcinoma, surgery, radiotherapy and chemotherapy. In the Cox regression model, risk of death was increased 2.1-fold in those who had experienced thrombosis (95% CI 1.64-2.88), adjusted for histological type of cancer, metastatic disease, surgery, radiotherapy and chemotherapy, and localization.

Discussion

This study shows a high risk of venous thrombosis in patients with upper gastro-intestinal tract cancer, with an annualized risk 3.9 %, and a cumulative incidence at one year of 5.6%. Patients with an adenocarcinoma of the upper gastrointestinal tract had a 2.6-fold higher risk of thrombosis than patients with a squamous cell carcinoma in the same organ site. Development of venous thrombosis also negatively affected survival.

Although many studies have shown an increased risk of thrombosis in malignancies (6) with in particular a high estimated relative risk in patients with an adenocarcinoma (8), estimations of the incidence for different types of cancer have rarely been made. In a previous study we showed an increased risk of venous thrombosis in patients with adenocarcinoma of the lung versus squamous lung tumors (9). In that study we also found reduced survival in those with thrombosis (9).

The mechanism of the thrombogenicity of adenocarcinomas may be the increased expression and shedding of aberrant mucins (10;11) which may bind to P- and L-selectins on platelets and leucocytes. This leads to platelet activation and aggregation and initiation of blood coagulation as suggested by the work of Wahrenbrock *et al*. (12). Another explanation may be that in the blood of adenocarcinoma patients circulating microparticles –of which some possibly arise from the tumor cells themselves- may express active tissue factor, the primary initiator of the coagulation cascade, and thus contribute to the coagulopathy observed in such patients (13).

Among the postulated mechanism for anti-cancer therapy-related venous thrombosis are the release of procoagulants and cytokines by chemotherapy-damaged tumor cells (14), or vascular endothelium damage by chemotherapy or radiotherapy (15;16) . In accordance with our previous findings in lung carcinoma patients (9), we found an increased risk of thrombosis during chemotherapy in the patients with an adenocarcinoma arising from the

stomach, but not in patients with a carcinomas arising from the esophagus. The latter may be explained by the small group of esophageal cancer patients (< 10 %) who were treated with chemotherapy, for in another study an increased risk during chemotherapy was shown for patients with a squamous cell carcinoma (17).

This study supports the widespread believe that patients with adenocarcinoma have a higher risk as compared to squamous cell carcinoma, and there are strengths but also limitations to the study. The large sample size and the single centre study are strong points of this study. However, the incidence of venous thrombosis may even have been underestimated by us as the diagnosis of a fatal pulmonary embolism may well have been missed at the time of death. Autopsy was only performed in a small number of patients and would have provided more precise information about the true incidence of pulmonary embolism

In conclusion, patients with an adenocarcinoma are more likely to develop thrombosis than patients with squamous cell carcinoma in the upper gastrointestinal tract. In recent years, adenocarcinomas are more frequently diagnosed than squamous cell carcinomas in the upper gastrointestinal tract. The incidence of thrombosis may thus increase due to this rise in incidence of adenocarcinoma in the upper gastro-intestinal tract and the more widely applied intensified treatment consisting of a combination of chemotherapy and radiotherapy. For this reason, the use of thrombosis prophylaxis should be reconsidered in those patients with the highest risk to develop venous thrombosis, and weighed against the risk of bleeding episodes in this group of patients.

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6 Microparticle-associated tissue factor activity: a link between cancer and thrombosis

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Abstract

Background*:* Cancer, in particular mucinous adenocarcinoma, is associated with venous thromboembolism (VTE). Tissue factor (TF), initiator of coagulation, plays a central role in the paradigm that clotting and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. Expression of TF in tumors is associated with poor differentiation and poor prognosis.

Patient/methods: We investigated the association between clinically manifest VTE and procoagulant properties of circulating microparticles (MP) isolated from blood of unselected pancreatic and breast adenocarcinoma patients, consecutive subjects, who presented with ultrasound or CT-scan confirmed VTE, and healthy subjects.

Results*:* Patients with disseminated breast and pancreatic cancer had significantly increased levels of MP-associated TF activity compared with healthy controls, subjects with idiopathic acute VTE and non-metastatic cancer patients. Patients with both high MP-associated TF-activity and MP-associated epithelial mucin (MUC1) had a lower survival rate at 3–9 months follow-up than those with low TF-activity and no MUC1 expression: the likelihood of survival was 0.42 (95% CI: 0.19–0.94) for an individual with these two predictor variables present, after adjustment for other factors (age cohort, type of cancer, VTE) in the Cox proportional hazards model.

Conclusions*:* Our results suggest an important role for MP-associated TF and MUC1 in the pathogenesis of thrombosis in disseminated mucinous adenocarcinoma patients. Future studies should reveal the mechanism underlying the observed associations.

Introduction

The link between activation of the blood coagulation system and malignancy dates from 1865, when Armand Trousseau first recognized the association between cancer and thrombosis (1). The incidence of thrombosis is high in adenocarcinomas such as ovarian, prostate and gastro-intestinal carcinoma (2,3), but is particularly high (up to 57%) in patients with pancreatic cancer (4–6). However, the cause of this association is still unknown. Thrombosis often precedes the diagnosis of cancer and is associated in cancer patients with a detrimental course of the disease (7). This supports the paradigm that coagulation and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. The strikingly poor prognosis of pancreatic cancer, and the high incidence of venous thromboembolism (VTE) support the hypothesis that local or systemic hypercoagulability confer a growth advantage to tumor cells (8).

Although abnormal coagulation profiles have been found in cancer patients, such abnormalities did not correlate with the development of thrombosis (9). Attention has been focused on tissue factor (TF), a transmembrane receptor protein, which is not only the primary initiator of coagulation but also promotes tumor growth, angiogenesis, and metastasis (10–12). Many tumor cells express high levels of TF and in several types of cancer, including breast cancer and pancreatic cancer, TF expression on tumor cells correlated with grade and tumor progression (13–15). Only Kakkar *et al*. (16) reported on the presence of circulating TF antigen in cancer patients, although the significance of this finding has not been clarified.

TF has been demonstrated on circulating microparticles (MP), small membrane vesicles that are released from cells following activation or during apoptosis (17–19). MP have been found in the circulation of healthy subjects (20). Elevated numbers of MP were found in patients with a variety of diseases associated with hypercoagulability and vessel wall injury (21–23). One of the most intriguing questions is whether specific MP express functionally active TF and form a critical determinant in thrombus formation.

We hypothesized that MP may initiate blood coagulation locally or at distant sites via functionally active TF expressed on these MP and that, in part, MP derived from malignant epithelial cells (24,25) may contribute to this process. We are the first to report on elevated MP-TF activity in cancer patients and the association with venous thrombosis (26).

Patients and methods

Cancer patients and controls

We investigated unselected cancer patients with non-resectable locally advanced (*n* = 4) or metastatic (*n* = 19) pancreatic cancer (11 males and 12 females, median age 59 years, range: 42–70), primary breast carcinoma before and 2 months after surgery of the breast tumor (*n* = 10, median age 50 years, range: 28–72), and breast carcinoma patients at the time of presentation of distant metastases (*n* = 17, median age 52 years, range: 28–72). In addition, we investigated 37 healthy subjects (16 males, 21 females, median age 43 years, range: 23–68) and seven subjects (three males, four females, median age 53 years, range: 24–72) who presented with ultrasound or CT-scan confirmed VTE, without a known history of cancer at the time of blood collection. Patients with a primary breast carcinoma had tumors of the histological type 'ductal adenocarcinoma' (one-third of patients had grade I, one-third had grade II and one-third had grade III tumors), and the tumors varied in size between 1 and 5 cm [staging according to the American Joint Committee on Cancer (AJCC) using TNM classification, in which T stands for tumor, N for lymph nodes and M for metastases]. In two of the 10 primary breast carcinoma patients, three axillary lymph nodes that contained tumor cells were found after surgery. In the other eight primary breast cancer patients, axillary lymph nodes did not contain tumor cells. Thus, the group of 10 patients with primary breast carcinoma was classified as AJCC stage I-II patients. The 17 patients with metastatic breast carcinoma were all found to have metastases at distant sites, either in lung, liver and/or bone and were classified as AJCC stage IV patients. The metastases were all established by conventional methods, including chest X-ray, liver ultrasound, bone scintigraphy and CT scan. Of the patients with pancreatic cancer, four patients had metastases in locoregional lymph nodes as well as in the superior mesenteric artery, whereas the other 19 patients had metastases at distance in various different organs. Thus, the pancreatic cancer patients were all classified as having AJCC stage III-IV pancreatic cancer. The pancreatic carcinomas were grade I, II and III respectively, each in about one-third of patients. Two patients with metastatic pancreatic cancer had primary surgery (Whipple procedure) for their tumor more than 1 year ago.

In all patients blood samples were collected before receiving any chemotherapy to preclude these agents affecting the number and type of MP. The number of platelets was always within the normal range and did not differ between the groups. None of the healthy subjects or patients used anti-inflammatory, antihypertensive or antineoplastic agents at the time of blood sampling.

Healthy controls and non-disseminated breast cancer patients were all alive at the end of the study, with a median follow-up of 36 months (32–48). Median survival after collection of the blood samples was 12 months (range: 1–44) in metastatic breast cancer patients and 3 months (range: 1–13) in pancreatic cancer patients.

All individuals signed an institutional review board approved consent. Tumor type was pathologically confirmed in all cancer patients; measurable disease was confirmed by CT scan.

MP isolation

Blood samples were taken from the antecubital vein, without tourniquet, into a 4.5-mL tube containing 0.105 mol L^{-1} citrate (Becton Dickinson, San Jose, CA, USA). Cells were removed by centrifugation for 20 min at 1550 × *g* at room temperature. In the supernatant plasma (source of MP) no platelets could be detected using a Sysmex 2100 Coulter counter and Phase contrast microscopy. The plasma was immediately snap frozen in liquid nitrogen and stored at −80°C for the isolation of MP as previously described (27). For the measurement of MP-associated TF activity (MP-TF activity), MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate. MP prepared from fresh and deep frozen plasma from the same donor did not differ in numbers, phenotype and associated TF activity. Therefore all assays were performed on MP isolated from deep frozen plasma. This allowed comparison of MP from different individuals in the same exercise.

Flow cytometric analysis of MP

Flowcytometric analysis of MP was performed using Allophycocyanin (APC)-labelled AnnexinV and cell-specific monoclonal antibodies (mAb) or isotype-matched control antibodies labelled with phycoerythrin (PE) or fluoresceinisothiocyonate (FITC): anti-CD61-PE (Y2/51, IgG_1) mouse IgG_1-PE (X40) and mouse IgG_1-FITC (X40) from BD Biosciences/Pharmingen (San Jose, CA, USA), anti-CD66e-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, the Netherlands), anti-CD14-PE (CRIS-6, IgG_1) from Biosource (Camarillo, CA, USA), anti-CD62e-PE (HAE-1f, IgG_1) from Kordia (the Netherlands, Leiden), antiglycophorin A-PE (JC159, IgG_1) from Dako A/S (Denmark, Glostrup), anti-TF-FITC (4508CJ) from American Diagnostics Inc. (Greenwich, CT, USA) and the negative control mouse IgG_1-FITC (clone X40) from BD Biosciences/Pharmingen (San Jose, CA, USA)

for anti-TF-FITC (the thresholds for measurement of MP-TF expression were set based on MP samples incubated with exactly the same concentration of the isotype-matched FITC-labelled mouse IgG_1control antibody) and antihuman epithelial membrane (MUC1) antigen-FITC (B24.1, IgG_1) that recognizes a multiple protein epitope (Biomeda, Foster City, CA, USA). MP were double- or triple-stained with AnnexinV-APC, PE-labelled cell-specific mAb, and anti-MUC1-FITC or anti-TF-FITC mAb, extensively washed to remove non-specific binding and analyzed for 1 min with Cell Quest software (Becton Dickinson, San Jose, CA, USA). The total MP population was defined on the basis of forward scatter and side scatter by which a gate was set to identify the single MP population. Scatter parameters were calibrated using polystyrene microspheres (size: 0.45, 0.70 and 1.09, 2 and 3 micron microspheres). Machine setting was optimized for optimal performance with respect to scatter analysis of small size particles. MP were defined as the population within the gate set on the basis of forward and side scatter, and subsequently the two and three color profiles of the selected populations recorded. The number of MP L^{-1} plasma was calculated as previously described (27).

Confocal laser microscopy of antibody-labelled MP

Isolated MP were incubated with anti-MUC1-FITC and anti-CD61-PE, centrifuged (30 min, 17 570 *g*, 20 °C) and analyzed by confocal laser scanning microscopy (Zeiss LSM 510; Zeiss, Jena, Germany) using established procedures.

Tissue factor activity assay

The TF activity in MP preparations was measured at room temperature by determining the FVII-dependent factor Xa (FXa) generation in the presence of excess negatively charged phospholipids under conditions that the TF concentration is rate limiting. Twenty-five micromolar dioleoylphosphatidylserine DOPS):dioleoylphosphatidylcholine (DOPC) (10:90) vesicles were incubated in 10 mM Hepes, pH 7.45, 137 mM NaCl, 4 mM KCl, 5 mg mL^{-1} ovalbumin, 50 nM hirudin and 6 mM $CaCl_2$ for 15 min. To 100 µL of this solution 20 µL MP-suspension was added and incubated for 15 min before 40 µL 5 nM FVII (or buffer) was added. After 10 min, 25 µL 2.5 mM S2765 was added and the reaction started by adding 40 µL 250 nM FX. The absorbance at 405 nm (expressed in mAbs) was recorded for 90 min, and plotted as a function of time (t) and, after correction for the absorbance in the absence of FVII, as a function of t^2. The slope of the latter curve is a measure for the rate of FXa generation and expressed as mAbs min^{-2} or as fM FXa min^{-1}. In all samples FXa

generation was measured both in the presence and absence of FVII and in the presence and absence of excess (125 μg mL^{-1}) polyclonal rabbit anti-huTF IgG (28) to establish the FVII- and TF-dependence of the FXa generation. Removal of DOPC/DOPS from the reaction mixture did not affect TF activity importantly. The MP-associated TF activity was calculated from the difference in the rate of Xa generation in the absence and presence of anti-TF antibodies and reported as the MP-associated TF-activity (fM Xa min^{-1}) in plasma. DOPS and DOPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Circulating mucins

Circulating mucins Ca 15.3 and Ca 19.9 (normal values ≤ 28 kU L^{-1} and ≤ 37 kU L^{-1}, respectively) were determined in plasma utilizing commercially available immuno-enzymatic assays on an IMx (Abbott Diagnostica, IL, USA). Both assays are routinely performed to measure different isoforms of these mucins.

Statistical analysis

Different groups of individuals (i.e. healthy controls vs. cancer patients, or vs. metastatic cancer patients) were compared with respect to numerical variables (number of total MP and MP subsets and MP-TF activity) by non-parametric Wilcoxon and Mann–Whitney tests. Numerical variables in primary breast cancer patients (preoperative and immediately postoperative) were compared using a paired Student's *t*-test. A Spearman's correlation test was performed to analyze the correlation between MP-TF activity and total number of MP, and MP-TF activity and presence of MUC1-expressing MP. *P* values < 0.05 were considered significant. Kaplan–Meier survival curves were created, groups compared on unadjusted survival by log-rank test and multivariate Cox proportional hazard models constructed. Analyses were performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

MP-associated TF activity in healthy subjects and cancer patients

TF activity associated with MP was studied by measuring FVII-dependent and anti-TF-IgG sensitive generation of FXa in isolated MP (Figure 1). The observation that MP-dependent FX activation was completely FVII and TF dependent indicated the absence of other FX activators such as the so-called cancer procoagulant in the MP preparation (29).

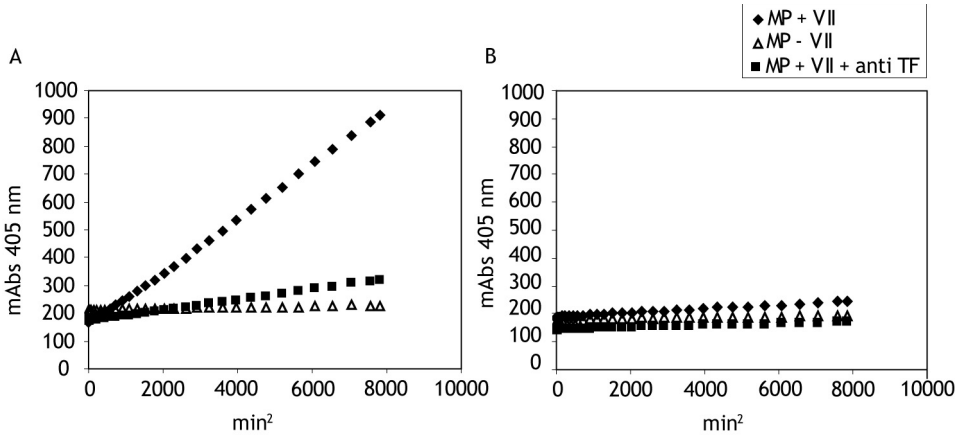

Figure 1. Tissue factor (TF) activity on microparticles (MP). Kinetic assay measuring factor Xa (FXa) formation, expressed as 405 nm absorbance units (mAbs or mAbs 405 nm). MP-associated TF activity in two patients with metastatic pancreatic carcinoma. One patient presented with venous thrombosis of the leg at the time of diagnosis and blood collection (A), while the other patient did not present with thrombosis (B). The slope of the line, which plots the mAbs at 405 nm against min^2 measures the rate of FXa formation (fM min^{-1}). MP + FVII (◆), MP – FVII (△), MP + anti-TF + FVII (■).

In MP isolated from 37 healthy subjects, a mean MP-associated TF activity level of 132 ± 47 fM Xa min^{-1} was found (Figure 2) and TF activities > 273 fM Xa min^{-1} (mean + 3 SD) were considered to be elevated. In patients with primary breast carcinoma, MP-TF activity was slightly elevated in 2/10 patients before and in 0/10 patients after surgery of the primary tumor (Figure 2, C + D). The two patients with elevated MP-TF activity before breast surgery both developed an active tumor following the initial surgery. One patient was diagnosed with a breast carcinoma in the other breast, whereas the other patient developed metastases at distant organs. All other primary breast cancer patients remained disease-free up to 4 years following surgery.

MP-TF activity in the metastatic breast and pancreatic carcinoma patients was increased compared with that in healthy controls ($P < 0.004$). In 5/17 disseminated breast carcinoma (Figure 2E) and in 8/23 pancreatic carcinoma patients (Figure 2F) elevated MP-TF activity was found; there was no correlation between MP-TF activity and the number of MP ($r = -0.22$, $P = 0.170$).

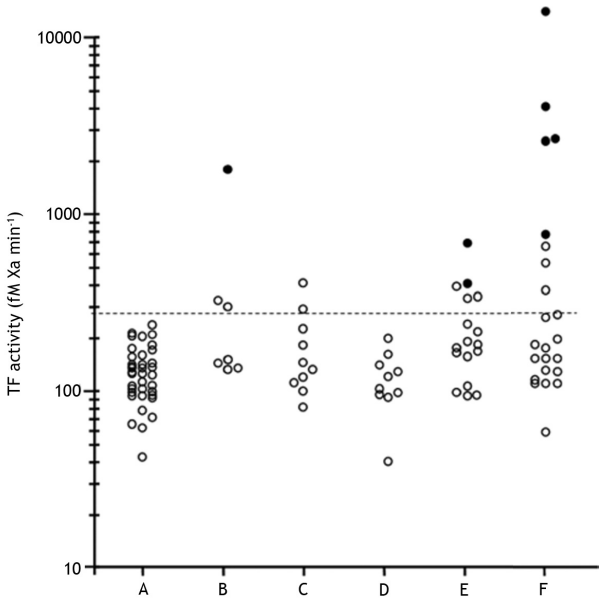

Figure 2. Microparticles (MP)-associated tissue factor (TF) activity in healthy subjects and cancer patients. (A) Thirty-seven healthy subjects. (B) Seven patients with idiopathic VTE without a history of cancer at the time of presentation and collection of blood sample. One of these patients (filled circle) was diagnosed with disseminated mucinous adenocarcinoma within the next month and thus no longer classified as having an idiopathic venous thromboembolism (VTE). (C) and (D) Ten early stage, non-disseminated breast cancer patients before (C) and after (D) surgical removal of the primary breast tumor. (E) Seventeen consecutive patients with metastatic adenocarcinoma of the breast. (F) Twenty-three consecutive patients with locally advanced or at distance metastasized adenocarcinoma of the pancreas. Filled circles (E + F) represent MP-associated TF activity in seven of these breast and pancreatic cancer patients who presented with VTE at the time of blood collection. The dotted line represents the upper limit of the normal range (99th percentile of > 273 fM Xa min^{-1}, i.e. mean plus 3 SD, as measured in 37 healthy subjects).

MP-associated TF activity and venous thrombosis

In the seven AJCC stage III and IV metastatic cancer patients who presented with VTE at the time of referral (six patients with deep VTE and one patient with PE), the mean and median MP-TF activity (Figure 2E,F, filled circles) was 3643 fM Xa min^{-1}, respectively 2620 fM Xa min^{-1}; range: 410–14 180 fM Xa min^{-1}. In the combined group of AJCC stage III and IV metastatic breast and pancreatic cancer patients, who did not develop thrombosis

(Figure 2E,F, open circles), the mean and median MP-TF activity was 209 fM Xa min^{-1} and 170 fM Xa min^{-1}, respectively, range: 59–665 fM Xa min^{-1}. The MP-TF activity per 10^6MP (expressed as fmol min^{-1} 10^{-6}) was up to eighteenfold higher in the disseminated cancer patients with clinically manifest VTE than in cancer patients without VTE ($P < 0.001$).

The median survival of metastatic breast and pancreatic cancer patients who presented with VTE was strikingly short (2 months; range: 1–2) compared with that of metastatic breast (13 months; range: 1–44) and pancreatic cancer patients (4.5 months; range: 1–13) without thrombosis ($P = 0.002$).

MP-TF activity was also measured in seven patients without a known history of cancer at the time of blood collection, who presented with an acute idiopathic thrombosis (two patients with deep vein thromboses and five patients with pulmonary embolisms) (Figure 2B). The median MP-associated TF activity in 6/7 patients was 148 fM Xa min^{-1} (range: 135–331 fM Xa min^{-1}). In the seventh patient with thrombosis of both legs, an elevated MP-TF activity of 1812 fM Xa min^{-1} was found (Figure 2B, filled circle). This patient was diagnosed with a disseminated mucinous adenocarcinoma within the next month, indicating that an elevated MP-TF activity might serve as a predictive marker for the presence of disseminated mucinous adenocarcinomas.

Number and cellular origin of MP

Table 1 shows the concentrations of circulating MP in healthy subjects and patients with cancer measured by two- or three-color flow cytometry. The median concentration of circulating AnnexinV-positive and Annexin V-negative MP together in patients with metastatic breast or pancreatic cancer was significantly higher than that in primary breast cancer patients and about 2-fold higher than that in healthy subjects ($P < 0.001$). In all individuals, > 90% of circulating MP binds to AnnexinV, indicating the presence of phosphatidyl serine on their membranes. Of the $AnnexinV^+$-MP, more than 90% expressed the platelet antigen CD61, whereas < 5% expressed the erythrocyte antigen glycophorin A. In about one-third of metastatic breast and pancreatic cancer patients and in 10% of healthy subjects and primary breast cancer patients the granulocyte antigen CD66e was found. In < 10% of the individuals, MP expressing the monocyte antigen CD14 were found, whereas in < 5% of the individuals, MP expressing the endothelium antigen CD62e were found. TF expressing MP were found in healthy subjects as well as in patients with disseminated breast and pancreatic cancer. We were able to study co-expression of TF antigen with CD61. The number of TF^+-$CD61^+$-MP varied between approximately 200 and 800×10^6 L^{-1} in the healthy subjects and the different groups of patients.

Table 1. Number, cellular origin and composition of microparticles (*10^6 L^{-1}) in healthy subjects and in cancer patients with different stages of adenocarcinoma of the breast or pancreas

	Controls	Breast cancer			Pancreatic cancer
		Early stage		Metastatic	
		Preoperative	Postoperative		
Total annexin V^+MP					
Median	1600	2560	1900	4900#	5600#
Range	720-9000	1650-9000	970-4500	1400-11 000	2350-13 200
Total annexin V^- MP					
Median	310	440	300	470	250
Range	130-650	100-800	130-530	140-1500	110-2100
Annexin V^+CD61^+-MP					
Median	1500	2400	2000	4100#	5600#
Range	700-7100	1300-9000	1000-4500	1300-9300	1800-12 700
Annexin V^+CD66e^+-MP					
Median	31	64	25	104	82
Range (% patients)	6-490 (13)	(10)	(10)	12-2060 (24)	13-1060 (38)
Annexin V^+TF^+-MP					
Median	255			290	460
Range (% patients)	130-560 (22)	< 1	< 1	115-2700 (29)	240-1550 (46)
Annexin V^+MUC1^+-MP					
Median		98		410#	310#
Range (% patients)	< 1	(10)	< 1	50-4100 (65)	100-500 (57)

The total number of microparticles (MP) in cancer patients was compared with that in healthy subjects; #indicates that MP are significantly increased ($P < 0.001$), whereas in all other cases MP are not significantly different from that in healthy subjects. In patients with early stage breast cancer the number of MP slightly decreased after surgical removal of the breast tumor ($P = 0.26$).

In 63% of the disseminated breast and pancreatic cancer patients, epithelial (tumor) cell antigen MUC1-expressing-MP were found. None of the healthy subjects or patients with idiopathic VTE had circulating $MUC1^+$-MP except the patient with VTE who was later diagnosed with a mucinous adenocarcinoma. Only in one patient were $MUC1^+$-MP detected pre- and postoperatively, which could indicate that this particular patient had a higher tumor load than the other patients. Furthermore, in this patient the postoperative blood sample was drawn before the start of adjuvant chemotherapy, which was administered

MP-TF activity, MUC1-positive MP and circulating mucin in relation to presence of VTE in cancer patients

Twenty-one (91%) of the pancreatic cancer patients had elevated CA19.9 (range: 91 to 191 207 kU L^{-1}, normal upper limit 37 kU L^{-1}) and 13 (76%) of the disseminated breast carcinoma patients had elevated CA15.3 (range: 45–638 kU L^{-1}, normal upper limit 28 kU L^{-1}) in their plasma. Similar levels were found before and after MP depletion. These antigens were not detectable in the resuspended MP pellets.

Elevated MP-TF activity correlated with the presence of $MUC1^+$-MP (r = 0.38, P = 0.01). All seven pancreatic cancer patients with elevated MP-TF activity also had $MUC1^+$-MP and five out of seven (71%) presented with thrombosis. Four out of five (80%) of the patients with disseminated breast cancer and elevated MP-TF activity had $MUC1^+$-MP, of whom two presented with thrombosis.

We compared the survival for patients using predictor variables like age, gender, type of cancer, MP-TF activity dichotomized on elevated (> 99th percentile) TF activity > 273 fM Xa min^{-1}, $CD61^+$-MP, $MUC1^+$-MP, and circulating mucins dichotomized on elevated CA15.3 and CA19.9 levels. After stratifying patients by type of cancer, or by age quartile, Kaplan–Meier survival curves were created, and multivariate Cox proportional hazard models constructed. Independent variables included were age, gender, occurrence of venous thrombosis, MP-TF activity, $CD61^+$-MP and $MUC1^+$-MP. In the analyses (overall chi-square of model $P < 0.0001$), the likelihood of survival at 3–9 months follow-up was lower for those with a TF activity > 273 fM Xa min^{-1}, and detectable mucin on MP (RR for survival 0.42, 95% CI: 0.19–0.94; and RR 0.41, 95% CI: 0.19–0.89, respectively) adjusting for the other factors (age cohort, type of cancer, VTE) in the Cox proportional hazards model. In the multivariate model, none of the variables mentioned above, namely age, gender, occurrence of venous thrombosis, $CD61^+$-MP and $MUC1^+$-MP, contributed significantly to the prediction of survival with the exception of MP-TF activity. The cumulative survival curve of patients with pancreatic carcinoma with either elevated or non-elevated MP-associated TF activity is shown in Figure 4; the survival of pancreatic patients with an elevated MP-TF activity was significantly decreased as compared with that in patients with non-elevated MP-TF activity (P = 0.0004).

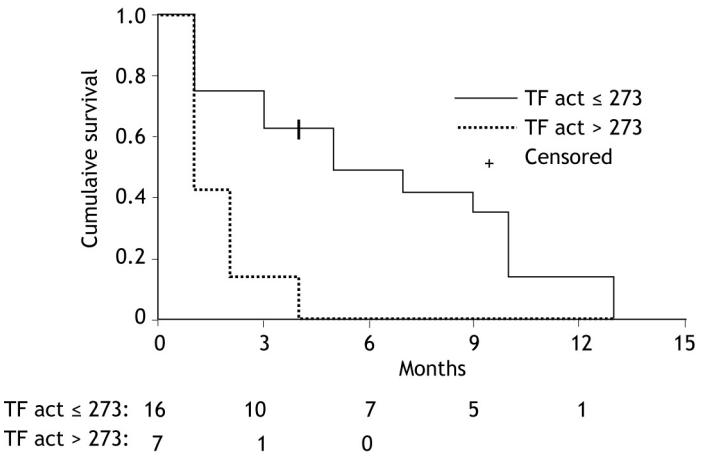

Figure 4. Cumulative survival of pancreatic cancer patients according to the presence of elevated or non-elevated microparticle-tissue factor (MP-TF) activity (> of ≤ 273 fM Xa min^{-1} MP-TF activity). The cross indicates the censored patient still alive at the time of analysis of the survival data. The likelihood of survival was least for pancreatic cancer patients with elevated MP-TF activity (> 273 fM Xa min^{-1}) as compared with those who did not ($P = 0.0004$).

Discussion

This is the first time that MP-TF activity was measured in cancer patients and that the association of MP-TF activity with venous thrombosis is reported. Patients with disseminated breast and pancreatic cancer, who presented with acute VTE, had higher levels of MP-TF activity than healthy subjects, cancer patients without VTE and subjects with idiopathic VTE. MP-TF activity correlated with the presence in the blood of MP expressing the epithelial antigen MUC1. Metastatic breast and pancreatic cancer patients with elevated MP-TF activity and detectable $MUC1^+$-MP had a lower survival rate at follow-up than those with normal MP-TF activity and $MUC1^+$-MP absent, suggesting that the properties of circulating MP are an important determinant of the link between cancer biology and thrombosis.

MP-associated TF activity is a quantitative estimate of the concentration of TF in the MP preparation, which can act as cofactor of FVIIa in FX activation. In contrast, the number of TF^+-MP is not a quantitative estimate of the TF concentration, as the number of TF molecules per MP may vary widely. Also, part of the TF antigen on MP might be

encrypted (not active as cofactor of FVIIa in FX activation, but detectable by the mAb used in the FACS analysis) (30–32).

The observed association between the levels of MP-TF activity and the development of VTE in metastatic adenocarcinoma cancer patients suggests that *in vivo*-generated MP, which can initiate coagulation via the TF-mediated pathway, contribute to the development of thrombosis. Such a hypothesis is supported by the observation that MP expressing TF and PSGL-1, the ligand for P selectin on platelets, accumulated in the developing thrombus in living mice as demonstrated by *in vivo* imaging in real time (33). The elevated MP-associated TF activity observed in pancreatic cancer patients corresponds to TF concentrations sufficient to shorten clot formation of plasma *in vitro* (34). Whether the minute amounts of MP-associated TF activity observed in the plasma of healthy volunteers (0.5–4 fM) are sufficient to stimulate fibrin formation is unknown; TF-dependent FXa generation might be too low to overcome the natural thresholds of the anticoagulant systems (35).

In patients with progressive mucinous cancer tumor-derived $MUC1^+$-MP may display enhanced binding to P and/or L selectin on platelets or other hematopoietic cells or on MP derived from such cells (36,37). Study of the co-expression of MUC1 and platelet antigen CD61 on MP by confocal immunofluorescence microscopy revealed that a small part of circulating MP seemed to result from fusion of cellular vesicles originating from malignant epithelial cells and platelets in patients with disseminated breast and pancreatic adenocarcinoma. Because of the requirement of anionic phospholipids for initiation and propagation of TF-dependent coagulation, it seems likely that only cell- or MP-bound forms of intravascular TF will support thrombin formation. Fusion of these MP may contribute to de-encryption of TF, especially in the presence of negatively charged phospholipids. MP-TF may be derived from tumor cells, but could also be transferred from monocytes to platelets or originate directly from platelet-derived MP (38).

$MUC1^+$-MP were mainly found in patients with disseminated breast and pancreatic cancer, who also had elevated levels of non-MP-bound mucins. Importantly, MUC1 is a glycoprotein that is overexpressed in aberrant forms in epithelial cancers like breast and pancreatic cancers. Mucins may be involved in adhesion and metastasis of tumor cells, whereas cell surface sialylation of tumor cells has been implicated in activation of leucocytes and aggregation of platelets. Wahrenbrock *et al*. (39) suggested that mucins play an important role in the development of VTE in cancer patients and provided a new explanation for the association between mucin-producing carcinomas and a specific

form of hypercoagulability (thrombophlebitis migrans) clinically presenting as platelet microthrombi. Our findings may indicate that besides soluble mucins (39) and tumor cell-bound mucins, also microparticle-bound mucin could play a critical role in the formation of thrombi.

In conclusion, patients with metastatic breast and pancreatic cancer who presented with thrombosis, carry MP that after isolation can initiate blood coagulation *in vitro* because of the presence of active TF on their membranes. The observed association between MP-associated TF-activity and the development of VTE in metastatic cancer patients underscores the possibility that *in vivo*-generated tumor-derived MP initiate coagulation via the TF-mediated pathway. Future studies should confirm our observations and reveal the precise mechanism underlying the observed associations. Greater understanding of the vicious circle between cancer and hypercoagulability may offer new targets for antithrombotic therapy.

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7 Case –control study identifying microparticle-associated tissue factor activity as a biomarker of cancer-specific thrombosis

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Submitted

Abstract

Objective

Cancer-related venous thrombosis (VT) is associated with poor survival. Microparticles (MP) released from cells bearing active tissue factor (TF), may be involved in the pathogenesis of thrombosis.

Methods and results

We designed a case-control study in which unselected cancer patients with VT (*n* = 51) were matched according to several criteria (age, sex, disease type and stage, and cancer-specific treatment) with a corresponding set of control cancer cases without VT (*n* = 49). MP were isolated from blood and MP-TF activity was measured as factor VIIa-dependent factor Xa generation (normal value < 273 fM Xa/min). Low MP-TF activity was found in 49 controls (median 150 fM Xa/min, range 23-535) and in 27 cases with known thrombosis risk factor, *i.e.* chemotherapy or compression of veins by the tumor (median 131 fM Xa/min, range 19-506). By contrast, high MP-TF activity was detected in the 24 cases with cancer-specific coagulopathy (median 1,131 fM Xa/min, range 395-12,333). Median survival was significantly shorter in patients with elevated MP-TF activity (3.5 months) than in patients with normal MP-TF activity (10 months).

Conclusions

Our results show both shorter survival time and markedly elevated TF activity associated with circulating MPs as features of cancer-specific thrombosis. We propose that TF-containing MPs could serve as a biomarker of cancer-specific coagulopathy and disease progression.

Introduction

Cancer is associated with venous thrombosis (VT) (1,2), which often precedes the diagnosis of cancer and cancer patients who develop VT have a worse survival than cancer patients with the same type of tumor who do not develop VT (3). The strong association with particular malignancies, and the more aggressive course of the disease in those who develop VT, points to a tumor-specific cause of the observed hypercoagulability in patients with cancer. However, the vast majority of cancer patients will not develop VT, while the occurrence of VT is particularly associated with certain types of malignancy. Although elevated plasma levels of markers of coagulation activation have been observed in cancer patients (4), studies failed to demonstrate a difference in clotting factor profile between cancer patients who developed thrombosis and those who did not. The pathogenesis of the hypercoagulability in cancer has not been elucidated. Cancer patients who are exposed to chemotherapy treatment are known to develop VT, most likely mediated by endothelial damage caused by the individual cytotoxic agents. Young male patients with germ cell tumors for instance, are only at increased risk for development of VT during the period of chemotherapy treatment (5), but not prior to diagnosis of their tumor or after treatment.

Tissue factor (TF), a transmembrane-receptor protein and initiator of coagulation, plays a central role in the theory that clotting and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa (6;7). TF acts both as receptor and co-factor for factor VIIa and thus initiates the (extrinsic) pathway of coagulation (8). Complex formation of TF with factor VIIa also triggers intracellular signals involved in angiogenesis, cell migration and inflammation (9). Expression of TF is associated with poor differentiation of tumors, increased angiogenesis and poor prognosis (10).

Microparticles (MP), membrane microvesicles produced during activation or apoptosis of cells (11), have been isolated from blood of healthy individuals. Recent data indicate that MP are cellular effectors involved in cell-cell cross talk and multiple MP-cell interactions seem to occur, indicating that MP may act locally at the site of origin as well as at a distance. Alterations in the numbers and origin of blood MP have been suggested to be involved in promoting thrombus formation in various clotting disorders and inflammatory conditions (12;13). MP may support prothrombinase activity (14) and express TF activity (15;16) and MP isolated from various patient populations have been reported to support in vitro coagulation (17;18). Small clinical studies demonstrated that

MP levels are elevated in individuals with thrombosis (19), whereas impaired membrane vesiculation in Scott syndrome is accompanied by decreased numbers of circulating MP and a bleeding tendency (20). MP isolated from human plasma, injected into rats were highly thrombogenic, and this effect was abolished by pre-incubation of MP with anti-tissue factor antibody.

In a previous study (7), we found that patients with adenocarcinoma arising from breast and pancreas, who presented with VT, had markedly elevated MP-TF activity and poor survival, compared to patients with the same type of cancer who did not develop thrombosis. The question then arises whether MP-TF activity is involved in the pathogenesis of cancer-related coagulopathy regardless of the origin of the tumor. To circumvent the need for a large sample size and cohort stratification we addressed this question by performing a case-control study in which MP-TF activity was analyzed in unselected cases with various types of malignancies who presented with a first episode of thrombosis. Cancer patients with VT were matched according to several criteria (age, sex, type of cancer, stage of the disease and subsequent cancer-specific treatments) with a corresponding set of control cancer cases without VT. Thus, pairs of patients (case and control) and consequently both groups of patients had identical a priori life expectancies.

Methods

Case- control design

In a case-control study design, we studied MP-TF activity in 100 cancer patients. Between mid 2003, and mid 2006, a total of 51 consecutive cancer patients who experienced a first episode of deep venous thrombosis of the leg or arm or pulmonary embolism and who did not have a family history of thrombosis were studied. VT was ascertained by echo Doppler and/or spiral computed tomography (CT).

For each cancer patient with VT, one eligible control was enrolled in the study who was matched for age ± 2 years, sex, type of cancer, stage of the disease and type of cancer-specific treatment, including the same chemotherapy regimen and previous cancer-specific treatments. To avoid genetic stratification, control cancer patients were matched for ethnicity and geographical area. We included 49 cancer patients as controls. One control was not matched for sex. For two cases with an adenocarcinoma of the lung we were not able to identify an appropriately matched control. Exclusion criteria were a

personal or family history of thrombotic disease, any other serious disease such as diabetes or renal insufficiency or use of anticoagulants.

Cancer patients who presented with VT were stratified based on the presence or absence of a known pre-specified risk factor for the development of VT, which were defined as: presence of compression of (large) veins due to tumor masses, administration of anticancer therapy i.e chemotherapy, hormonal therapy or antiangiogenic therapy, the presence of an indwelling venous catheter, and recent surgery or immobilization.

The Medical Ethics Committee approved investigation of blood MPs in patients with various types of cancer and different stages of their disease. All individuals gave informed consent. Thirty seven healthy subjects served as controls for blood cell count and MP measurements.

Patients

The diagnosis, classification and staging of the different types of cancer were based on standard diagnostic evaluation, including CT and magnetic resonance imaging of the body and histopathological examination of tumor tissue specimens. All cancer patients were staged using the final version of the American Joint Committee on Cancer (AJCC) staging system and followed-up in our department of Clinical Oncology with regular intervals until death or end of study (May 2007) with no patients lost to follow-up, thus enabling us to precisely assess the time of death. In all cases, mortality was due to cancer-specific death.

The 51 consecutive cancer patients who presented with thrombosis suffered from different malignancies: 27 had gastro-intestinal adenocarcinomas (esophageal carcinoma, colorectal carcinoma, pancreatic carcinoma and cholangiocarcinoma), 12 had genito-urinary tract tumors (renal cell carcinoma, prostate carcinoma and germ cell tumors of the testis), 8 had adenocarcinoma originating from various other organs including ovary, breast, lung, and adrenal gland, 2 had squamous cell carcinomas of the head and neck and 2 patients had an osteosarcoma (Table 1). Forty-one (80%) of the patients who presented with thrombosis had an adenocarcinoma originating from different organs, whereas 10 patients did not have an adenocarcinoma (2 patients with squamous cell carcinoma, 6 with a malignant germ cell tumor of the testis and the 2 patients with osteosarcoma).

Table 1. Demographics and MP-TF activity in 51 cancer patients with VT, 49 cancer patients without VT and 37 healthy subjects

	Cases			Controls	Healthy Subjects
	All cases	Pts with cancer-related VT	Pts with chemotherapy- or vein compression- related VT		
	n = 51	*n* = 24	*n* = 27	*n* = 49	*n* = 37
Sex (M/F)	28/23	12/12	16/11	25/24	16/21
Age (yr)					
median	62	63	61	60	43
range	30-83	46-77	30-83	20-82	23-68
Tumor type					
gastro-intestinal tumors	27	17	10	27	NA
genito-urinary tumors	12	1	11	12	
various adeno CA	8	5	3	6	
squamouscell CA	2	1	1	2	
osteosarcoma	2	0	2	2	
Adenocarcinoma	43 (84%)	23 (96%)	20 (74 %)	40 (82 %)	NA
Distant metastasis	46 (90%)	20 (83 %)	26 (96 %)	44 (90 %)	NA
Survival (months)					
median	7	2	13	12	NA
range	1-48^{+}	1-29	1-48^{+}	1-48^{+}	
Platelet counts (10^9/L)					
median	258	302	227	258	ND
range	44-678	118-678	44-389	123-521	
Total MP (10^6/L)					
median	4.3	4.2	4.7	4.1	1.9
range	1.4-15.6	2.2-15.6	1.4-13.5	1.3-13.6	0.9-9.8
MP-TF activity(fM Xa/min)					
median	168	1,131	131	150	132
range	19-12,333	395-12,333	19-506	23-535	85-179

Laboratory investigations

Blood samples were obtained from cases at the time of VT diagnosis and before the start of anticoagulant therapy and from controls for measurement of MP number and MP-TF activity. Blood samples were collected from healthy subjects as reported previously (7). Plasma samples were coded after collection and stored as described below. The average storage time for plasma of cases, controls and healthy subjects was similar. The samples were analyzed blinded as to case control status.

MP isolation

Blood was collected in 1/10 volume of 3.2 % trisodium citrate (Becton Dickinson, San Jose, CA) and platelet-poor plasma prepared immediately by centrifugation for 20 min at 1550 × g at room temperature. Platelet-poor plasma (source of MP) was carefully removed, snapfrozen in liquid nitrogen and stored at −80ºC for future analysis.

MP-containing plasma was thawed in melting ice, centrifuged at 17,570 x g at 20ºC, washed, and resuspended in 1/10 of the original volume buffer (154 mM NaCl, 1.4 mM sodium phosphate, 10.9 mM trisodium citrate, pH 7.4). This MP preparation was used for FACS analysis. For the measurement of MP-associated TF activity, MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate.

Enumeration of MP by Flowcytometry

Flowcytometric analysis of MP was performed using APC-labeled Annexin V (BD Biosciences Pharmingen, San Jose, CA). MP were stained with Annexin V and analyzed for 1 minute with Cell Quest software (Becton Dickinson, San Jose, CA), and identified by their characteristic forward and side scatter, and by annexin V binding to anionic phospholipids. Annexin V bound to the majority (>90 %) of circulating MP of patients and healthy subjects. The number of MP/L plasma was calculated as previously described (21).

Tissue factor activity assay

The TF activity in MP preparations (MP-TF activity) was measured in 96 wells plates at room temperature by determining the FVII-dependent factor Xa (FXa) generation as previously described (7). In all samples FXa generation was measured both in the presence and absence of FVII and in the presence and absence of excess polyclonal rabbit anti-

huTF IgG (22) to establish the FVII- and TF-dependence of the FXa generation. Results are reported as MP-TF activity/ ml plasma (fM Xa/min). MP-TF activity is reported as the FVII-dependent, anti-TF sensitive factor Xa generation. The median and mean TF activity in the MP isolated from the plasma of healthy subjects did not differ (129 versus 132 fM Xa/min) and MP-TF activities higher than the mean + 3 SD (> 273 fM Xa/min) were considered to be elevated.

Statistical Analysis

We compared means and proportions and constructed confidence intervals based on normal and binomial distributions. Kaplan-Meier curves were constructed to assess survival and Cox models were used to calculate hazard ratios. All analyses were performed using SPSS for Windows, version 14.0 (SPSS Inc, Chicago, IL)

Results

Patient characteristics

In Table 1 characteristics of the 100 cases and controls, and 39 healthy subjects are shown. None of the patients (cases or controls) received hormonal therapy or antiangiogenic therapy which is known to be associated with increased risk for VT. None of the patients had an indwelling venous catheter, recent surgery or immobilization. Of the 51 cases who developed thrombosis, 24 patients did not have any pre-specified risk factor for the development of VT like chemotherapy or tumor related vein compression. In these 24 patients the occurrence of thrombosis was assumed to be attributable to a cancer-specific coagulopathy. In the remaining 27 patients the VT was attributable to chemotherapy (*n* = 22) or tumor related vein compression (*n* = 5; Table 1). There was no difference with respect to age or presence of distant metastases between cases with cancer-specific coagulopathy or chemotherapy c.q. vein-compression-related thrombosis (Table 1). Furthermore, platelet levels were within the normal range and did not differ between cases and controls, although platelet counts were somewhat lower in the cases with a pre-specified risk factor for thrombosis (Table 1).

Forty-six (90%) of the patients who presented with thrombosis had distant metastases at the time of VT and blood collection (Table 1). The percentage of patients with an adenocarcinoma was higher in the 24 cases without (96%) than in the 27 cases with a pre-specified risk factor for thrombosis (74%, Table 1).

Number of MP and MP-associated TF activity in healthy subjects and cancer patients

Table 1 shows the concentrations of circulating MP in cases and controls. The total number of MP did not differ between the cases and controls, but was more than 2-fold higher than in healthy subjects (Table 1). The median MP-TF activity in the 49 cancer patients without VT (controls) was 150 fM Xa/min, range 23-535 fM Xa/min (Table 1; Figure 1). The majority of these patients had a normal MP-TF activity. The four control patients (8%), who had slightly elevated MP-TF activity (290, 350, 336 and 535 fM Xa/min) all received chemotherapy treatment. The median MP-TF activity in the 51 cancer cases with VT was 168 fM Xa/min, range 19-12,333 fM Xa/min (Table 1).

The median MP-TF activity in the 27 cases with chemotherapy treatment or vein compression as pre-specified risk factors for thrombosis was 131 fM Xa/min, range 19-506 (Table 1, Figure 1). Twenty three had normal MP-associated TF activity levels, whereas 4 had elevated MP-TF activity (288, 342, 355 and 506 fM Xa/min) and all 4 received chemotherapy.

In contrast, all 24 cases with VT only related to the malignancy had markedly elevated MP-TF activity (median MP-TF activity 1,131 range 395-12,333 fM Xa/min) (Table 1; Figure 1). The 2 patients with lung adenocarcinomas for whom no control could be found, had MP-TF activity of 574, respectively 1812 fM Xa/min. MP-TF activity was 10.2-fold higher in these 24 cases than in the 27 cases with pre-specified risk factors or patients without VT (HR=10.24 CI 95 6.24-16.35). There was no association between MP-TF activity, the total number of MP, nor with platelet counts in the groups of cancer patients.

Survival of cancer patients

Twenty eight (28 %) of the 100 cancer patients were still alive at the end of follow-up with a median follow-up of 35 months (range 12-48^{+} months). All deaths were cancer-specific. Survival in 51 cancer cases with VT was 7 months (range 1-48 months), and in 49 cancer controls 12 months (range 1-48^{+} months) (Table 1).

Median survival in patients with elevated MP-TF activity (n = 32) was 3.5 months (range 1 to 43 months) and significantly shorter when compared to that of patients with non-elevated MP-TF activity (median 10 months, range 1 to 48^{+}).

Survival of 24 of the 51 cases in which thrombosis seemed directly related to the presence of their malignancy was much shorter (median 2 months, range 1-29) than that of 27 of the 51 cases with a pre-specified risk factor for thrombosis (median 13 months,

range 1 – 48+) and of the 49 matched cancer controls without thrombosis (median 12 months, range 1 – 48+) (Table 1; Figure 2).

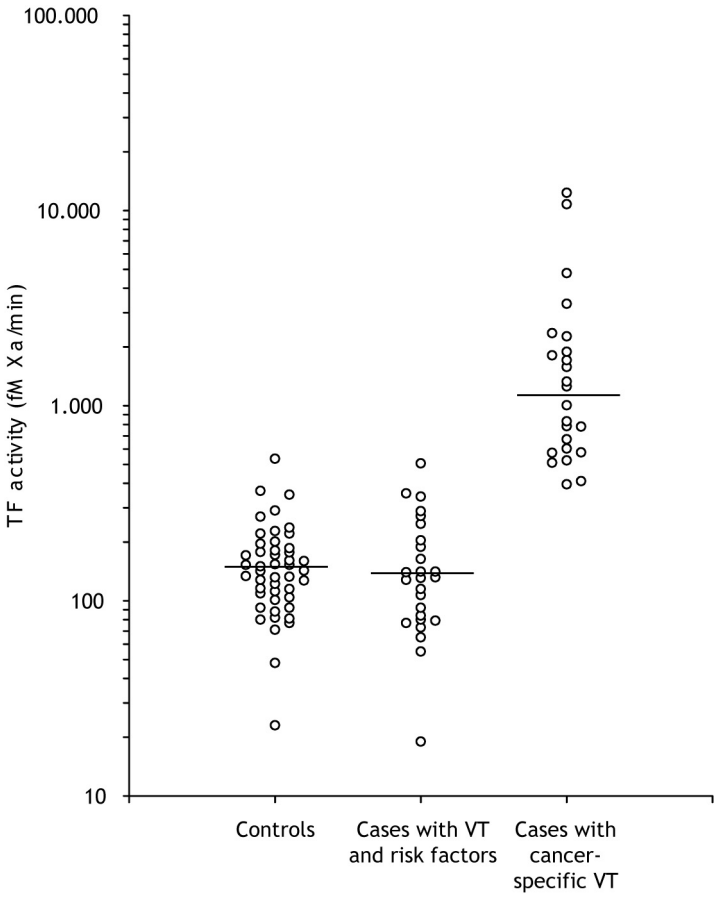

Figure 1. MP-associated TF activity in cancer controls without VT and cancer cases with VT. MP-associated TF activity in 49 cancer patients without VT (Controls), 27 cases with pre-specified risk factors for VT (Cases with VT and risk factors) and 24 cases with VT only related to the malignancy (Cases with cancer-specific VT).

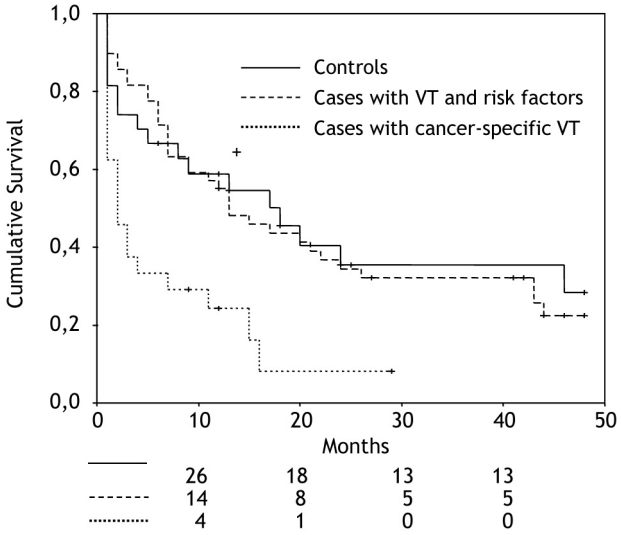

Figure 2. Kaplan-Meier survival curve of patients with cancer. Cumulative survival of 49 control cancer patients without VT (Controls), 27 cases with VT attributable to pre-specified thrombosis risk factors, either chemotherapy or tumor-related venous compression (Cases with VT and risk factors) and 24 cases with cancer-specific coagulopathy (Cases with cancer-specific VT). A cross indicates censored patients still alive at the time of analysis of the survival data. The likelihood of survival was reduced for cases with cancer-specific coagulopathy (Cases with cancer-specific VT) as compared to cases with chemotherapy or tumor-related vein compression as cause for coagulopathy (Cases with VT and risk factors) and control cancer patients (Controls).

Discussion

To our knowledge, this is the first case-control study of the causes of venous thrombosis in unselected cancer patients in a single institution.

Markedly increased microparticle-associated TF activity was only observed in those patients in whom the malignant process seemed to be the cause of the hypercoagulability and who had a strikingly poor survival. This suggests a role for MP bearing active TF in triggering thrombotic events and a direct pathogenic mechanism with regard to the release of such MP and their mode of action.

Although it has been suggested that elevated levels of platelets may play a role in cancer-associated thrombosis (23), there was no clear difference in numbers of circulating platelets between the various groups of cancer patients and no association between numbers of platelets and MP-associated TF activity.

The high percentage (96%) of adenocarcinomas among the cases with VT follows from the high incidence of thrombosis for such malignancies. Of all patients referred to our centre about 50% have an adenocarcinoma. An explanation may be that the source of TF are epithelial cancer cells themselves, or that expression of mucins by adenocarcinoma cells or other factors also contribute to the coagulopathy (24;25).

In our population of cancer patients, the high MP-TF activity and poor survival in cases with cancer-related thrombosis is intriguing, particularly since microparticles carrying active TF may play a causative role in the development of thrombosis and perhaps also in the poor survival. In this study we did not address the size and origin of TF positive MP responsible for the MP-TF activity, but the observation that elevated MP-TF activity was found in all patients who presented with cancer-specific coagulopathy which was not attributable to chemotherapy or venous compression, points to a contribution of cancer cells themselves as cause of elevated MP-TF activity. Experimental studies suggest that cancer-specific genetic lesions (*e.g.* activation of K-ras and inactivation of p53) may impact the level of TF expression in tumor cells and affect the numbers of circulating MPs containing TF originating from cancer cells themselves (26). On the other hand it was proposed that circulating TF originates mainly from TF-expressing stromal cells surrounding the tumor cells (10). Another hypothesis is that tumor-derived MP display enhanced binding to platelet- or other hematopoietic or vascular cell-derived MP, perhaps via mucin-lectin interactions, leading to initiation and propagation of coagulation, fusion of MP and formation of MP rich in decrypted TF.

Cancer patients who develop venous thrombosis have been reported to have a poor prognosis (3;27) with thrombosis as an independent risk factor for survival in cancer patients. Levitan *et al*. (2) demonstrated that the mortality in cancer patients with thrombosis was higher than that of patients with cancer or thrombosis alone. One explanation could be that thrombosis occurs more frequently in patients with advanced disease. However, Blom *et al*. (28) showed that development of thrombosis is a risk factor for dying in lung carcinoma patients independent of the presence or absence of metastases. Recently, Chew *et al*. (29) also reported that VT is an independent predictor of decreased 2-year survival in breast cancer patients and -when stratified by initial

cancer stage- the effect was highest with localized or regional stage cancer compared with patients with metastatic disease.

Elevated MP-TF activity may serve as biomarker for cancer-related thrombosis and poor outcome of the disease but not for thrombosis related to chemotherapy or to tumor compression of veins. The detrimental course of the disease observed in cases without known thrombosis risk factor other than cancer itself, supports the hypothesis that hypercoagulability facilitates the aggressive biology of cancer and vice versa.

This study suggests that MP-TF activity might serve as a biomarker for cancer-related thrombosis and poor prognosis but the mechanism by which MP carrying active TF could contribute to thrombosis in cancer patients remains to be elucidated. The data underscore that occurrence of thrombotic events in cancer patients are not unrelated events, as the increasing likelihood of overt and symptomatic VT is inversely related to worsening of the underlying malignancy.

To further understand the pathophysiology of MP-TF activity in cancer patients with hypercoagulability, additional studies are required to unravel the intriguing relationship between cancer, thrombosis, MP-TF activity and poor prognosis.

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8 Microparticle-associated tissue factor activity, venous thrombosis and poor survival in pancreatic cancer patients

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In preparation

Summary

Background

Patients with pancreatic adenocarcinomas have a high incidence of venous thrombosis (VT). Histological findings in pancreatic carcinoma indicates that high grade tumours are associated with expression of tissue factor (TF) in tumour cells, suggesting that TF could indeed be involved in processes such as clotting, angiogenesis and metastasis, which determine the clinical outcome. Microparticles (MP), released from tumour cells expressing TF, might be a mediator of thrombosis in patients with pancreatic carcinoma.

Methods

62 patients with ductal adenocarcinoma of the pancreas, 22 patients with locoregional disease and 40 with distant metastatic disease were studied. VT was confirmed by ultrasound or CT-scan. MP were isolated from blood and MP-associated TF activity determined. In 27 patients, immunohistochemistry was performed to assess expression of TF in tumour tissue.

Results

Thrombotic events developed in 12 of the 62 patients (7 cases with deep vein thrombosis, 3 cases with pulmonary embolism and 2 cases with both). There was no difference between the total number of MP, platelet-derived MP and platelets in pancreatic cancer patients with or without thrombosis. All patients with VT had elevated MP-TF activity with a median of 1891 fM Xa/min (range 510-12,344), whereas only 6 (12%) patients without VT had elevated MP-TF activity (median 463 fM Xa/min; range 334-855). There was no clear association between TF expression on the tumour and MP-TF activity or development of VT. Median survival following diagnosis of ductal pancreatic adenocarcinoma was 4 months (range 1-20) and 6 month-mortality was 30 % (n = 17). MP-TF activity and clinically manifest venous thrombosis were the only factors associated with poor survival.

Conclusions

Microparticles bearing active TF may play an important role in the pathogenesis of cancer-related thrombosis and may serve as prognostic marker for thrombosis and survival in patients with adenocarcinoma of the pancreas.

Introduction

Venous thromboembolism (VT) is common in patients with malignant disease (1-3) and numerous studies documented the association between cancer and VT. Although abnormalities in various proteins involved in the clotting cascade have been investigated, only known risk factors such as FV Leiden or prothombin gene mutation has been shown to increase the risk of VT two-fold (4), whereas elevated levels of FVIII has not been shown to play a major role in cancer-related VT.

The cause of the excessive risk in certain cancers has not been elucidated, but differences with regard to the excess risk for thrombosis exist between the various types of malignancies (4). Carcinoma of the pancreas, a relatively rare tumour of which in the last decades the incidence is increasing, seems to have an inherent and unique ability to induce a hypercoagulable state that leads to clinically significant thrombosis (5). Ogren *et al*. (6) reported a large series of 23,796 standardised autopsies performed between 1970 and 1982 and representing 84% of all in-hospital deaths in an urban Swedish population.The overall PE prevalence was 23%, and 10% of the population had a fatal PE. Forty-two per cent of pancreatic cancer patients had PE (OR 2.55; 95% CI 2.10-3.09). Adenocarcinoma and metastatic cancer were independently associated with PE risk (OR 1.27; 95% CI 1.16-1.4 and OR 1.10; 95% CI 1.01-1.20 respectively). They concluded that the risk of PE in cancer patients largely depends on cancer site and spread of the disease, but also on histological type and in particular reported an intriguing excess independent risk for pancreatic cancer warranting further research.

Recently, Tissue Factor (TF), initiator of coagulation, has been suggested to play a central role in the association between cancer and thrombosis. Studies indicated that perhaps TF as well as membrane-bound TF, *i.e*. microparticle **(**MP**)**-associated TF (7), may be one of the mechanism by which tumour growth and clotting form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. Expression of TF is associated with poor differentiation of tumours and poor prognosis (8;9) but also cancer patients who develop thrombosis have a poor survival as compared to those that do not (10;11). Patients with adenocarcinoma of breast or pancreas were found to have significantly increased levels of MP-associated TF activity in their circulation and MP-associated TF activity correlated with VT and the presence of circulating MP expressing the epithelial antigen MUC1 -most likely derived from malignant cells (7). Patients with high level of TF-activity on MP that also expressed mucin had a significantly lower survival

rate than those with low TF-activity and no mucin expression. Thus, TF and MUC1 on MP may be factors which play a decisive role in the pathogenesis of the prothrombotic state in disseminated mucinous adenocarcinoma patients.

Patients with mucinous adenocarcinomas of the pancreas are thus at high risk of venous thromboemblism, have a poor prognosis and short survival once the diagnosis is made, whereas effective systemic treatment options are of very limited value. For this reason, this patient population provides an unique opportunity to study the biological role of tumour-related compounds without intervention of other factors which may affect the occurrence of thrombosis, such as chemotherapy.

Interestingly, the endocrine, but not the exocrine, cells of the pancreas were found to synthesise and secrete active TF (12). Aadenocarcinomas arising from ductal exocrine, which often produce large amounts of mucins, are the most common malignancy in the pancreas, and in particular high grade pancreatic cancer have been reported to express tissue factor (13;14). The extracellular domain of TF is involved in initiating the clotting cascade, whereas the intracellular domain has been reported to be involved in angiogenesis and the process of cell invasion (15). Therefore, importantly, TF expressed in pancreatic ductal carcinoma cells may be involved in tumour progression, which may be related to the clotting activity of TF, but also to TF-mediated activation of angiogenesis and tumour cell invasion. Production and release of tissue factor may thus provide an explanation for the aggressive biological behaviour of pancreatic carcinoma.

We therefore decided to study the expression of TF in invasive pancreatic cancer, circulating MP bearing active TF and thromboembolic events in relation to survival time in pancreatic cancer patients.

Materials and methods

Patients

Sixty-two consecutive patients diagnosed between 2003 and 2007 with pancreatic adenocrcinoma were identified and evaluated to document the incidence of VT and the predisposing factors. Of these patients, 13 underwent pancreatic resections for pancreas ductal adenocarcinoma and plasma samples were collected pre-operatively with the tumour still in situ. Another 9 pancreatic cancer patients were diagnosed with locally advanced irresectable tumours, while 40 patients were diagnosed as metastatic disease.

In one patient we collected two samples, one sample before surgery and one sample at the time of metastatic disease. Staging of the diseases occurred by CT scan and in some cases also during exploratory surgery. Plasma samples were always collected before the start of any systemic treatment or before surgery. Tumour type was pathologically confirmed in all patients. All individuals signed institutional review board approved consent.

MP isolation

Blood was collected in 1/10 volume of 3.2% trisodium citrate (Becton Dickinson, San Jose, California) and prepared immediately by high-speed centrifugation as described previously (7). Platelet-poor plasma (source of MP) was carefully removed, snap frozen in liquid nitrogen and stored at −80ºC for future analysis. MP-containing plasma was, washed, and resuspended in 1/10 of the original volume buffer (154 mM NaCl, 1.4 mM sodium phosphate, 10.9 mM trisodium citrate, pH 7.4). For the measurement of MP-associated TF activity, MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate. Assays were performed on MP isolated from deep frozen plasma, allowing comparison of MP from different individuals in the same exercise as previously described.

Flow cytometric analysis of MP

Flowcytometric analysis of MP was performed using Allophycocyanin (APC)-labeled AnnexinV from BD Biosciences/Pharmingen (San Jose, CA) and cell-specific monoclonal antibody (mAb) or isotype-matched control antibody labeled with phycoerythrin (PE): anti-CD41-PE (clone P2, IgG_1), mouse IgG_1-PE (X40) from BD Biosciences/Pharmingen (San Jose, CA). MP were double-stained with Annexin V and anti-CD41-PE, analyzed for 1 minute with Cell Quest software (Becton Dickinson, San Jose, CA), and identified by their characteristic forward and side scatter, and by their ability to bind Annexin V and cell-specific moAb. The number of MP/L plasma was calculated as previously described.

Tissue factor activity assay

The TF activity in MP preparations was measured in 96 wells plates at room temperature by determining the FVII-dependent factor Xa (FXa) generation as previously described (7). Results are reported as the MP-associated TF-activity/ ml plasma (fM Xa/min). For all samples the FVII dependent FXa generation was found to be sensitive to anti-tissue factor antibodies. The median and mean TF activity in the MP isolated from the plasma

of healthy subjects (MP-associated TF activity) did not differ (129 versus 132 fM Xa/min) and TF activities higher than the mean + 3 SD (> 273 fM Xa/min) were considered to be elevated.

Circulating mucins

Circulating mucin Ca 19.9 (normal values ≤ 37 kU /L) were determined in plasma utilizing commercially available immunoenzymatic assays on an IMx (Abbott Diagnostica, IL, USA). The assay was routinely performed to measure different isoforms of these mucins.

Immunohistochemistry

Tissue sections of formalin-fixed, paraffin-embedded tumour biopsies or resection specimen of pancreatic tumour tissue were deparaffinised, after an overnight drying step, with xylene and rehydrated in increasing percentages of ethanol. After an antigen retrieval step, sections were incubated with the following mAbs or isotype-matched control antibodies: anti-mouse IgG_1 from BD Biosciences/Pharmingen (San Jose, CA), mouse-anti-human TF (4509) from American Diagnostica, (Stamford, CT) or mouse-anti-human epithelial membrane antigen (clone E29) from DakoCytomation (Glostrup, Denmark). Antibodies were diluted in Dako Real Antibody diluent to decrease background staining and avoid the need for additional blocking steps. After incubation, sections were washed in PBS and endogenous peroxidase was blocked in Peroxidase block solution (DakoCytomation, Glostrup Denmark) for 15 min. Thereafter, EnVision immunoenzymatic system was used. All sections were reviewed by two independent persons who were blinded with respect to the results of the MP-TF activity assays. TF and mucin expression was scored based on percentage of positively staining tumour cells as well as intensity of the staining.

Statistical Analysis

We compared means and proportions and constructed confidence intervals based on normal and binomial distributions. Kaplan-Meier curves were constructed to assess survival and cox models were used to calculate hazard ratios. All analyses were performed using SPSS for Windows, version 14.0 (SPSS Inc, Chicago, IL).

Results

Table 1 shows the patients characteristics of the 62 pancreatic cancer patients with and without VT. Twenty-one (33%) patients received chemotherapy after collection of plasma samples. In 13 (21%) pancreatic cancer patients, VT (*i.e.* in 8 patients deep vein thrombosis, in 3 pulmonary embolism, and in 2 both) was observed. Median survival following in the whole group of ductal pancreatic adenocarcinoma patients was 4 months (range 1-20 months) and mortality at 6 months was 31% (*n* = 19). Survival was not associated with age, sex, or chemotherapy.

Table 1. Characteristics of pancreatic carcinoma patients who did or did not develop VT.

	Patients with VT		**Patients without VT**	
	***n* = 12**	**(%)**	***n* = 50**	**(%)**
Surgical resection	1	(8.3)	12	(24.0)
Distant metastases	11	(91.7)	30	(60.0)
Alive at 3 months	2	(16.7)	32	(64.0)
Alive at 6 months	1	(8.3)	18	(36.0)
Overall survival				
median (mo)	1		5	
range (mo)	1-11		1-20^{+}	
CA 19.9 (UK/L)				
median	37,751		593	
range	5-197,000		1-56,365	
Platelets (x 10^9/L)				
median	323	(100)	238	(100)
range	168-678		120-581	
MP total (x 10^6/L)				
median	4,933	(100)	5,890	(100)
range	1,849-23,386		1,500-13,897	
P-MP (x 10^6/L)				
median	4,281	(100)	5,370	(100)
range	1,762-17,859		1,000-12,673	
MP-TF activity (fM Xa/min)				
median	1,891	(100)	134	(100)
range	102-12,344		120-581	

There was no difference between the total number of circulating MP and platelet-derived MP as well as absolute platelet count. Total number of circulating MP was not associated

with MP-TF associated activity, but there was a strong correlation between MP-TF activity and VT $P < 0.000$. Furthermore in all patients with VT, mucin-bearing MP were found whereas this was found in 28 % of the pancreatic cancer patients without VT. More pancreatic cancer patients with VT had metastatic disease as compared to those without VT (OR 7.97 CI 95% 0.95-66.5)

Patients with distant metastases in other organs and/or lymph nodes had a much shorter survival as compared to patients in whom no metastases were detected (estimated median survival 4.0 CI 95% 2.4-5.6 versus 12.0 CI 95% 9.67-14.32; $P < 0.001$); HR for death 3.0. Furthermore, survival was significantly reduced in patients with both elevated levels of circulating MP-associated TF activity and a thrombotic event, MP-associated TF activity and VT were independently associated with a short survival. Furthermore, mucin-producing potential as evidenced by expression of mucin in the primary tumour and/or secretion of CA19.9 mucin in the circulation, were found to be significant predisposing factors with respect to occurrence of VT or survival.

In the 27 available tumour specimens, in mucin was expressed by the tumour cells in almost all cases, but TF in slightly less than fifty percent of the tumours. In most cases a low number of tumour cells were found to express TF. In the 7 patients with elevated MP-TF activity of whom tumour specimens were available, in 55% tissue factor was expressed by tumour cells. Thus no clear correlation between MP-TF activity in a later stage of the disease and tumour TF expression in the original biopsies could be found in this small series. Only one tumour was poorly differentiated, precluding assessment of a correlation between tumour grade and TF expression. In 6 (50%) of the 12 patients who presented with VT and who all had elevated MP-TF activity, tumour specimens were available for TF expression; 2 (33%) of these six patients had TF expression in their tumour. In 3 patients with elevated MP-TF activity but no thrombosis, tumour specimens were available, and in 1 of the 3 tumour samples TF expression was observed in the tumour ducts.

Discussion

We found a high incidence of VT in patients with pancreatic adenocarcinoma and those patients who developed VT presented with markedly elevated levels of circulating MP-TF activity. The majority of patients with thrombosis had metastatic desease and only the malignancy itself as risk factors of thrombosis. Furthermore, MP-TF activity and occurrence of VT were associated with poor survival.

In contrast to Khorana *et al*. (13) and Nitori *et al*. (14) we found TF expression in a low percentage of pancreatic tumours. This discrepancy may relate to the limited number of tumour specimens available and sampling error due to low numbers of malignant cells present in most of the specimens. Mostly, tumour biopsies were rather small, and marked tumour cell heterogeneity present, accounting for a failure to find TF expressing cells if present. Similar to what has previously been described by Moberg *et al*. (12), we always found marked TF expression in the pancreatic islets, precluding that our staining method failed to detect TF. Perhaps, differences in semi quantitative scoring system applied by Khorana *et al*. (13) and Nitori *et al*. (14) explains the lower expression rate of TF in pancreatic carcinoma in our data. Independent of the TF expression by tumour cells in the original specimens, still selection of predominantly TF-expressing tumour cells may have occurred and those cells could have an advantage in the process of metastasis. When released into the circulation, such cells would contribute further to formation of microparticles and perhaps also to the observed thrombotic events and poor survival of the patients.

The role of tissue factor and its inhibitor in thrombosis is further substantiated by other findings, in which elevated circulating TF and tissue factor pathway inhibitor was found in patients with other diseases (16,17) such as cardiovascular diseases and diabetes mellitus. Elevated numbers of TF-expressing microparticles correlated with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Involvement of TF in the pathogenesis of increased risk to develop thrombosis also comes from observations made in women without cancer. Interestingly, Smith *et al*. (18) recently observed that out of 24 coagulation, anticoagulation, fibrinolysis and antifibrinolysis candidate genes, only the tissue factor pathway inhibitor gene was globally associated with the risk of VT in postmenopausal women. Although the findings of Smith *et al*. (18) cannot be generalized to men or younger women, their findings underscore that TF and its inhibitor protein may be important factors also in the well-recognized increased risk of VT in patients with cancer.

Future therapeutic intervention of in the clotting cascade that may be initiated in cancer patients by MP which carry active TF, possibly arising from pancreatic carcinoma cells themselves, could lead to abrogation of the clotting cascade and perhaps improvement of survival. One way could be by blocking the active site of TF with specific antibodies or by administration of candidate drugs for inhibition of TF activity *in viv*o, such as reactive site-inactivated factor VIIa. Patient selection should be done based on

the MP-TF activity assay of plasma. Furthermore, further investigation of polymorphism of genes involved in the clotting cascade is relevant to determine whether this modulates the risk to develop serious thromboembolic complications and poor survival.

In conclusion, this study indicates that unique intrinsic tumour procoagulant characteristics and perhaps tumour-derived microparticles bearing active tissue factor are present in pancreatic adenocarcinoma patients who suffer from thromboembolic complications. Measurement of circulating MP-TF activity identifies patients at high risk for VT and poor survival. Although we could not reproduce the TF expression on pancreatic cancer, TF represents an attractive therapeutic target in pancreatic cancer patients to serve as anti-tumour agent and anti-thrombotic agent and to approve survival in those patients.

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9 Summary and general discussion

The incidence of cancer is growing with increased ageing of people and cancer is now the leading cause of death in the West European countries and the US. As described in the introduction of this thesis, hypercoagulability seems to contribute to the two most frequent causes of death in cancer patients, metastasis and venous thrombosis. The poor prognosis of cancer patients who develop thrombosis forms a challenge to clinicians to select cancer patients at highest risk for development of thrombosis and to develop effective prophylactic strategies to prevent thrombosis and hopefully also improve survival.

Although the relationship between cancer and thrombosis has been known for more than a century, the mechanism by which tumour predispose to thrombosis has not been elucidated. The activation of blood coagulation in patients with cancer may well have several causes. Prothrombotic mechanisms may be related to the host's response to cancer and other factors to procoagulant properties of the cancer cells themselves.

The aim of this thesis is to investigate risk factors for cancer-related thrombosis (part I) and the role for micropaticles in the pathogenesis of cancer-related thrombosis (part II).

Part I

Chapter 2. We investigated the risk factors for venous thrombosis in cancer patients with implantable ports (arm or chest ports) who underwent chemotherapy in our hospital between 1994 and 2003. In this 10-year period, clinically overt thrombotic events, which were all confirmed by ultrasound or angiography, were counted. Catheter-related thrombosis cumulative incidence in patients who did not receive anticoagulants was 28% in patients with arm ports and 33% in patients with chest ports. In contrast, in patients who did receive anticoagulants the incidence was 32% in patients with arm ports and only 1% in patients with chest ports (odds ratio (OR) 34.8 95% confidence interval (CI) 7.3–165). Left-sided placement as compared to right-sided and catheter tip position in the superior *vena cava* as compared to placement in the right atrium were associated with a 3.5- respectively 2.6-fold increased risk of thrombosis. Blood samples were obtained from 101 patients for analysis of plasma concentrations of factor VIII, IX, XI, and for gene mutation analysis, namely FV G1691A and FII G20210A mutation. Thrombosis was associated with elevated homocysteine levels (OR = 3.8, 95% CI 1.3–11.3), but not

with factor V Leiden or prothrombin 20210A gene mutations, or high concentration of factor VIII, IX or XI. Based on our data, we concluded that prophylaxis with anticoagulants should be recommended when using chest ports, and that the use of arm ports for the administration of chemotherapy in cancer patients should be avoided. Determination of plasma homocysteine levels may identify patients at an increased risk for thrombosis but further research is needed to further substantiate these data and its clinical relevance.

Chapter 3. This chapter concerns a review of the incidence and risk factors of catheter-related thrombosis. Cancer as underlying disease, but also type and material of catheters are risk factors in the development of catheter-related thrombosis. Since the introduction of other materials for the production of central venous catheters, which seem to be less thrombogenic, and the increased use of oral anti-cancer agents precluding the need for permanent venous catheters, the incidence of catheter-related thrombosis is likely to decline. The issue of prophylactic anticoagulants therefore becomes less important in cancer patients.

Chapter 4. Risk factors of venous thrombosis in lung cancer patients are reviewed in this chapter. Lung cancer is the second most common cancer in Western countries and the leading cause of cancer death in men, but strikingly few papers on the phenomenon of venous thrombosis in lung cancer patients are found and data on mortality due to thrombosis are limited. Risk factors of venous thrombosis which were identified in lung cancer patients are histology, namely adenocarcinoma, presence of metastatic disease, pneumonectomy and anti-cancer therapy including chemotherapy and the use of anti-VEGF targeted drugs. Other risk factors identified include platelet counts (prior to systemic treatment) and in one case report active TF expressing circulating microparticles.

Chapter 5. Although adenocarcinoma was identified by us as a risk factor for venous thrombosis in lung cancer patients, no other studies were ever performed to estimate the risk of venous thrombosis in patients with either adenocarcinoma or squamous carcinoma originating in the same organ site. We therefore performed a follow-up study in patients with upper gastro-intestinal cancer, i.e. gastric and oesophageal carcinoma, to study the incidence of venous thrombosis in adenocarcinomas and squamous carcinomas arising in this organ site as described in this chapter. We performed a study in 1000 consecutive patients diagnosed with upper-gastrointestinal cancer in our hospital in the

period between 1980 and 2000. In these 535 oesophageal carcinoma patients, of whom 216 (40%) had an adenocarcinomas and 319 (60%) a squamous cell carcinoma, and 465 patients with adenocarcinomas originating in the stomach, the incidence of thrombosis was 10 respectively 15 times higher than in the Dutch population. The risk of thrombosis in patients with adenocarcinoma was 2.6-fold increased (HR 2.63, 95% CI: 1.38-5.00) compared to patients with squamous cell carcinoma. Survival was markedly decreased in patients who developed thrombosis (HR 2.13, 95 % CI: 1.64-2.88), irrespective of whether the tumour originated in the stomach or the oesophagus as compared to that in patients who did not develop thrombosis.

Part II describes several studies on microparticles, isolated from blood of cancer patients, and their role in the pathogenensis of cancer-related thrombosis. Micropatricles or apoptotic bodies vary in size between 100-1000 nm, and their numbers, cellular origin and chemico-physical properties seem to be dependent on the type of disease and in cancer patients on type of cancer, stage of the as well as factors contributing to the thrombotic event. For a long time, microparticles were considered to be cellular debris reflecting cellular activation or damage, but these microparticles are now known to interact with other cells and acquire a pathophysiologic potential. There are several lines of evidence supporting the procoagulant activity of microparticles and several small studies have demonstrated that microparticles levels are elevated in individuals suffering from thrombotic events. So far little is knownabout the role of microparticles in cancer-related thrombosis. Most of the data on numbers and cellular origin of microparticles have been obtained using flow cytometry. This technique offers the possibility to label microparticles with several fluorochrome-labelled antibodies and annexin-V and thus determine the cellular origin of the microparticles. However, different laboratorie use different methods for isolation and preservation of microparticles, precluding direct comparison of data obtained, which may in part explain the sometimes seemingly inconsistent or conflicting data. More recently, various groups are exploring the use of other techniques to detect MP isolated from blood of different individuals in order to quantify MP number and determine their characteristics.

Chapter 6. We investigated procoagulant properties of circulating microparticles isolated from blood of unselected pancreatic and breast adenocarcinoma patients, from consecutive subjects without cancer who presented with ultrasound or CT-scan confirmed

venous thrombosis, and from healthy subjects. Microparticles were examined by FACS analysis and microparticle-associated tissuefactor (TF) activity measured by determination of factor VIIa-dependent factor generation of Xa. Patients with disseminated breast and pancreatic cancer had increased levels of microparticles-associated TF activity as compared to healthy controls, subjects with idiopathic acute venous thrombosis and cancer patients without metastatic disease. Patients with elevated microparticles-associated TF-activity and microparticles-expressing the epithelial mucin antigen MUC1 had a lower survival at 3–9 months follow-up than those with low MP-TF activity and no expression of mucin antigen on their MP: the likelihood of survival was 0.42 (95% CI: 0.19–0.94) for an individual with these two predictor variables present, after adjustment for other factors (age , type of cancer, venous thrombosis) in a Cox proportional hazards model. Our results suggest an important role for microparticles-associated TF and mucin in the pathogenesis of thrombosis in disseminated mucinous adenocarcinoma patients.

Chapter 7. In a case-contol study, Microparticle-associated TF activity in blood isolated from 100 consecutive cancer patients was investigated. Cases were 51 unselected cancer patients who presented with radiologically confirmed venous thrombosis and controls were 49 cancer patients without thrombosis, who were matched for age, type and stage of cancer and type of cancer-specific treatment, including the precise chemotherapy regimens as well as previous cancer-specific treatments. An additional risk factor for development of venous thrombosis was defined as concurrent chemotherapy or compression of veins by tumour. Of the 51 cases, i.e. the cancer patients with venous thrombosis, 24 patients did not have an additional thrombosis risk factor, whereas 27 patients did have an additional risk factor. The median microparticle-associated TF activity in patients with additional risk factors was low and did not differ from that in the 49 control cancer patients without venous thrombosis. In contrast, microparticle-associated TF activity was elevated in all 24 cancer patients without additional risk factors and differed significantly from that found in all other cancer patients. Median survival in patients with thrombosis but without additional risk factor was significantly shorter than in patients with additional risk factors and control cancer patients, namely 1.9, respectively 13.1 and 12.2 months.

Chapter 8. The role of tissue factor was further investigated in 62 patients with pancreatic cancer in whom we assessed circulating MP-TF activity. We also assessed expression of TF by immunohistochemistry in the original tumours, of 27 patients of whom

tumour biopsies were available to us. Both factors were correlated with development of venous thrombosis and survival. Of the 62 patients, all with ductal adenocarcinoma of the pancreas, 22 patients had locoregional disease and 40 distant metastases. Thrombotic events developed in 12 (19%) of the 62 pancreatic carcinoma patients and was confirmed in all cases by ultrasound or CT-scan. Circulating microparticle-associated TF activity were analysed to assess predictors of survival following diagnosis. There was no difference between the total number of microparticles, platelet-derived microparticles and platelets in pancreatic cancer patients with or without thrombosis. All patients with venous thrombosis had elevated MP-TF activity, whereas only 6 (13.6%) patients without VTE had elevated microparticles, and MP-TF activity was significantly higher in patients who presented with VTE than in the other patients. In the 27 tumour specimens available for immunohistochemistry, only in a small percentage of tumours TF expression was observed and the extent of TF expression was not clearly associated with the grade of malignancy. Furthermore, no clear association was found between TF expression in the tumour and MP-TF activity. Mortality was associated with elevated microparticleassociated-TF activity and both MP-TF activity and occurrence of venous thrombosis significantly predicted poor survival.

In summary, in this thesis risk factors of thrombosis such as central venous catheters, the use of chemotherapy, FV Leiden and prothrombin gene mutation and abnormal levels of proteins involved in the clotting cascade were investigated in various groups of cancer patients. Although none of the studies performed in **part I** of this thesis explored the use of thrombosis prophylaxis, this should be considered in patients identified to be at highest risk for development of thrombosis, namely patients with an adenocarcinoma of the lung or gastro-intestinal tract, patients with a central venous catheters undergoing anti-cancer therapy, and the advantage of such a strategy should be weighed against the risk for bleeding episodes in this group of patients. In part II of this thesis, the presence of epithelial cell-derived, mucin-bearing microparticles in the circulation of cancer patients has been demonstrated and microparticles bearing active tissue factor were linked to cancer-related thrombosis and poor prognosis in patients with various types of carcinoma. In the coming years, additional studies in larger cohorts of cancer patients may help to define the role of circulating microparticle–associated TF activity as a potential biomarker for cancer-related thrombosis risk and poor prognosis in cancer patients. Such research is also needed to investigate whether active TF-bearing microparticles in cancer

patients are indeed the cause of thrombosis or a marker of a prothrombotic state and to unequivocally demonstrate whether the source of TF-bearing microparticles in cancer patients is the tumour cells themselves or other cells, including platelets, monocytes, activated endothelial cells or even fibroblasts.

10 Nederlandse samenvatting

Jarenlang werd aangenomen dat Armand Trousseau de eerste was die het verband tussen veneuze trombose en kanker beschreef, recentelijk werd echter aangetoond dat dit al in 1823 door Bouillaud geschiedde. Trousseau beschreef dat trombose vaak voorkomt bij patiënten met gastro-intestinale tumoren. Pas een eeuw later verschenen de eerste grote cohort studies, die deze observatie ondersteunen. Bij patiënten met trombose wordt vaker de diagnose kanker gesteld, terwijl omgekeerd, patiënten met kanker vaker trombose zullen ontwikkelen dan patiënten die geen kanker hebben. Verder blijkt dat kankerpatiënten met trombose een slechtere prognose hebben, dan kankerpatiënten die geen trombose ontwikkelen. Dit alles wijst er op dat stolling (stolsels) de groei van tumorcellen kan bevorderen.

Bekende risicofactoren voor het ontwikkelen van trombose zijn ondermeer bedrust, operatie, de pil, zwangerschap, en de periode van het kraambed, langdurige vliegreizen, en het hebben van aangeboren risico factoren zoals een mutatie in het factor V Leiden gen of een prothrombine 20210A genmutatie. Bij kankerpatiënten kunnen veranderingen van de bloedviscositeit door ontstekingsfactoren of hemodynamische veranderingen zoals stasis van bloed door tumorcompressie van buiten af op het bloedvat, een rol spelen. Verondersteld wordt dat ook verschillende andere mechanismen bij kankerpatiënten een rol kunnen spelen. Het betreft activatie van de stollingscascade wellicht geïnitieerd door de tumorcel zelf, specifieke effecten van bloedplaatjes, witte bloedcellen en/of endotheelcellen, en hoge concentraties van diverse cytokines (IL-1, TNF, VEGF). Het geheel kan resulteren in een toename van stollingsbevorderende eiwitten of afname van eiwitten die normaliter de afbraak van de trombus bewerkstelligen. Kankercellen zelf kunnen echter ook eiwitten produceren die rechtstreeks de stolling op gang kunnen brengen of bevorderen. Een goed voorbeeld hiervan is weefselfactor (“tissue factor”), de initiator van de stolling, of het zogeheten ‘cancer procoagulant’ eiwit.

De twee meest frequente oorzaken van het overlijden van kankerpatiënten zijn het hebben van metastasen en trombose. Het zou heel goed kunnen zijn, dat de verhoogde neiging tot stolling in het lichaam zelf een milieu vormt, waarin tumorcellen ook makkelijker kunnen uitzaaien in het lichaam (metastaseren). Weefselfactor en andere eiwitten zoals trombine zijn niet alleen betrokken bij de stollingscascade, maar lijken ook betrokken te zijn bij het metastaseringsproces van tumorcellen zelf. Anti-kanker therapie zoals cytostatica, hormonale therapieën, maar ook de nieuwere ‘targeted drugs’ kunnen de hemodynamische balans verder verstoren door veranderingen in de bloedvaten of directe beïnvloeding van de stollingscascade-eiwitten.

De incidentie van veneuze trombose in kankerpatiënten varieert in de literatuur en is in het bijzonder hoog bij patiënten met een pancreastumor (in de literatuur wordt een cumulatieve incidentie tot wel 57% gerapporteerd). Ook in patiënten met andere (adeno) carcinomen zoals tumoren uitgaande van de ovaria, long, prostaat en overige tractus digestivus tumoren, is de incidentie van kanker-gerelateerde trombose hoog. De gedachte is dat patiënten met een adenocarcinoom een hoger risico voor het ontwikkelen van trombose hebben dan patiënten met een andere histologische type tumor. Een directe vergelijking tussen de verschillende tumor typen is, met uitzondering van de studie van Blom et al, nooit eerder gemaakt.

Andere risicofactoren bij kankerpatiënten zijn het, bij deze groep patiënten vaker toegepaste gebruik van centraal-veneuze lijn, de vaak langdurige bedrust als gevolg van de slechte algemene conditie van de kankerpatiënt, en langere en intensievere operaties vergeleken met patiënten die geen kanker hebben.

Ondanks het feit dat veel kankerpatiënten (tot wel 80%) een abnormaal stollings-profiel hebben (bijvoorbeeld verhoogde concentraties van stollingsfactoren als fibrinogeen, factoren V, VIII, IX en XI en trombocytose), ontwikkelen zij niet allemaal trombose en is het mechanisme verantwoordelijk voor kanker-gerelateerde trombose tot op heden nog steeds niet goed opgehelderd.

Micropartikels, kleine subcellulaire fragmenten, werden in 1967 door Wolf voor het eerst aangetoond, en destijds ook wel “platelet dust” genoemd. Inmiddels is bekend dat circulerende bloedcellen, maar ook endotheelcellen in staat zijn micropartikels te vormen door cel-activatie of cel-dood (apoptose). Deze micropartikels variëren in grootte van 100 tot 1000 nanometer en worden gekenmerkt door de aanwezigheid van dezelfde antigenen op hun oppervlak als de cel waar ze van afkomstig zijn (de moedercel). Heel lang is gedacht dat micropartikels slechts celdebris zijn en geen pathofysiologische betekenis hebben. Er komen echter steeds meer aanwijzingen dat dit niet het geval is.

Bij patiënten met een aangeboren bloedziekte, die gekenmerkt wordt door een gestoorde membraan-vesiculatie (‘Scott syndroom’), wordt in het bloed een sterk verlaagd aantal micropartikels gevonden en juist deze patiënten presenteren zich met een verhoogde neiging tot bloeden. Daartegenover staan ziektebeelden die geassocieerd zijn met vaatwand beschadiging en verhoogde neiging tot stolling (hypercoagulabliteit), waarbij de aantallen micropartikels duidelijk hoger zijn dan die van gezonde mensen.

Flowcytometrie is wereldwijd de meest gebruikte methode om micropartikels te karakteriseren. Micropartikels kunnen worden geïsoleerd uit plaatjes-vrij plasma voordat

ze worden geïncubeerd met specifieke antilichamen of Annexine-V (dat specifiek bindt aan negatief geladen phospholipiden in aanwezigheid van calcium ionen), en waaraan een fluorochroom is bevestigd. Op deze manier kunnen antigenen, respectievelijk phosphatidylserines, die zich op het micropartikel oppervlak bevinden, gekwantificeerd worden met behulp van de flowcytometer. Het merendeel van de micropartikels bij zowel gezonde als zieke mensen, bindt Annexine-V en brengt CD61 of CD41 tot expressie en is dus afkomstig van bloedplaatjes. De door de verschillende auteurs gerapporteerde aantallen MP bij gezonde mensen lopen sterk uiteen. Deze grote verschillen lijken te worden verklaard door de verschillende technieken die de diverse laboratoria gebruiken voor de isolatie (wel of niet gesedimenteerd, centrifugatie snelheid), detectie en karakterisering van micropartikels (keuze en het gebruik van de verschillende cel-specifieke antilichamen). Helaas is er tot op heden nog geen richtlijn voor de beste techniek om aantallen MP betrouwbaar te meten. Door de verschillen in toegepaste methode is onderling vergelijken van de resultaten van verschillende laboratoria niet goed mogelijk. Soms leidt dit tot inconsistente of zelfs tegenstrijdige resultaten.

In 1999 toonden Giesen et al. aan dat *ex vivo* gevormde trombi niet alleen bloedplaatjes en fibrine, maar ook een aanzienlijke hoeveelheid weefselfactor bevatten. Door middel van elektronenmicroscopie met goudgelabeld anti-weefselfactor antilichaam konden zij grote clusters van weefselfactor antigeen-positieve membraan vesikels aantonen, die zich bevonden in de nabijheid van bloedplaatjes. Het bleef echter onduidelijk waar dit zogeheten “blood-born” weefsel factor vandaan kwam. De auteurs suggereerden destijds dat de weefselfactor wellicht afkomstig was van weefselfactor-positieve leukocyten of monocyten. Muller et al toonden door middel van elektronenmicroscopie aan dat weefselfactor antigeen aanwezig was in de alpha granula van bloedplaatjes, en dat - na stimulatie van bloedplaatjes – weefselfactor aanwezig was op het oppervlak van de plaatjes. Voorts onderzochten zij direct uit bloed geïsoleerde micropartikels en ook micropartikels afkomstig van bloedplaatjes, verkregen door middel van *in vitro* stimulatie. Zij toonden aan dat MP van bloedplaatjes functioneel inactief weefselfactor bevatten, maar na stimulering van de bloedplaatjes of bij toediening van MP, bleek dat de MP zelf weefselfactor-procoagulante activiteit bezaten. Zij suggereren dat dergelijke micropartikels afkomstig van gestimuleerde bloedplaatjes wellicht ook *in vivo* voorkomen en weefselfactor kunnen “vervoeren” naar verschillende delen van het lichaam, waardoor zij de stolling zowel locaal als op afstand kunnen initiëren. Rauch et al toonden aan dat monocytaire cellijnen die weefselfactor tot expressie brengen,

weefselfactor kunnen overbrengen op geactiveerde bloedplaatjes via micropartikels, en het mogelijk maken de trombus vorming te initiëren.

De eerste artikelen over een mogelijke rol van micropartikels bij de ontwikkeling van trombose bij kankerpatiënten, dateren uit de tachtigerjaren van de vorige eeuw waarin Dvorak aantoonde dat micropartikels afkomstig van tumorcellijnen stollingsactiviteit bezitten. Het lukte hem niet een relatie aan te tonen tussen de pro-coagulante activiteit van de micropartikels enerzijds en het ontwikkelen van trombose anderzijds. Na een "windstilte" van enkele tientallen jaren verschijnen daarna de eerste artikelen over micropartikels in relatie tot kanker in het begin van de eenentwintigste eeuw. In 2004 toonden Yu et al weefselfactor activiteit aan in micropartikels afgesnoerd van twee tumorcellijnen. Daarna toonden wij aan dat er een sterke associatie is tussen mircopartikels, trombose en kanker bij de mens. De toekomst zal moeten uitmaken in hoeverre micropartikels daadwerkelijk een belangrijke rol spelen bij het ontstaan van kanker-gerelateerde trombose.

Hoofdstuk 2. In dit hoofdstuk worden de resultaten getoond van een studie naar risicofactoren van centrale veneuze lijn trombose in kankerpatiënten, die chemotherapie krijgen toegediend via deze centraal veneuze lijn. In totaal zijn er 255 centraal veneuze lijnen ingebracht in 243 kankerpatiënten; 84 zogehete 'arm-poorten', ingebracht via een ader in de arm, en 171 zogehete 'borst-poorten', ingebracht via de vena jugularis of vena subclavia. Van de totale groep kregen 181 patiënten een vorm van antistolling, als profylaxe dan wel therapeutisch. Bij verdenking op een centraal veneuze lijn trombose, werd de trombose altijd bevestigd door middel van echografie of flebogram. Van de patiënten die geen antistolling gebruikten, ontwikkelden 28% respectievelijk 33% een arm-poort cq borst-poort centrale veneuze lijn trombose. Het gebruik van antistolling had geen invloed op het optreden van trombose bij patiënten met een arm-poort, maar was geassocieerd met een veel lagere incidentie van trombose bij de patiënten met een borst-poort (slechts 1% van deze patiënten ontwikkelde een trombose). Verder bleek er vaker trombose op te treden bij patiënten bij wie de borst-poort aan de linker zijde van het lichaam was ingebracht, en bij patiënten bij wie de tip van de katheter niet in het rechter atrium zat. Bij 101 patiënten werden bloedmonsters afgenomen voor de bepaling van factor V Leiden en prothrombine 20210A mutatie, de concentratie van fibrinogeen, de stollingsfactoren VIII, IX en XI en homocysteine. Bij deze 101 patiënten bleek er geen relatie te zijn tussen het optreden van trombose en een genmutatie van factor V Leiden

of een prothrombin 20210A, verhoogde spiegels van de stollingsfactoren VIII, IX of XI. Wel was er een relatie tussen verhoogde spiegels homocysteine en het optreden van centraal veneuze lijn trombose.

Hoofdstuk 3. Dit overzichtsartikel beschrijft de problemen rondom centraal veneuze lijn trombose in de algemene praktijk. Central veneuze lijnen worden frequent gebruikt door nefrologen in het kader van hemodialyse, hemato-oncologen voor een continue toegang tot de bloedbaan voor het geven van diverse bloedproducten, cytostatica en parenterale voeding, maar ook door cardiologen voor pacemakers bij ritme stoornissen. De oorzaak van centraal veneuze lijn trombose is multicausaal waarbij erfelijke factoren zoals factor V Leiden mutatie een rol spelen. Ook kankerpatiënten die chemotherapie krijgen toegediend blijken een grotere kans te hebben op het ontwikkelen van symptomatische trombose. De belangrijkste complicaties van centraal veneuze lijn trombose zijn longembolie en het ontwikkelen van een post-trombotisch syndroom. Het is onduidelijk of profylactische antistollingsbehandeling het optreden van veneuze lijn trombose doet verminderen, maar ook de behandeling van een lijn trombose zelf is controversieel. Zo zijn er geen gerandomiseerde studies waarin wordt onderzocht wat de beste behandeling is van een centraal veneuze lijn trombose. In de meeste cohort studies wordt er enige vorm van antistolling gegeven. Het gebruik van stolsels oplossende agentia (thrombolytica) wordt in de meeste studies ontraden gezien de hoge complicaties die daar bij optreden en wordt gereserveerd voor levensbedreigende situaties. Het is niet goed uitgezocht of het beter is de centraal veneuze lijn indien er sprake is van trombose, verwijderd dient te worden. Er is dan ook behoefte aan een studie waarin de optimale behandeling van een centraal veneuze lijn trombose wordt onderzocht.

Hoofdstuk 4. Longkanker is een van de meest voorkomende tumoren in de Westerse wereld, met de hoogste mortaliteit bij zowel mannen als vrouwen. In dit hoofdstuk worden de risicofactoren van veneuze trombose beschreven bij patiënten met longkanker. Risico factoren op het ontwikkelen van trombose bij longkanker patiënten zijn: het stadium van de ziekte (patiënten met vergevorderd stadium van hun ziekte hebben vaker trombose), uitgebreide longchirurgie zoals pneumectomie (chirurgische verwijdering van een long), antikanker therapie zoals chemotherapie, of de nieuwere “targeted drugs”, en het hebben van een adenocarcinoom. Ook het aantal trombocyten voor start van de antikanker behandeling en de aanwezigheid van MP die weefselfactor tot expressie brengen in de bloedcirculatie lijken geassocieerd met een hogere kans op trombose.

Hoofdstuk 5. De hypothese dat patiënten met een adenocarcinoom vaker trombose ontwikkelen dan patiënten met een ander histologisch type kanker, wordt ondersteund door bevindingen van grote studies, waaronder de Leidse MEGA studie, waaruit blijkt dat trombose het vaakst optreedt bij patiënten met een tumor uitgaande van de ovaria, long, prostaat en tractus digestivus, tumoren meestal van het type adenocarcinoom. Hoofdstuk 5 bekijkt het risico op het ontwikkelen van trombose bij 1000 patiënten met een tumor in het bovenste deel van de tractus digestivus. Het betreft 535 patiënten met slokdarmkanker, waarvan 216 (40%) patiënten een adenocarcinoom hebben en 319 (60%) een plaveiselcel carcinoom, en 465 patiënten met een adenocarcinoom uitgaande van de maag. Het risico op trombose in patiënten met slokdarm of maagkanker was 10 respectievelijk 15 keer hoger dan je zou verwachten in de Nederlandse populatie. Het risico op veneuze trombose bij patiënten met een adenocarcinoom (59/681) was 2.6-voudig verhoogd (HR 2.63, 95% CI: 1.38-5.00) vergeleken met het risico bij patiënten met een plaveiselcel carcinoom (11/319). De overleving van patiënten die trombose ontwikkelden was veel slechter dan van de patiënten die geen trombose kregen. Dit verschil bleek onafhankelijk van waar de tumor zich bevond, maag dan wel slokdarm.

Hoofdstuk 6. In dit hoofdstuk wordt de rol die micropartikels (MP) spelen bij de ontwikkeling van kanker-gerelateerde trombose onderzocht. Bloed van gezonde vrijwilligers (*n* = 37), patiënten met trombose maar zonder kanker (n=7) en patiënten met kanker (*n* = 80) is geanalyseerd met behulp flowcytometrie op aantallen MP en de expressie van bepaalde eiwitten (antigenen) op de MP. Met een functionele essay is onderzocht of de uit bloed geïsoleerde MP, weefsel factor (TF) activiteit (MP-TF activiteit) bezitten. De kankerpatiënten zijn in 4 groepen verdeeld, groep 1+2 betreft patiënten met een vroeg stadium mammacarcinoom bij wie voorafgaande aan en na de operatieve verwijdering van hun tumor, bloed werd afgenomen. Groep 3, patiënten met een vergevorderd stadium mammacarcinoom en groep 4, patiënten met een vergevorderd stadium pancreas carcinoom. Van deze laatste 2 groepen presenteerden 2 respectievelijk 5 patiënten zich met een veneuze trombose.

Patiënten met een vergevorderd stadium kanker hebben een verhoogd aantal MP in hun circulatie en een deel van de MP brengt het epitheliale antigen MUC1 tot expressie. Verder bleken zij een hogere MP-TF activiteit te bezitten in vergelijking met gezonde vrijwilligers, patiënten met trombose maar zonder kanker en kankerpatiënten in een vroeg stadium van hun ziekte. Kankerpatiënten met trombose hadden een sterk verhoogde

MP-TF activiteit. Patiënten met zowel MUC1 antigen-positieve MP als een verhoogde MP-TF activiteit hadden een slechte overleving in vergelijking tot de andere individuen.

Hoofdstuk 7. Naar aanleiding van de bevindingen in hoofdstuk 6, wordt in dit hoofdstuk verder gekeken naar de MP- TF activiteit bij verschillende type kankerpatiënten met en zonder trombose. Hiertoe zijn MP uit bloed van 51 kankerpatiënten met een trombose vergeleken met dat van controle kankerpatiënten, zonder trombose. De controle patiënten waren geselecteerd op leeftijd, geslacht, type tumor, stadium van de ziekte en therapie. Voor 2 patiënten met veneuze trombose kon geen geschikte controle patiënt worden gevonden. Naast het hebben van kanker, identificeerden wij risicofactoren voor het krijgen van trombose ('trombose risico factor'), zoals compressie van het bloedvat door de tumor en het ondergaan van een behandeling met chemotherapie.

Van de 51 kankerpatiënten met trombose hadden 24 patiënten geen 'trombose risico factor', terwijl 27 patiënten dit wel hadden. Kankerpatiënten (n=27) met 'trombose risico factoren' en de 49 controle kankerpatiënten zonder trombose hadden een normale of minimaal verhoogde MP-TF activiteit. De 24 kankerpatiënten met trombose, maar zonder 'trombose risico factor', hadden allen een verhoogd tot sterk verhoogde MP-TF activiteit. Opvallend was dat de meeste patiënten met trombose een adenocarcinoom hadden. De overleving van de kankerpatiënten met trombose en zonder 'trombose risico factor' was veel korter (2 maanden) dan die van de kankerpatiënten met trombose maar met een 'trombose risico factor' (13 maanden) en die van de controlegroep (12 maanden). Er is dus een duidelijke relatie tussen de MP-TF activiteit en overleving.

Hoofdstuk 8. Vooral patiënten met een pancreascarcinoom staan bekend om hun hoge incidentie van veneuze trombose. Histologisch onderzoek van pancreascarcinomen heeft aangetoond dat slecht gedifferentieerde tumoren vaker TF tot expressie brengen, wat suggereert dat TF betrokken is bij stolling, angiogenese en het biologische gedrag van de tumor. In dit hoofdstuk kijken we naar MP-TF activiteit, het voorkomen van veneuze trombose en de expressie van TF in de tumor. Twaalf van de 62 patiënten met een ductaal adenocarcinoom uitgaande van de pancreas ontwikkelden trombose. Immunohistologisch onderzoek kon bij 27 patiënten worden verricht. Er bleek geen verschil te zijn tussen de patiënten met en zonder trombose voor wat betreft de aantallen MP, de hoeveelheid MP afkomstig van bloedplaatjes en de totale concentratie bloedplaatjes in de circulatie. Alle 12 patiënten met trombose hadden een verhoogde MP-TF activiteit. Er was geen associatie

tussen TF expressie van de tumor zelf en het optreden van trombose. De mediane overleving van de patiënten was 4 maanden en de overleving was niet geassocieerd met leeftijd of geslacht, maar wel sterk geassocieerd met de MP-TF activiteit en het optreden van veneuze trombose.

Curriculum vitae

De auteur van dit proefschrift, geboren 5 oktober 1963 te Breda, voltooide in 1982 haar VWO diploma aan het Mencia de Mendosa Lyceum te Breda. Hierna begon ze aan haar studie geneeskunde aan de Rijksuniversiteit Leiden. In 1988 werd het doctoraal examen gehaald. Na een verlengde stage van haar co-schappen in de Dr. Daniel den Hoed kliniek te Rotterdam, werd het artsexamen (1991) behaald en werd er aansluitend gestart met de opleiding tot internist. Van 1992-1996, de eerste 4 jaren, in het Leijenburg ziekenhuis te 's Gravenhage (opleider Dr. J.C.M van der Vijver) en van 1996-1998 in het Academisch Ziekenhuis te Leiden (opleider Prof. Dr. A.E. Meinders). In 1999 werd zij opgeleid in het aandachtsgebied klinische oncologie (waarnemend opleider Dr. S. Osanto), resulterend in de registratie als medisch oncoloog in 2000. Eind 2002 is er gestart met het beschreven onderzoek, naast haar werkzaamheden als internist-oncoloog en latere staflid op de afdeling Klinische oncologie van het Leids Universitair Medisch centrum. Vanaf maart 2008 zal zij werkzaam zijn als internist-oncoloog in het Antoni van Leeuwenhoek ziekenhuis te Amsterdam. De auteur heeft samen met Olivier Mensonides 3 kinderen; Martijn, Boris en Eline.

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