Risk factors of thrombosis in cancer : the role of microparticles

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1 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.

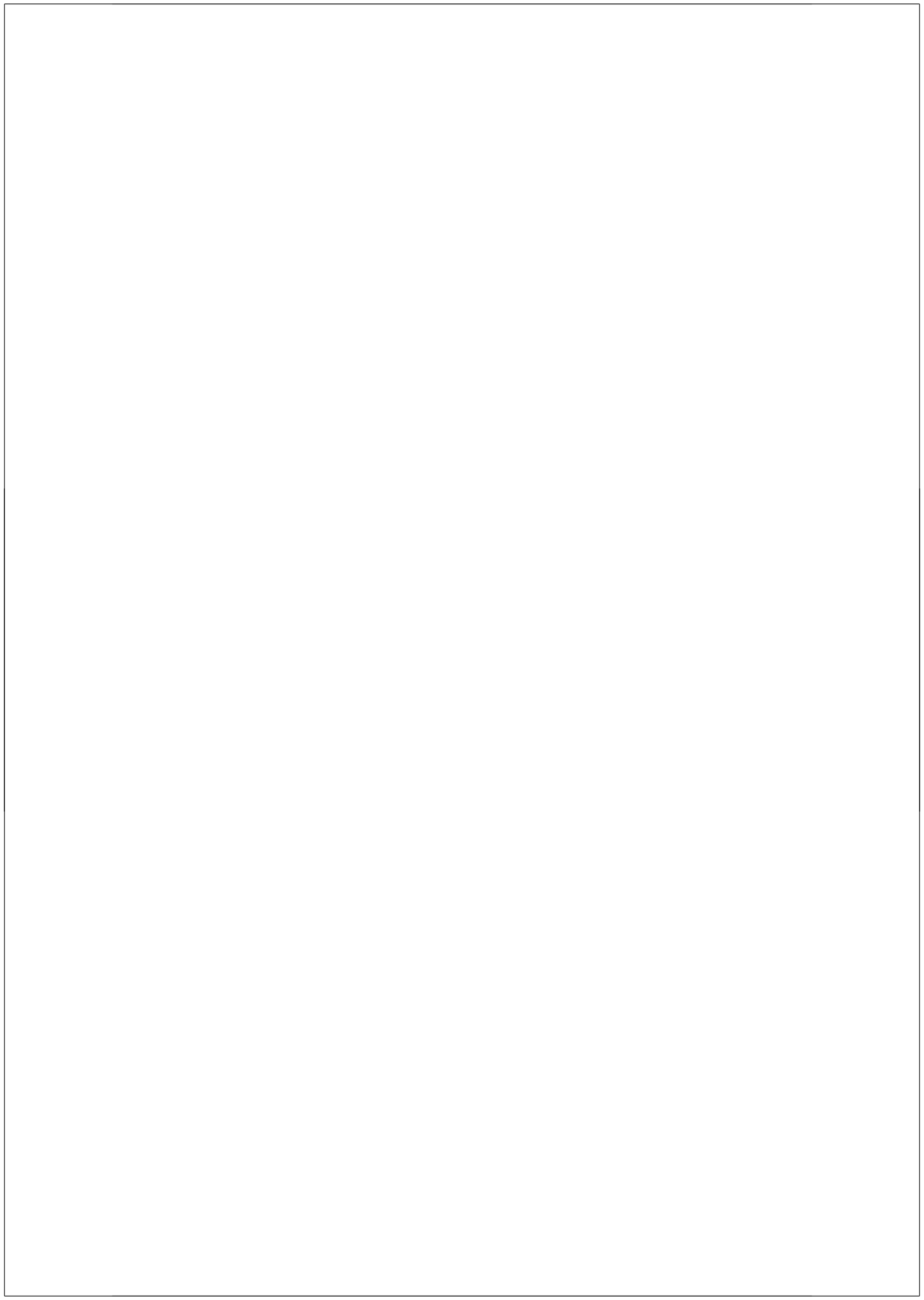
Central venous catheters

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 1β, 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 1β 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.

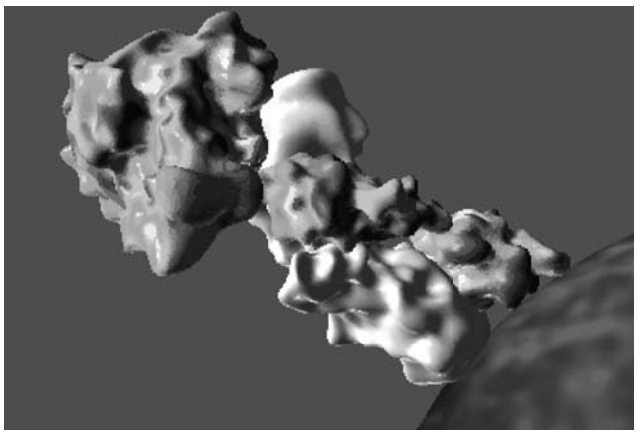
Treatment options include vitamin K antagonists and low-molecular-weight heparins, and the long-term use of these heparins in prevention of venous thrombosis may improve the outcome in comparison with oral anticoagulants. Further research is needed to better understand the morbidity and mortality associated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