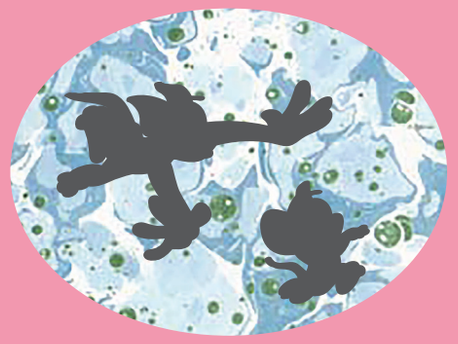
Interplay between cancer and thrombosis; identification of key factors

Ünlü, B.



Interplay between cancer and trombosis; identification of key factors Betül Ünlü 2019

Interplay between cancer and trombosis; identification of key factors

Betül Ünlü

Propositions

Affiliated with the thesis

Interplay between cancer and thrombosis; identification of key factors

By Betül Ünlü

1. Physiologic expression of alternatively spliced Tissue Factor has no effect on the coagulant property of full-length Tissue Factor when co-expressed in endothelial cells, since these proteins are expressed in different cellular compartments. (*This thesis*)

2. Tissue Factor signaling affects epithelial-to-mesenchymal transition and cancer stemness via modulation function of β1- and β4 integrins, that results in increased metastasis. (*This thesis*)

3. Patients with cancer-associated thrombosis show a different tumor expression profile compared to those with cancer. (*This thesis*)

4. The spontaneous thrombosis model will allow us to study the underlying mechanism behind thrombosis and disseminated intravascular coagulation -an extreme form of dysregulated hemostasis- in mice with cancer. (*This thesis*)

5. In the bloodstream, Tissue Factor expression on circulating tumor cells is important in order to protect itself against attack from Natural Killer cells. (*Palumbo JS, et al. Blood 2007; 110:133-41*)

6. Risk factors of venous thromboembolism cannot be extrapolated to patients with cancer, because cancer itself is the largest risk factor.

7. In order to better understand the evolution of cancer-associated thrombosis, the genetic background -both germline and somatic mutations- of the patient should be studied as well. (Inspired by *Carter H, et al. Cancer Discovery 2017; 7:410-423*)

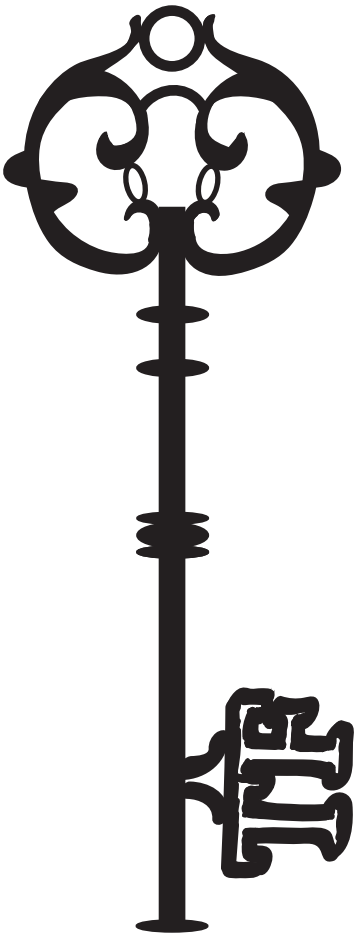
8. In order to stimulate good and innovative science, scientists should less be accountable for all their expenses to grant providers and should be given more 'free-time' in the laboratory.

9. Making a lot of mistakes is good for the character development of a PhD-student, as he/she learns to be patient and improves to temper the frustration.

10. A scientist is not cluttered, just very well organized in a tremendously chaotic manner.

11. "Yesterday I was clever, so I wanted to change the world. Today I am wise, so I am changing myself." (*Rumi 1207-1273*) A young scientist starts naive and with big goals, and along the way learns that academia is more challenging than expected.

12. "I believe in pink." (*Audrey Hepburn 1923-1993*) Pink is more than just a color, it will make a huge difference in life and academia.



Interplay between cancer and thrombosis; identification of key factors

Betül Ünlü

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Proefschrift

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“Büyük başarılar, değerli anaların yetiştirdikleri seçkin çocukların yardımıyla meydana gelir”

“Great achievements occur with the help of elite children raised by precious mothers”

Mustafa Kemal Atatürk (1881-1938)

Hayatımdaki kahramanıma,

Anneme

Voor Levent, Nejla & Ceyla

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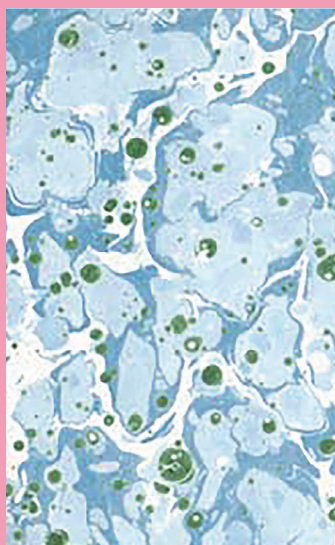
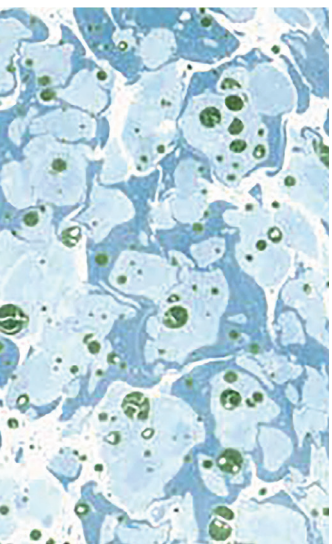


Chapter 1

General introduction



In 1823 the link between cancer and thrombosis was first reported by Jean Baptiste Bouillaud [1]. Although being the first, Armand Trousseau was the one who got recognized for the association between gastric cancer and thrombosis in 1865 [2]. Ironically, this French internist diagnosed himself with venous thromboembolism (VTE) and predicted his own death, which took place in 1867. For these reasons cancer-associated thrombosis (CAT) is now referred to as Trousseau's Syndrome.

Blood clots in the deep veins (formation of which is termed deep vein thrombosis; DVT) can detach and ultimately travel through the bloodstream and occlude an artery in the lung, which is called a pulmonary embolism (PE). Among all patients with VTE (DVT + PE) one in five is diagnosed with cancer [3, 4]. Furthermore, cancer patients have on average a 7-fold increased risk for developing VTE when compared to healthy subjects [5, 6]. CAT contributes to high morbidity and mortality [7]. After cancer itself, CAT is the second cause of death in cancer patients [8]. Unfortunately, the mechanism underlying CAT is poorly understood. Until now only a few clinical and patient-related factors have been uncovered that may contribute to VTE in cancer patients, such as high age, obesity, and prior history of VTE [7, 9]. Risk factors directly associated with the presence of a tumor further contribute to an increased risk of VTE, like the location of the primary tumor, increase in tumor stage and cancer treatment [10, 11]. Assessment tools to estimate the risk of CAT have been developed, however they do not perform well. Furthermore, thromboprophylaxis of cancer patients increases the risk of spontaneous bleedings. Therefore, it is key to unravel the mechanism behind cancer-associated thrombosis in order to select those patients that are at high risk for CAT and those who could benefit from prophylactic anticoagulant treatment.

Nowadays, it is well accepted that the transmembrane protein Tissue Factor (TF), the physiological activator of the coagulation cascade, is the linking pin between coagulation and cancer. Under normal conditions, TF is expressed on subendothelial cells. Upon external stimuli (e.g. injury), TF is exposed to the bloodstream and forms a binary complex with its ligand Factor VII (FVII) that eventually results in thrombin activation, fibrin deposition and platelet activation in order to close the site of the wound [12]. Apart from its function in blood coagulation, TF is expressed by tumors and has been shown to play a pivotal role in tumor progression. Overexpression has been associated with reduced survival, increased angiogenesis, migration and invasive capacity of tumor cells [13].

TF influences cancer progression through two distinct pathways. First, it activates cellular signaling events to promote tumor growth via activation of Protease activated Receptor-2

(PAR2), facilitating the angiogenic switch and affecting migration of cancer cells. Upon entering the blood vessel, coagulant properties of TF become important, leading to the formation of a platelet and fibrin shield around the tumor cell to prevent shear stress and immune cell mediated apoptosis, thereby promoting survival of metastatic cells [14].

A soluble alternatively spliced isoform of TF has been discovered in 2003, the so-called alternatively spliced Tissue Factor (asTF). This isoform lacks the transmembrane region of TF – as exon 5 is spliced out – and due to a frameshift it contains a unique C-terminal tail that differs from that of full-length TF protein (flTF) [15]. Studies have shown involvement of asTF in angiogenesis and tumor growth in an integrin-dependent manner [16, 17]. Although the first 166 amino acids are identical between flTF and asTF, the procoagulant properties of asTF have remained controversial as it lacks the binding site for macromolecular coagulation factors IX and X [18-20].

Outline

In this thesis the focus is on the interplay between coagulation factors and cancer. First, the role of asTF in flTF activity modulation is addressed. Second, the contribution of TF signaling in breast cancer metastasis and its underlying mechanism has been elucidated. Finally, tumor-expressed contributors to VTE were studied and an ***in vivo*** model was developed with spontaneous thrombosis in combination with cancer.

Chapter 2 shows that co-expression of asTF has a negligible impact on flTF coagulant activity of endothelial cells and extracellular vesicles. Analysis shows that asTF and flTF do not localize to the same cellular compartments, explaining why asTF does not influence flTF function. **Chapter 3** summarizes the current literature on tumor-expressed coagulation factors in cancer progression and the elevated risk of VTE. Whether TF signaling promotes metastasis in breast cancer is described in **Chapter 4.** By using ***in vivo, ex vivo*** and ***in vitro*** models it is shown that inhibition of TF signaling reduces i) epithelial-to-mesenchymal transition and cancer stem cell transcriptional programs, ii) primary tumor-resident cancer stem cells and iii) subsequent metastasis ***in vivo***. In addition, metastasis is significantly associated with TF expression in estrogen receptor negative tumors in a large breast cancer cohort. Furthermore, the underlying mechanism, i.e. TF-dependent modulation of integrin function, is elucidated. In **Chapter 5** a spontaneous ***in vivo*** model was developed in order to provide insight in the evolution of cancer-associated thrombosis. Due to elevated platelet counts in mice with breast cancer, a severe phenotype with consumption of coagulation factors is reduced. **Chapter 6** reveals new genes that were associated with VTE in colorectal

cancer patients. Through the use of laser capture microdissection method to isolate RNA from tumor cells only, a comparison in tumor cell-expression profiles could be made between individually matched colorectal cancer patients with and without VTE. In **Chapter 7** current knowledge on links between genetic alterations and risk of cancer-associated thrombosis is reviewed. In addition, future directions are discussed in order to increase the accuracy of cancer-associated thrombosis prediction models. Finally, **Chapter 8** provides a general summary and discussion of this thesis.

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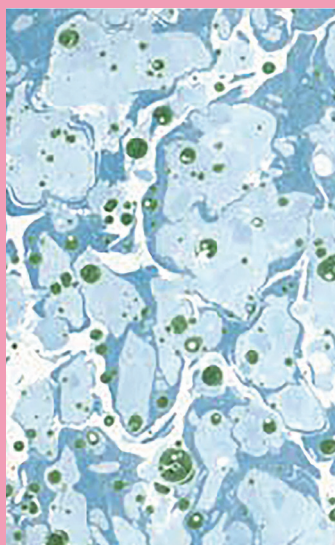
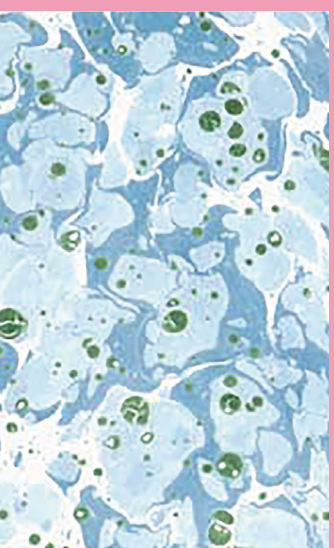
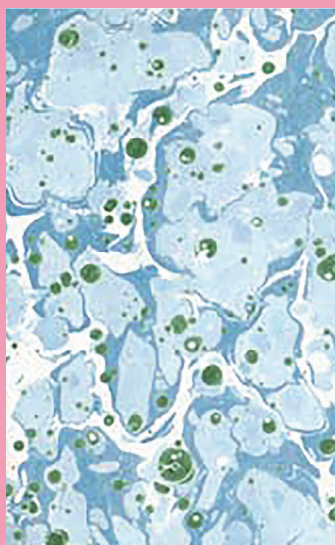


Part I



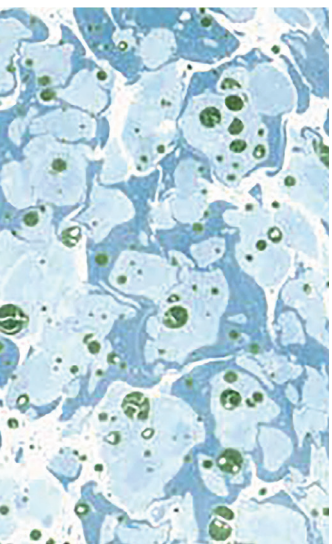


Chapter 2

Interplay between alternatively spliced Tissue Factor and full-length Tissue Factor in modulating coagulant activity of endothelial cells.

Betül Ünlü
Vladimir Y. Bogdanov
Henri H. Versteeg

Thromb Res **2017** *Aug*; **156: 1-7.**



Highlights

- Interplay between asTF and flTF in endothelial cells is unknown
- Concomitant expression of asTF and flTF does not alter the coagulation potential of cells and/or MVs.
- The two TF protein isoforms do not co-localize in endothelial cells.
- High levels of asTF decrease flTF expression, mainly in non-lipid raft plasma membrane fractions.
- High levels of asTF increase ER stress.

ABSTRACT

Background: Full-length Tissue factor (flTF) is a key player in hemostasis and also likely contributes to venous thromboembolism (VTE), the third most common cardiovascular disease. flTF and its minimally coagulant isoform, alternatively spliced TF (asTF), have been detected in thrombi, suggesting participation of both isoforms in thrombogenesis, but data on participation of asTF in hemostasis is lacking. Therefore, we assessed the role of asTF in flTF cofactor activity modulation, using a co-expression system.

Objective: To investigate the interplay between flTF and asTF in hemostasis on endothelial cell surface.

Methods: Immortalized endothelial (ECRF) cells were adenovirally transduced to express asTF and flTF, after which flTF cofactor activity was measured on cells and microvesicles (MVs). To study co-localization of flTF/asTF proteins, confocal microscopy was performed. Finally, intracellular distribution of flTF was studied in the presence or absence of heightened asTF levels.

Results: Levels of flTF antigen and cofactor activity were not affected by asTF co-expression. asTF and flTF were found to localize in distinct subcellular compartments. Only upon heightened overexpression of asTF, lower flTF protein levels and cofactor activity were observed. Heightened asTF levels also induced a shift of flTF from non-raft to lipid raft plasma membrane fractions, and triggered the expression of ER stress marker BiP. Proteasome inhibition resulted in increased asTF – but not flTF – protein expression.

Conclusion: At moderate levels, asTF appears to have negligible impact on flTF cofactor activity on endothelial cells and MVs; however, at supra-physiological levels, asTF is able to reduce the levels of flTF protein and cofactor activity.

Key words: alternatively spliced Tissue Factor, full-length Tissue Factor, hemostasis, coagulation

INTRODUCTION

Venous thromboembolism (VTE) belongs to the top three most common cardiovascular diseases in industrialized nations [1]. VTE mainly comprises deep vein thrombosis and pulmonary embolism, which occurs with an incidence rate of approximately 1-2 events per 1000 individuals per year. Thrombosis is initiated after changes in blood flow, composition of blood, and/or damage to the vessel wall, also known as the triad of Virchow [2]. Hypercoagulability may be caused by increased microvesicle (MV) levels in blood and is associated with VTE in cancer patients, as reviewed elsewhere [3, 4].

MVs can be shed via e.g. blebbing of the cell membrane and fall within a size range of 10-1000 nm [5]. In numerous disease settings these MVs can be shed from platelets, monocytes, and/or endothelial cells, and are believed to contribute to pathological processes such as cancer and VTE [6]. To date, it is unclear whether MVs are a cause or a consequence of VTE. Depending on the origin of the cell, these MVs may contain negatively charged phosphatidylserine (PS) and/or Tissue Factor full-length form (TF/flTF) in the outside leaflet of their lipid bilayer, which increases the procoagulant potential of these vesicles [7].

flTF, a glycosylated transmembrane protein, is the only initiator of the extrinsic coagulation cascade ***in vivo*** [8], and a key player in thrombosis [3, 9]. flTF is also required for vessel formation and maturation, as flTF deficiency results in the abrogation of mouse embryonic development between days 8.5 and 10.5 due to defects in the yolk sac vasculature [10, 11]. In 2003, the structure of an alternative isoform of TF termed alternatively spliced Tissue Factor (asTF), was reported [12]. During TF pre-mRNA processing, exon 5 is spliced out, resulting in a frameshift that yields a soluble TF isoform with a unique C-terminal domain. We have previously shown that recombinant asTF induces angiogenesis in an integrin-dependent manner. asTF binding to αvβ3 integrins promotes endothelial cell migration, while capillary formation is induced by asTF via α6β1 integrin activation [13]. In cancer, asTF induces tumor growth and metastasis in a β1 integrin dependent manner, and recruits monocytes to the tumor stroma [14-16]. asTF also increases the coagulant potential of pancreatic ductal adenocarcinoma cells and MVs via an indirect mechanism [17].

Even though asTF promotes cancer progression non-proteolytically, coagulant properties of asTF and thus its involvement in thrombosis and/or hemostasis are controversial [18-20]. Both flTF and asTF accumulate in occlusive thrombi [21], suggesting a role for asTF in thrombus formation. asTF may in principle influence coagulation as it retains the first 166 residues of flTF critical to forming a complex with FVII(a), including the 165-166 lysine

doublet involved in the binding of FVII(a) and FX [22, 23], but asTF lacks a complete binding site for the macromolecular substrates FIX and FX [12, 24]. Initial functional studies showed that high concentrations of recombinant asTF shorten clotting times in the presence of PS-containing phospholipid vesicles [12], but these studies did not uncover how and whether asTF influences flTF-dependent clotting. In arterial lipid-rich plaques, the functional activity of asTF likely contributes to thrombus formation in a slow and long-term manner; however, in these settings asTF more likely serves to recruit monocytes that destabilize the plaque (reviewed in [25]). Another study showed that coagulant activity in supernatants from cytokine-stimulated endothelial cells decreased upon asTF depletion [20], but again this study did not explore whether asTF and flTF may synergize, or alternatively, have opposing functions in coagulation initiation. Finally, a study by Böing and colleagues found that asTF expressed in flTF-null HEK293 cells did not influence coagulation initiation, but again, this study did not evaluate the possible effects of asTF on flTF function [18].

While the above studies investigated TF isoforms in isolation, ***in vivo***, ***F3*** expression results in simultaneous biosynthesis of flTF and asTF. Although the relative abundance of asTF can vary widely, asTF is never exclusively expressed [25]. The lack of functional data on TF isoform-dependent coagulation activation prompted us to study the effects of flTF/asTF co-expression on TF cofactor activity of endothelial cells and endothelial cell-derived MVs.

METHODS

Reagents

Full-length tissue factor (flTF)-specific mouse monoclonal antibody mAb TF9-10H10 and alternatively spliced tissue factor (asTF)-specific rabbit monoclonal antibody RabMab1 were described previously [14]. Anti-BiP (C50B12) and anti-GAPDH (14C10) rabbit monoclonal antibodies were purchased from Cell Signaling. B-actin (N-21)-specific rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology.

Cell culture and adenoviral transductions

The immortalized human endothelial cell line ECRF was cultured in medium 199 (Life Technologies), supplemented with 20% FCS (PAA), 0,5% bovine pituitary extract (Gibco), 1 ml heparin Na LEO 5000 IE/ml (LEO Pharma Inc.) and Penicillin/Streptomycin in a 5% CO_2 incubator at 37 °C. ECRF cells were transduced with GFP (mock), flTF (NM_001993)

and/or asTF (NM_001178096) adenoviral constructs as previously described [26]. Briefly, ECRF cells were transduced, and the experiments were performed 2 days later. To induce MP shedding, cells were serum-starved [27] in M199 medium for 3 h at 37 °C, after which conditioned media was harvested and centrifuged for 5 min at 1,000 × *g* to remove cell debris. To pellet MVs, the supernatant was spun at 20,000 × *g* for 1 h as described [28].

Adenovirus production

Adenoviral constructs encoding asTF or flTF were generated using AdenoX Expression system (Clontech). For large-scale adenovirus production, HEK293 cells were transduced with adenoviral constructs for 3-4 days at 37 °C. Cells were freeze-thawed three times, after which supernatant was collected and stored at -80 °C. Numbers of viral particles were determined via QuickTiter Adenovirus Titer ELISA kit (Cell biolabs).

Reverse transcriptase-PCR analysis

Total cellular RNA was isolated with TRIzol (Life Technologies) according to the manufacturer's instructions. 1 µg RNA was converted to first strand cDNA using Super Script II kit (Life Technologies). PCR amplifications for β-actin and flTF were performed at 95 °C for 5 min, 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (25 cycles for β-actin; 29 cycles for flTF), 72 °C for 10 min. PCR conditions for asTF were performed at 95 °C for 5 min, 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min (40 cycles), 72 °C for 5 min. A common asTF/flTF forward primer was used: forward, 5'-TTACACACAGACACAGAGTGTGA-3', asTF reverse primer, 5'-GAATATTTCTTTCTTTCCTGAACTTGAAG-3', flTF reverse primer, 5'-TTGAACACTGAAACAGTAGTTTTCTCC-3'. The β-actin primers were: forward, 5'-AAGAGATGGCCCACGGCTGCT-3', and reverse, 5'-CCTTCTGCATCCTGTCGGCA-3'.

Western blotting

For western blotting, cells and MVs were lysed in 2× sample buffer (Life Technologies), proteins were denatured at 95 °C for 5 min and cell lysates were sonicated for 10 s. For lipid raft collection, cells were lysed in Brij58 buffer (50 mM Tris, 150 mM CaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Brij58), centrifuged for 10 min at 800 × *g* to pellet cytoskeletal fractions and nuclei, and the supernatant was spun at 16,000 × *g* for 30 minutes to pellet lipid rafts. Lysates were loaded on 4-12% Bis-Tris PLUS Gels (Thermo Fischer Scientific) and transferred to 0.2 µm pore size PVDF membranes. Membranes were blocked in 5% milk/TBST and incubated with flTF- or asTF-specific primary antibodies O/N in blocking buffer; mAb TF9-10H10 (flTF-specific), RabMab1 (asTF-specific) [14]. After multiple TBST washing steps, the membranes were incubated with horseradish peroxidase-conjugated secondary

antibodies for 1h. Bands were visualized with Western lightning Plus ECL (PerkinElmer) on X-ray film (Santa Cruz).

FXa generation

TF-dependent coagulant activity on the surface of ECRF cells and MVs were performed in HBS with 1.5 mM $CaCl_2$. The reaction was started by the addition of 1 nM FVIIa (Novo Nordisk) and 50 nM FX (Stago). After 30 min at 37 °C the reaction was quenched, after which the generated FXa was measured using the kinetic chromogenic substrate Spectrozyme FXa (Sekisui) as described before [26].

Immunofluorescence

ECRF cells were grown on glass coverslips, transduced with flTF or asTF adenovirus and serum starved, as described above. The cells were washed and fixed with 2% formaldehyde in PBS for 20 min at RT. In selected experiments, cells were subsequently permeabilized with 0.1% Triton-X100 for 5 min and blocked with 5% BSA/PBS for 30 min. Primary custom rabbit polyclonal antibody specific for human asTF (pAb-1979) [14] was applied at 5 µg/ml in the blocking buffer and incubated O/N at 4 °C. The following day, cells were incubated with goat anti-rabbit-Alexa488 and Alexa594-conjugated 10H10 for 1 h in the dark. Coverslips were mounted with ProlongGold containing DAPI (Thermo Fischer Scientific) for nuclear staining. Images of immunofluorescent labeled cells were captured using a Leica SP5 confocal microscope.

RESULTS

asTF exhibits minimal coagulant activity when expressed in human endothelial cells

The role of asTF in coagulation initiation has previously been assessed, but the interplay between flTF and asTF has never been elucidated. To gain insight into such an interplay, we studied asTF's effect on TF cofactor function when expressed in endothelial cells. In our ***in vitro*** model, asTF and flTF antigen levels were much higher compared to those under TNFα and LPS stimulation conditions (data not shown). Immortalized endothelial ECRF cells were transduced to express asTF and/or flTF and, as expected, expression of the two TF isoforms was observed in these cells, both on mRNA (Fig. 1A) and antigen (Fig. 1B) levels. We found that flTF expression levels did not significantly alter asTF expression levels, and vice versa.

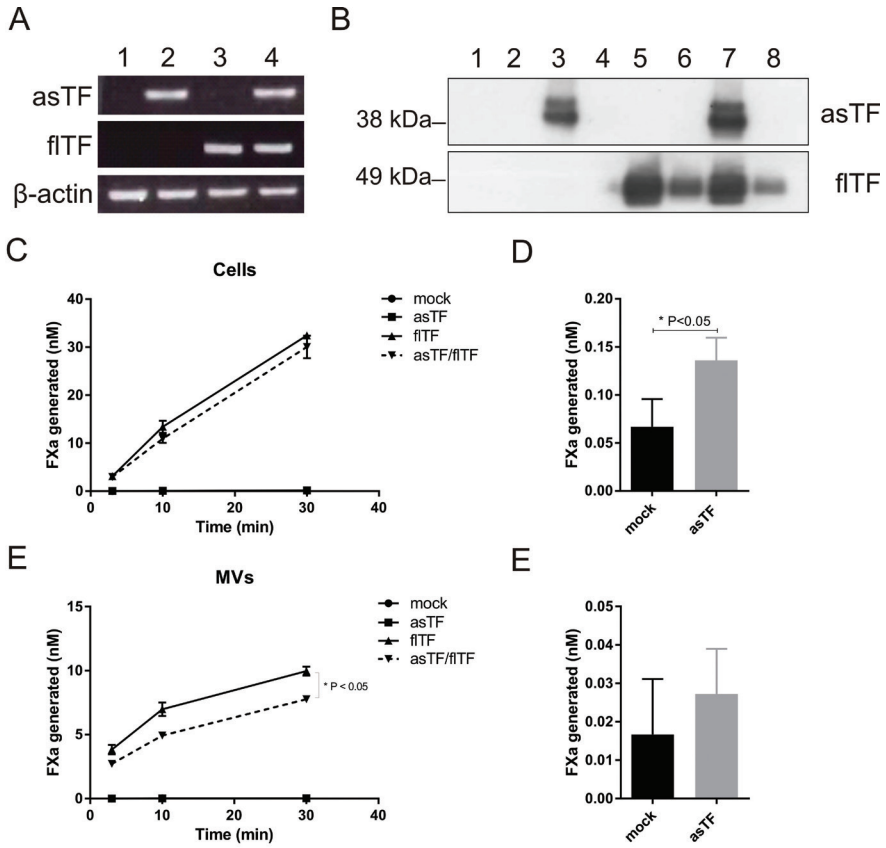


Figure 1 Characterization of asTF and flTF in ECRF cells after adenoviral transduction.

(**A**) mRNA levels of asTF and flTF were determined in mock-transduced (1), asTF-transduced (2), flTF-transduced (3) and asTF/flTF transduced cells (4) via RT-PCR. (**B**) asTF and flTF protein expression in cell lysates and MVs assessed using western blot. Odd numbers represent total lysates, and even numbers – MVs. (**C**) and (**E**) FXa generation on cells and MVs in the presence of 1 nM FVIIa and 50 nM FX. (**D**) and (**F**) Bar graphs represent FXa generation on mock (black) versus asTF (grey) ECRF cells and MVs. (*P < 0.05).

Antigen analysis on MVs indicated the presence of flTF, but not asTF, suggesting that asTF does not associate with endothelial MVs at appreciable levels (Fig. 1B; upper panel). A chromogenic FXa generation assay was performed to address whether asTF influences cofactor activity of flTF in an endothelial setting. Mock transduced ECRF cells generated < 1 nM FXa,

while asTF expression led to a very minor yet statistically significant increase in TF activity (Fig. 1D). flTF-transduced ECRF generated 30 nM FXa after 30 min (Fig. 1C). In cells co-expressing asTF and flTF, asTF did not influence flTF cofactor activity. Similar results were obtained with MVs shed from these cells, but the levels of TF cofactor activity and flTF antigen were lower than those observed in ECRF cells (Fig. 1E). We conclude that asTF – by itself and without exogenously added phospholipids – has minimal coagulant activity, which is consistent with prior reports on endothelial human asTF [17]; when co-expressed with flTF, asTF does not significantly alter TF cofactor activity of human endothelial cells – neither on cell surfaces, nor on MVs derived from them.

asTF and flTF proteins do not co-localize in human endothelial cells

It is unknown whether asTF and flTF can physically interact. Therefore, we co-expressed asTF and flTF in ECRF cells, and immunofluorescence staining was performed. The majority of the flTF pool was found on the surface of plasma membrane, while asTF predominantly homed to intracellular ECRF compartments (Fig. 2A). Confocal analysis of multiple z-stacks confirmed that asTF and flTF proteins do not co-localize in ECRF cells (supplementary Fig. 1). Similarly, asTF and flTF did not co-localize in cell protrusions where MV shedding takes place (Fig. 2B). Lack of co-localization was also observed in HUVEC cells when both TF isoforms were co-expressed (Fig. 2C). We conclude that, in endothelial cells, asTF and flTF proteins home to distinct sub-compartments and are thus not in close proximity to each other.

Heightened overexpression of asTF reduces flTF expression levels

As we did not observe asTF/flTF protein co-localization, we then studied whether supra-physiological asTF levels may impact TF cofactor activity. A 3-fold higher dose of asTF adenovirus hampered flTF adenovirus expression when co-transduced with asTF (Fig. 3A. left panel). As expected, this decrease in flTF protein levels also yielded a significantly lower FXa conversion rate on endothelial cells and MVs (Fig. 3B and C, and supplementary Fig. 2). Cofactor activity and/or protein levels of flTF were not affected by increasing GFP expression (supplementary Fig. 3), demonstrating that this effect was not due to high protein expression and the resultant loading of the ER. To study if FVIIa is sequestered by binding to asTF, increasing amounts of recombinant FVIIa were added to the cells and MVs; higher FVIIa concentrations did not restore TF cofactor activity (Fig. 3D and E). In untransformed HUVECs, supra-physiological levels of asTF also lowered flTF-dependent FXa generation (Fig. 3F and G). Because of the unaltered asTF protein levels after escalating virus doses, we hypothesized that excess asTF protein might be targeted to the proteasome. Inhibition of

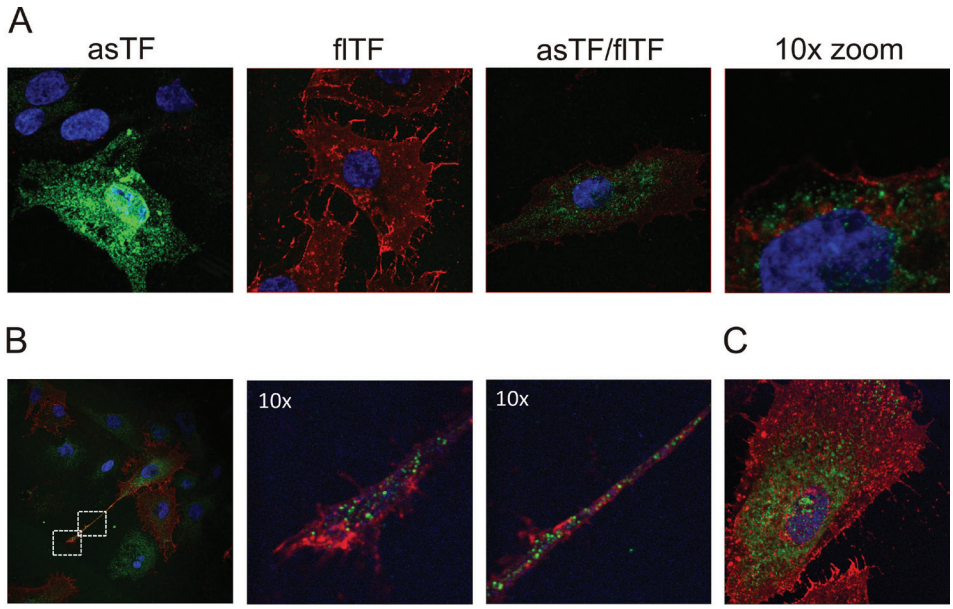


Figure 2 asTF and flTF do not co-localize when concomitantly expressed.

(**A**) After transduction, ECRF cells were fixed and stained with pAb-1979 (asTF, green) and 10H10 (flTF, red). A 10-fold magnified image (right panel) shows asTF and flTF in different cellular sub-compartments. (**B**) Localization of asTF/flTF in cellular protrusions. (**C**) asTF and flTF localization in HUVEC cells.

the proteasome showed increased asTF protein levels, while flTF protein levels remained unaffected (Fig. 3H). As high asTF levels were cleared by the cells, we gathered that high asTF levels were likely to induce stress within the endoplasmic reticulum (ER). Indeed, BiP, a marker for ER stress, was upregulated as a consequence of high asTF (co-)expression (Fig. 3H). We conclude that heightened overexpression of asTF leads to proteosomal degradation of asTF protein, yet not flTF protein, and concomitant ER stress.

asTF impacts distribution of flTF into non-lipid raft fractions of endothelial cell plasma membrane

As flTF protein levels and TF coagulant activity are decreased due to heightened asTF, the question emerges as to whether asTF is able to induce changes in flTF sub-compartment distribution in the plasma membrane. Therefore, plasma membrane was fractionated into the material containing lipid rafts, and that free of lipid rafts. asTF levels were equally distributed between raft and non-raft fractions (Fig. 4A, upper panel). asTF localization pat-

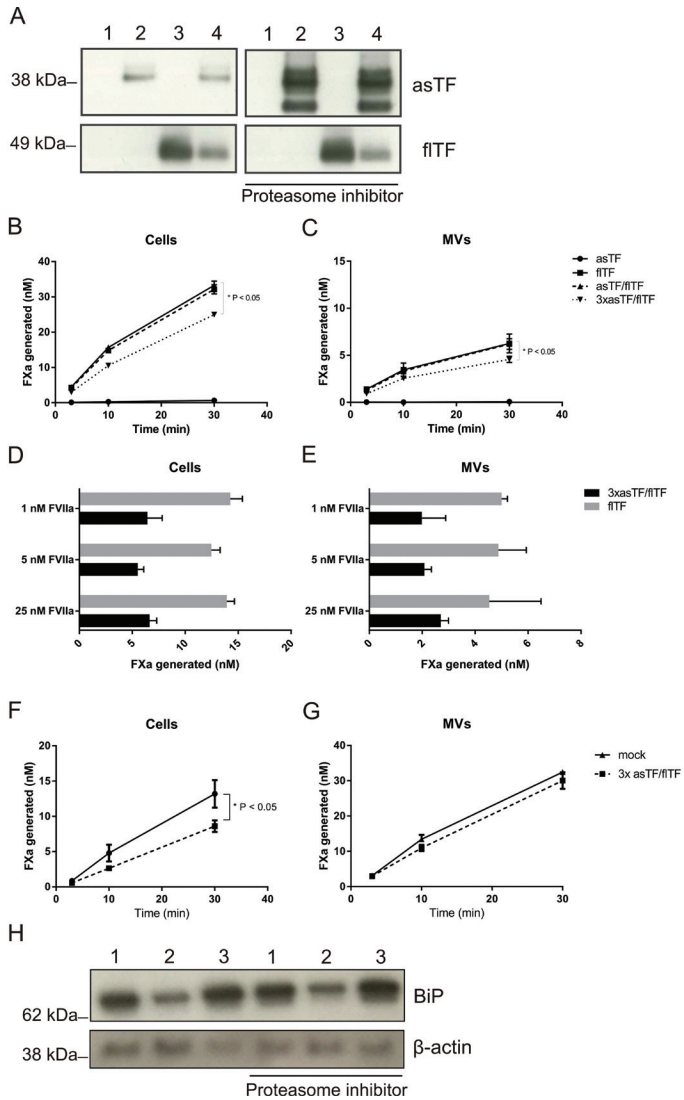


Figure 3 The effects of supra-physiological asTF levels on TF cofactor activity.

(**A**) Western blot analysis of total lysates of mock (1), asTF (2), flTF (3) and asTF/flTF transduced (4) ECRF cells. Cells were incubated for 12 h with 5 μM MG132, to inhibit the proteasome. (**B**) and (**C**) FXa generation on cells and MVs in the presence of 1 nM FVIIa and 50 nM FX. (**D**) and (**E**) FXa generation on flTF and asTF/flTF cells and their MVs in the presence of 1 nM, 5 nM or 25 nM FVIIa and 50 nM FX. (**F**) and (**G**) FXa generation on HUVEC cells and MVs with heightened overexpression of asTF, respectively. (**H**) Western blot analysis for the ER-stress marker BiP in ECRF cells and asTF (1), flTF (2), and asTF/flTF (3) transduced cells.

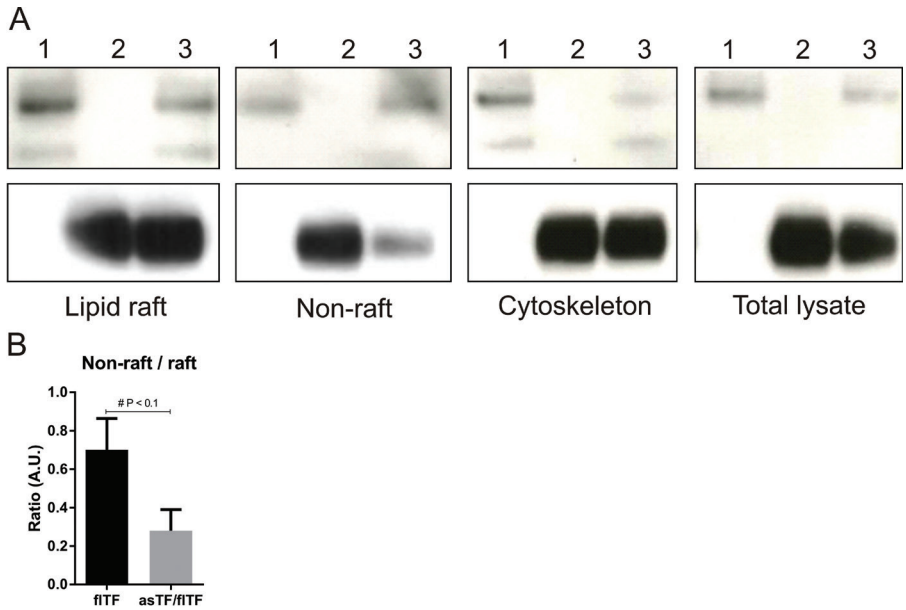


Figure 4 Effects of asTF expression on sub-cellular distribution of flTF.

(**A**) Protein levels of asTF (1), flTF (2) and asTF/flTF (3) transduced cells in lipid raft fraction, non-raft fraction, cytoskeleton, and total cell lysate. (**B**) The flTF ratio in non-raft versus raft fractions in the absence and presence of asTF, respectively; analyzed via ImageJ.

terns were not changed upon co-expression of flTF. The majority of flTF was found in the lipid raft fraction, yet a smaller fraction of the total flTF pool was still present in non-raft fractions (Fig. 4A, lower panel). Strikingly, flTF distribution was changed when asTF was co-expressed, showing a decrease of flTF protein present in the non-raft fraction (Fig. 4B). These results suggest that the decrease in flTF expression, when flTF is co-expressed with asTF, can be attributed to asTF-triggered downregulation of flTF present in non-raft fractions. Separately, these results suggest that coagulant-active flTF is mostly located in non-lipid raft plasma membrane fractions of human endothelial cells.

DISCUSSION

In this study, we investigated the interplay between asTF and flTF in coagulation initiation. Our data indicate that simultaneous expression of asTF and flTF has no effect on TF cofactor activity, but heightened asTF overexpression modulates flTF expression in human endothelial cells. We base our conclusions on the following observations: i) low asTF levels did not alter flTF cofactor activity on cells and/or MVs; ii) asTF and flTF proteins did not co-localize in cellular sub-compartments; iii) heightened levels of asTF decreased the levels of flTF protein in non-raft plasma membrane fractions, thereby reducing TF cofactor activity.

Previous studies of coagulant properties of asTF are fairly inconclusive [19, 20]. This is the first study that reports on asTF expressed concomitantly with flTF in an endothelial cell setting, to study asTF's possible function in hemostasis. While FXa generation on cells expressing only asTF was very low, a significantly higher FXa conversion rate was observed on asTF expressing cells compared to mock-transduced cells (Fig. 1C and D). Although Fig. 1D seems to demonstrate a lowered rate of FXa generation by asTF/flTF MVs compared to flTF MVs, the slope of the asTF/flTF MVs curve is identical to that of the flTF MVs curve, likely revealing an artifact during MV isolation and/or processing; we note that this artifact was not consistently observed (Fig. 3C). Western blot analysis on MVs and supernatants revealed that asTF is not secreted from non-activated endothelial cells (data not shown). Also, stimulation of HUVEC cells with IL-1α did not result in asTF secretion, as previously reported by Böing et al. [18]. However, other studies that utilized endothelial or malignant cell lines were able to demonstrate asTF in conditioned media and/or plasma of cancer patients [14, 29]. Up till now, asTF cofactor activity was only observed in cell supernatants under inflammatory conditions or in a cancerous setting [17, 20]. Thus, it might very well be that it is only in unique disease settings, e.g. cancer, that the soluble asTF protein is secreted by endothelial cells whereby it can modulate coagulation. Studies to measure the quantities of MVs and their phosphatidylserine content will provide insight as to how asTF might be involved in direct or indirect modulation of flTF-containing MV shedding / coagulant activity.

When asTF was co-expressed with flTF, asTF did not change total TF cofactor activity. When higher adenovirus titers were used, we hardly detected increases in asTF protein levels. Only after treatment with a proteasome inhibitor, we observed restored asTF levels after overexpression. asTF increased ER stress, as BiP levels were elevated (Fig. 3H). Interestingly, it has been shown that overexpression of BiP reduces cofactor activity of flTF [30, 31]. However, this does not completely explain the reduced FXa conversion rates in asTF/flTF co-expressing cells, as we observe reduced flTF protein levels. BiP is an ER resident chaperone protein

and plays a role in the quality control and folding of proteins. The two TF isoforms share the large N-terminal domain, but differ in their relatively small C-terminal domains, whereby the highly hydrophobic alpha-helical region is present only in flTF. Because asTF already increases ER stress, during protein synthesis BiP may also increasingly recognize flTF as an incorrectly folded protein and lock it in an unfolded state in the presence of asTF, and this might explain lower flTF expression [32]. These results indicate that, like many other alternatively spliced soluble proteins [33], asTF may be an unstable protein degraded via a proteasome-dependent pathway.

Our study has an important limitation. The asTF and flTF expression levels used in our study exceed those observed after stimulation with inflammatory agents such as TNFa and LPS (results not shown). Thus, our model may not fully mimic endothelial cell biology. Nevertheless, our intention was not to create a biologically faithful endothelial model with physiological asTF and flTF expression levels per se, but to investigate the effects of asTF/flTF co-expression on total TF cofactor activity. Our finding that supra-physiological asTF levels never observed in endothelial cells – even under inflammatory conditions – do not influence FXa generation, further supports our conclusion that asTF does not influence TF cofactor function in this cell type.

In conclusion, asTF appears to have a very limited role in normal hemostasis. It has minimal cofactor activity and does not alter the cofactor function of flTF expressed in human endothelial cells and/or TF^+ MVs derived from them.

Acknowledgements

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Conflicts of interest

None.

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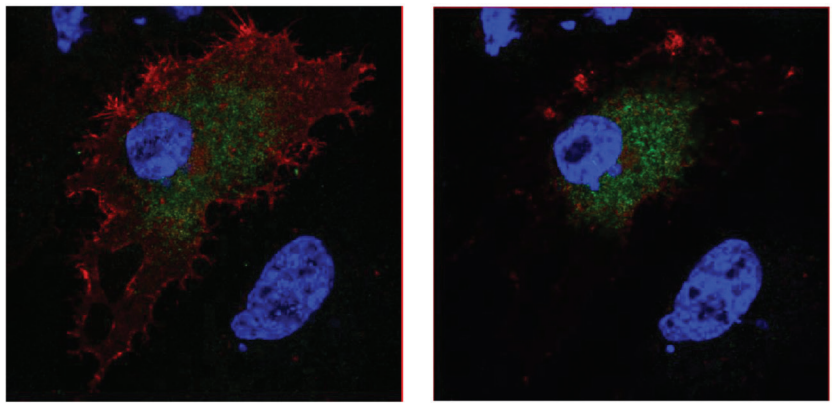
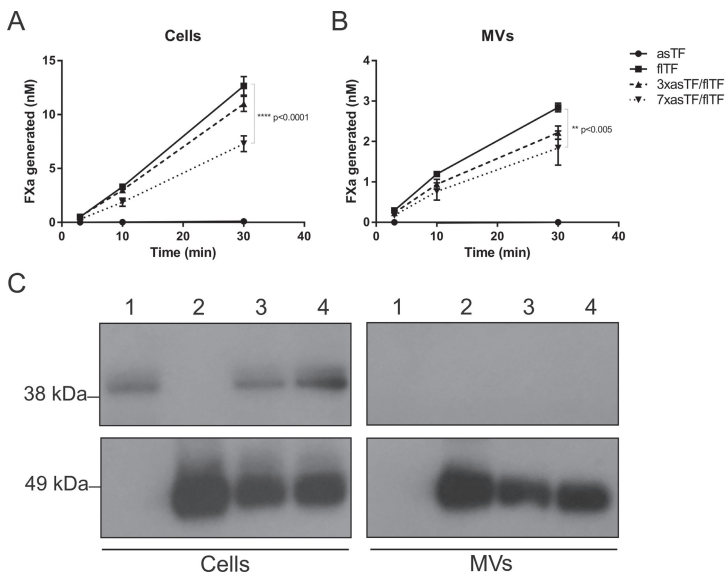
SUPPLEMENTARY MATERIALS

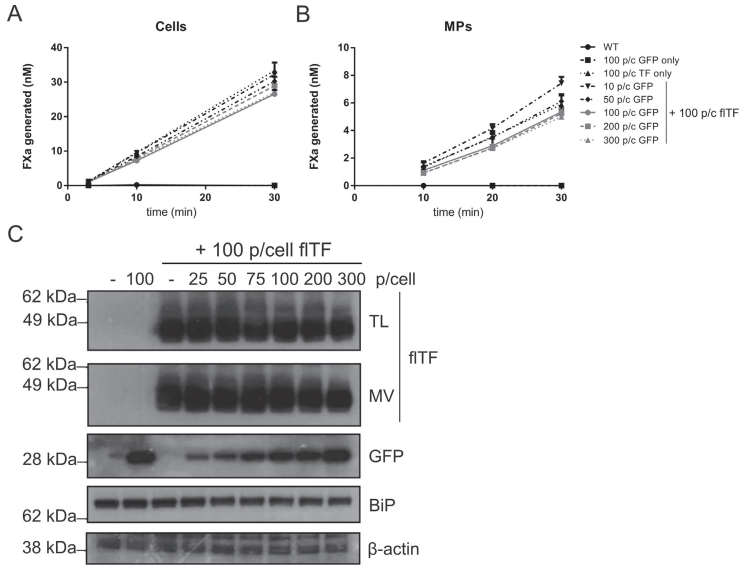


Supplementary figure 1 Confocal images of ECRF cells co-expressing asTF (green) and flTF (red). Images were captured in different z-planes.



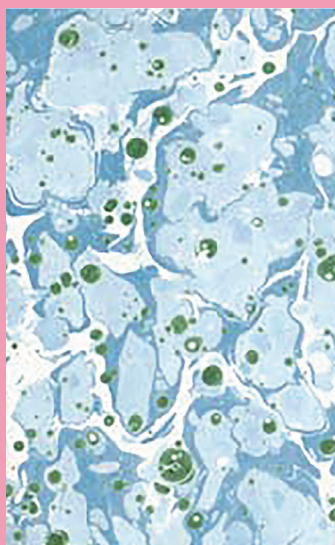
Supplementary figure 2 Titration curve: escalating doses of asTF expressing adenovirus in ECRF cells.

(**A**) and (**B**) Rates of FXa generation on cells and MVs in the presence of 1 nM FVIIa and 50 nM FX. asTF virus particle numbers were increased 3- or 7-fold. (**C**) Protein levels on cells and MVs transduced with 3× asTF (1); flTF (2); 3× asTF with flTF (3), and 7× asTF with flTF (4).

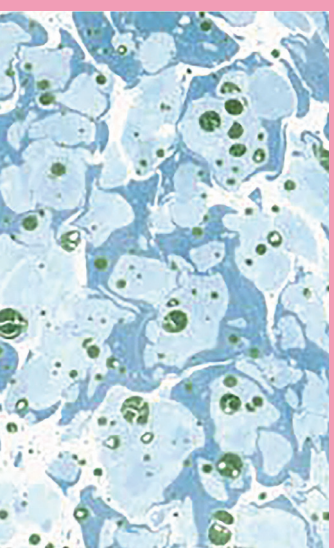


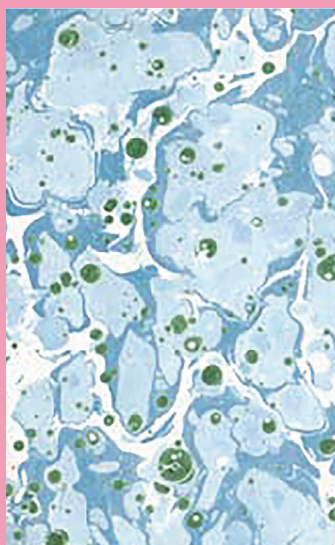
Supplementary figure 3 Titration curve: escalating doses of GFP expressing adenovirus in ECRF cells.

(**A**) and (**B**) Rates of FXa generation on cells and MVs in the presence of 1 nM FVIIa and 50 nM FX. GFP virus particle numbers were increased to 300 particles per cell. (**C**) Protein levels on cells and MVs transduced with increasing levels of GFP virus particles in the presence or absence of flTF.



Part II



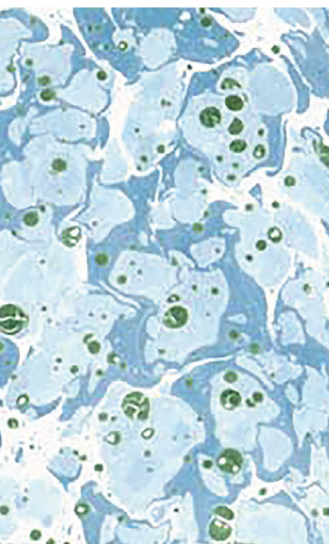


Chapter 3

Effects of tumor-expressed coagulation factors on cancer progression and venous thrombosis: is there a key factor?

Betül Ünlü
Henri H. Versteeg

Thromb Res. **2014** *May*; **133 Suppl 2:S76-84**



ABSTRACT

Since the 19th century an association between cancer and thromboembolic events has been described, with a poorer survival prognosis. Production and secretion of procoagulant factors affect tumor biology and cancer-associated thrombosis. Tissue factor (TF) exerts coagulant and protease activated receptor (PAR)-dependent signaling effects, both of which can contribute to tumor progression. Tumor cells are also capable of shedding TF-positive microparticles, suggesting a contribution to cancer-associated thrombosis at a distance from the tumor. Selected tumors are capable of ectopically expressing FVII and/or FX, which may lead to increased procoagulant features of tumors. Alternatively spliced TF (asTF) may affect tumor progression by inducing tumor growth and angiogenesis in an integrin dependent manner. Ectopic thrombin production also affects tumor progression by influencing proliferation rate, angiogenesis, invasion and metastasis. However, the roles of these coagulation factors in tumor progression and cancer-associated thrombosis are still not fully understood. In this review we will discuss several coagulation factors and their contribution on cancer progression and venous thromboembolism.

Keywords: Tissue factor, ectopic FVII, asTF, TF^+ MPs, cancer, thrombosis

INTRODUCTION

A relationship between coagulation and cancer has been described in the 19th century by Armand Trousseau [1]. Trousseau's Syndrome therefore describes the association of the presence of active cancer with elevated thromboembolic disorders and hypercoagulability. These thromboembolic events have been observed in patients with various tumors like glioblastoma, pancreas, stomach, lung and colon [1] and are the second leading cause of death in cancer patients, after metastasis [2]. Furthermore, patients known to have a prior history of thrombosis have an even further increased risk for cancer-associated thrombosis [3-4]. Reciprocally, hypercoagulability is also capable of increasing cancer progression in these groups of patients [5]. Patients diagnosed with cancer only have a first year survival rate of 36%, compared to patients with both cancer and thrombosis of whom only 12% survives in their first year [6]. Similarly, a higher mortality is observed in the first six months after diagnosis in patients suffering from both cancer and thrombosis compared to patients suffering from thrombotic events in the absence of active cancer [7]. This lower survival rate of cancer patients with thromboembolism may be explained by several factors; first, cancer patients experiencing thrombosis frequently have elevated cellular and circulating procoagulant factors, which is a hallmark of more malignant tumors. Second, production and secretion of coagulation factors has been shown to influence tumor-associated processes such as angiogenesis, metastasis and modulation of the immune system [8-9]. In this narrative review we will discuss tumor-specific production and secretion of coagulation factors, such as full-length tissue factor (flTF or TF), alternatively spliced TF (asTF), factor VII (FVII) and thrombin, as well as expression of the cellular receptors they activate. Finally, we will elaborate on the impact of tumor-specific coagulation factor production on the cancerous process.

TF and TF-dependent signaling in cancer

TF is a 47 kDa transmembrane glycoprotein that is expressed on subendothelial cells. Disruption of the endothelium exposes TF to the bloodstream enabling binding of its natural ligand coagulation FVII [10-11]. Calcium-dependent binding of FVII to TF converts the ligand into FVIIa, initiating the coagulation cascade by proteolytically activating factor X (FX) into FXa [10, 12]. Subsequently, thrombin is generated and this leads to platelet activation and a fibrin network that closes the damaged vessel [12-13].

Nowadays TF has been well accepted as the linking pin between coagulation and cancer (reviewed in [14-16]). Elevated TF expression in cancer has been associated with poor survival [1, 17-19]. Additionally, high TF expression in tumor specimens enhances the risk of

venous thromboembolism (VTE) in patients suffering from various cancers such as lung cancer, neck squamous cell carcinoma and pancreatic cancer [15]. For instance, an approximate 5-fold upregulation of VTE incidence was observed in pancreatic cancer patients with high TF expression, compared to patients with low TF levels [20]. TF expression is generally increased in tumors with specific mutations in oncogenes (e.g. ***K-ras***, ***c-Met***) and tumor suppressors (e.g. ***TP53***, ***PTEN***), in cells undergoing epithelial-to-mesenchymal transition (EMT), and tumor cells experiencing hypoxia [1].

Apart from its role in coagulation, the TF:FVIIa complex also promotes intracellular signaling. The TF:FVIIa complex triggers signaling cascades via Protease activated Receptor-2 (PAR2). PAR2 is a seven span-transmembrane G protein-coupled receptor and belongs to a family consisting of four members. This receptor can be activated by TF:FVIIa, the ternary TF:FVIIa:FXa complex and FXa, while thrombin activates PAR1, PAR3 and PAR4 [21]. A strong correlation was shown between overexpression of PARs and aggressive behavior of cancer cells [22]. Proteolytic cleavage of the extracellular N-terminus creates the formation of a pseudo-ligand that folds back onto the second extracellular loop of this receptor, activating downstream signaling [23]. PAR2 activation causes migration and invasion of tumor cells as well as secretion of factors such as interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) [24]. Secretion of IL-8 regulates endothelial cell survival, proliferation and matrix metalloproteinase (MMP) production, leading to increased angiogenesis [25]. In colon cancer TF:FVIIa-dependent PAR2 signaling affects the decomposition of the extracellular matrix by enhancing expression of MMP2 and MMP9, all of which promote metastasis and invasion [26]. In breast cancer tumor models, activated PAR2 impacts tumor growth, angiogenesis and invasion of cancer cells [27]. TF cytoplasmic tail (TF-CT) phosphorylation further stimulates PAR2-dependent signaling, causing increased induction of angiogenesis [28-29]. In mice harboring a mammary tumor virus promoter-driven polyoma middle T antigen (PyMT) cassette, PAR2 deficiency resulted in a delay of the angiogenic switch and a concomitant reduction in tumor growth [30]. Interestingly, similar results were observed when the TF-CT was deleted. The combination of TF-CT deletion and PAR2 deficiency did not result in an additional reduction in tumor size, which suggests TF-CT/PAR2 crosstalk and an overlapping role in breast tumors [28].

Apart from primary tumor growth, TF is also critically involved in metastasis. TF expression in tumors associates with metastasis in various cancer types such as pancreatic, breast, lung, melanoma and esophagus cancer [1]. While TF-dependent tumor growth is critically dependent on PAR2 activation, TF-dependent metastasis is dependent on the formation

of thrombin [31], which will be discussed below. Nevertheless, TF itself also contributes to metastasis, as in a CHO cell model TF-CT deletion results in reduced metastasis compared to that observed with TF harboring a CT [32]. Although it is currently unknown how the TF-CT affects metastasis, TF regulates integrin function [33] and the CT may be critical in integrin-dependent metastatic processes. TF mediated PAR2 signaling is to some extent also dependent on β1 integrins, as PAR2 signaling could be diminished using a β1 integrin inhibiting antibody [31]. We will not extensively review the details of β1 integrin mediated TF/PAR2 signaling. Rather, we refer to a recent review by Kocatürk et al. [16].

TF-positive microparticles in cancer

TF may play a role in cancer-associated thrombosis, and TF-positive microparticles (TF^+ MPs) may form a causal link between cancer and thrombosis. Microparticles (MPs) are formed through blebbing of the cell plasma membrane, with MPs falling into a size range of 0.1-1 μm. MPs usually contain a negatively-charged phosphatidylserine-rich surface [34]. In erythrocytes, monocytes, platelets, leukocytes and endothelial cells MP shedding is induced upon cell activation or the presence of apoptotic signals [14]. These MPs can be positive for TF and in conjunction with the presence of PS, which is required for the assembly of the coagulant-active complex, thrombin can be formed [35,36]. Upon vascular damage MPs interact with activated platelets via P-selectin binding. Due to transfer of TF to platelets via these TF^+ MPs, an additionally procoagulant state can be obtained [37]. Of note, cancer cells are also capable of shedding TF^+ MPs, suggesting a link with cancer-associated thrombosis at sites distant from the tumor.

MP participation in tumor biology has been shown; they may regulate processes such as blood coagulation, angiogenesis and metastasis [22, 38]. A study demonstrated that platelet-derived MPs (P-MPs) can elevate proliferation, migration and angiogenesis. Interaction of lung cancer cells with P-MPs showed activation of proliferation pathways (e.g. the p42/44 MAP kinase pathway), migration and invasion by means of MMP secretion. P-MP fusion with cancer cells resulted in transfer of integrins, leading to increased adhesion to endothelial cells and fibrinogen. However, stimulation of several angiogenesis markers in lung cancer cells were also observed, like VEGF, IL-8, and hepatocyte growth factor (HGF) [39]. Similarly, a study on tumor-derived MPs demonstrated that these MPs can induce metastatic cell homing and growth, but also suppression of anti-tumoral immune responses [40]. Based on these results it is tempting to speculate on a similar mechanism for TF^+ MPs in cancer progression.

Various clinical studies have determined associations between TF^+ MP activity or antigen and cancer-associated VTE. As may be expected, activity was highest in patients that developed VTE [41]. Additionally, TF activity on MPs was increased in plasma from metastatic breast and pancreatic cancer patients when compared to non-metastatic cancer patients [41-42]. This suggests a correlation between TF^+ MP coagulant activity and lower survival rate. A different study shows that P-selectin mediates fusion of TF^+ MPs with activated platelets and endothelial cells [43]. Thus, TF^+ MPs can mediate cancer progression via fusion to other (tumor) cells and activate several processes to induce angiogenesis and metastasis.

Ectopic FVII in cancer

The TF:FVIIa complex appears to plays a key role in pro-oncogenic mechanisms like angiogenesis [20, 29, 44-45], cell survival [46-47], migration and invasion [48]. The plasma protein FVII is, under normal conditions, constitutively expressed in the liver, mainly by hepatocytes [49]. However, this zymogen is also expressed by monocytes and macrophages in inflammatory settings, and in atherosclerotic vessels [50-53]. In cancer, expression of ectopic FVII is also observed in hepatocellular carcinoma cells (HCC) [54], bladder cancer [55], ovarian cancer [56], and laryngeal carcinoma tissues [57]. Blood coagulation initiated by the TF:FVIIa complex is under control of tissue factor pathway inhibitor (TFPI) [58]. When coagulation is initiated by forming the ternary TF:FVIIa:FXa complex, TFPI-1 binds to this complex to form a quaternary structure and inhibits FXa generation [58]. TFPI-2, a poor inhibitor of the TF pathway, has been shown to inhibit plasmin and MMPs, resulting in reduced invasion and migration of endothelial and cancer cells [59]. Paradoxically, in HCC, binding of TFPI-2 to the TF:FVIIa complex induced invasion [54], perhaps by stabilizing ternary complex signaling.

As FVII expression in the previously mentioned studies was primarily detected by means of immunohistochemical staining, it remained unclear whether FVII-positivity was caused by ectopic FVII or FVII taken up from the circulation [55]. Therefore, Koizume and coworkers have studied mRNA expression of FVII in 46 different cancer cell lines, showing frequent expression of endogenous FVII in various cancer cells [60]. In support, Tang et al. have shown FVII expression in six colon cancer cell lines [26]. Ectopic FVII(a) was functionally active due to cancer cell expression of γ-glutamyl carboxylase, which facilitates key post-translational modifications required for proper positioning of FVII on the cell membrane [60]. In the liver the gene encoding FVII (*F7*) is under transcriptional control of hepatocyte nuclear factor-4 (HNF-4) and Sp1 [49, 61] (Fig. 1). In breast cancer cells Sp1 is similarly crucial for the transcription of ectopic FVII, while HNF-4 has no effect on FVII expression in

breast cancer cells. To activate the promoter and start transcription several histone acetyltransferases (HATs) are recruited by HNF-4, like p300/CBP-associated factor (PCAF), p300, cyclic AMP-responsive element binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) [62-63]. In breast cancer mainly p300 and CBP were found at the promoter site [62]. For endogenous expression of FVII in breast cancer Sp1 is essential, as several studies have shown interaction with p300, thus causing transcription of the target gene [62, 64]. This specific expression of FVII by p300/CBP could be suppressed by the spice curcumin [62] (Fig. 1). The anticancer effects of this compound have been widely investigated and were shown to decrease proliferation and induce apoptosis in cancer [65]. Interestingly, in ovarian cancers hypoxia-inducible factor-2α but not Sp1, was found to regulate ectopic FVII expression [66]. This suggests different ectopic FVII expression pathways in various cancer types.

Ectopic FVII expression was shown to induce metastasis and invasion in ovarian, colorectal and breast cancers [26, 60], but the exact mechanism of FVII-facilitated tumor growth and metastasis has not been unraveled yet. It is unclear if metastasis in breast cancer is mainly dominated by FVII, because both FVIIa-dependent PAR2 activation and PAR1/PAR2 activation via TF:FVIIa:FXa may potentially play pivotal roles [24, 67]. In addition, thrombin-dependent coagulation and PAR1 activation have been shown to play a key role in metastasis [68], and it may well be that the role of ectopic FVII in metastasis is limited to enhanced invasion or enhanced coagulation activation in cancer.

Magnus and coworkers showed in glioblastoma multiforme (GBM) cancers that the ligand-independent epidermal growth factor receptor (EGFRvIII) mutant elevated proteins belonging to the TF signaling pathways, such as TF, PAR1, PAR2 and ectopic FVII. These tumor cells show increased coagulant activity and hypersensitive TF signaling in GBM cells [69]. Interestingly, different subtypes of GBM cells had different protein expression levels, pointing towards distinct regulation of proteins and risk factors for VTE in different patients. More specifically, ectopic FVII was upregulated, TF and PAR1/PAR2 downregulated in proneural GBM cells, while in classical GBM TF, PAR1 and PAR2 gene expression was upregulated and FVII down regulated [70]. Exposure of MDA-MB-231 breast carcinoma cells to FVII facilitates cell growth and cell survival through transcription of chemokines, cytokines and growth factors [71]. Hypoxia regulates ectopic FVII expression, making these cells more invasive [60]. Primary ovarian cancer cells and ovarian cancer cells in ascites are constantly exposed to hypoxia, thereby enhancing the expression of ectopic FVII [66, 72]. Coagulant active ectopically expressed TF:FVIIa is not only found in cells, but also on

TF^+ MPs [66]. As cancer cell-derived MPs - which are in general TF positive - contribute to hypercoagulability in those cancer patients [73], it is plausible that ectopic FVII in complex with MP-exposed TF plays a role in the increased risk of VTE at distant sites, which has a high incidence in ovarian cancer patients [74-75].

As mentioned before, TFPI-1 inhibits the TF:FVIIa:FX complex by binding simultaneously to FVIIa and FX. This inhibitor consists of three Kunitz domains; while the second Kunitz domain binds and inhibits to the active site of FXa, the first Kunitz domain binds to TF:FVIIa complex, preventing the formation of newly generated FXa [12]. Ten years ago, a TF inhibiting protein from the saliva of the tick ***Ixodes scapularis***, ixolaris, was discovered. This protein, very much like TFPI, forms a quaternary complex, existing of TF:FVIIa:FX/ixolaris to inhibit the activation of FX [58]. However high concentrations of ixolaris are also capable of blocking the TF:FVIIa binary complex. In glioblastoma and melanoma models ixolaris inhibits primary tumor growth and angiogenesis initiated by the TF:FVIIa:FX complex [76]. A recent study shows inhibition of tumor growth in breast cancer cells by ixolaris through blockade of both TF:FVIIa-dependent coagulation as well as TF:FVIIa-mediated PAR2 signaling with ixolaris [11]. This suggests that the ectopic TF:FVIIa binary complex could be a potential druggable target.

asTF in cancer

In 2003 a new isoform of TF was discovered and termed alternatively spliced tissue factor (asTF). asTF lacks the transmembrane domain normally found in full-length – normally spliced - TF (flTF) and has a unique C-terminus due to a frameshift resulting from exclusion of exon 5. The expression of this protein has been detected in $CD14^+$ macrophages and organs like heart, brain, lung, placenta and pancreas [77]. Interestingly, high asTF expression has also been observed in several cancers, such as breast, colorectal, glioblastoma, melanoma, as well as gastric and squamous cell carcinoma cell lines [78]. Enhanced tumor progression and angiogenesis was observed in asTF-expressing tumor cell lines [1, 79-80]. In esophageal cancer no association between asTF and thrombosis was established, as mRNA levels of asTF were comparable to those in healthy patients [81]. In contrast, an association between asTF mRNA expression and advanced tumor stage in patients with non-small cell lung carcinoma (NSCLC) exists, indicating variable asTF expression levels in various stages of cancer [82]. The observed effects of asTF on angiogenesis and proliferation rates appear to be integrin-dependent [78, 80]. A study of our own laboratory shows a role for asTF-induced angiogenesis in an integrin-dependent manner. Angiogenesis was induced by asTF through binding to β1 and β3 integrins [80]. asTF mediates endothelial cell migra-

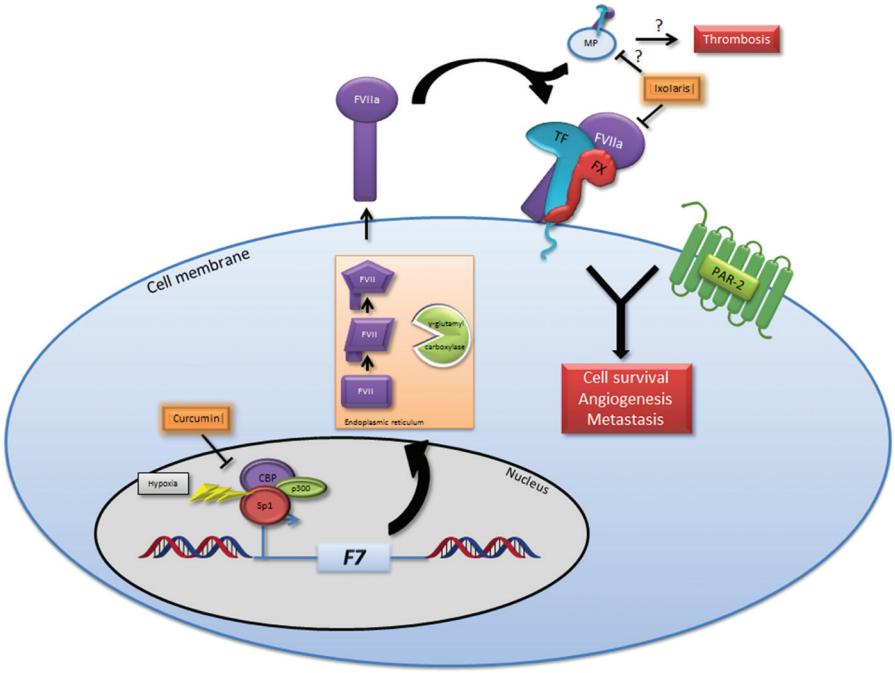


Figure 1 Regulation of ectopic FVII in breast cancer.

Hypoxia induces the assembly of transcription factors Sp1, p300 and CBP at the promoter site of *FVII* and leads to ectopic FVII expression. This transcription can be inhibited by curcumin as it suppresses the p300/CBP complex. Endogenous FVII can be folded into a functioning protein as γ-glutamyl carboxylase is also expressed, which facilitates key posttranslational modifications. This ectopically expressed FVII can interact with TF and subsequent PAR-2 dependent signaling may lead to cell survival, metastasis and angiogenesis. The TF:FVII complex may also be shed by the tumor cell on the surface of MPs thus causing thrombosis at a site distant from the tumor. Ixolaris inhibits coagulant and signaling properties of both the TF:FVIIa binary complex and the TF:FVIIa:FX ternary complex. The inhibitory function of ixolaris on TF:FVIIa and TF:FVIIa:FXa positive MPs has not been studied yet.

tion, capillary formation and aortic sprouting via ligation to αvβ3 and α6β1 integrins respectively. The different stages of angiogenesis require different integrin subsets. For instance, tip cell directional migration requires αvβ3 while capillary formation, which is dependent on non-directional migration, requires β1 integrin [83-84]. In support, asTF activates αvβ3 integrin, p38 MAPK and PI3-kinase to induce endothelial cell migration, while asTF-in-

duced capillary formation involved α6β1, p42/44 MAPK and PI3-kinase [80]. Although flTF also induces angiogenesis in a β1 integrin-dependent manner, the involvement of integrins differs mechanistically; association of flTF with integrins facilitates flTF:FVIIa/PAR2-dependent signaling [28, 31, 33]. In a second study, we showed that tumoral flTF expression correlates only with histological grade, whereas asTF correlates with histological grade, tumor size and age in a cohort of approximately 600 breast cancer patients [78]. This suggests different roles for flTF and asTF in breast cancer progression; as asTF expression associated with tumor size, we hypothesized that asTF influences cell proliferation rates. Using MCF-7 cells overexpressing flTF or asTF we showed that asTF, but not flTF expression conferred a proliferative advantage to these cells in a murine orthotopic breast cancer model. While low PAR2 expression, a requirement for flTF-dependent tumor growth, might lie at the basis of absent flTF-dependent tumor growth, subsequent experiments with different versions of the MDA-MB-231 breast cancer cell line, excluded this possibility. Normal MDA-MB-231 cells express high levels of both flTF and PAR-2 but low asTF levels. In contrast, the more aggressive MDA-MB-231-mfp subline expresses high asTF protein levels. Both asTF-expressing MCF-7 and MDA-MB-231-mfp cells showed higher proliferation rates ***in vivo***, while flTF expression in flTF-MCF-7 cells or flTF inhibition in MDA-MB-231-mfp cells did not affect proliferation [78]. This is consistent with a role for flTF in angiogenesis rather than proliferation. Notably, asTF inhibition in MDA-MB-231-mfp cells did not significantly affect angiogenesis while asTF expression in MCF-7 cells enhanced angiogenesis, most likely because secreted asTF levels are low in MDA-MB-231-mfp cells, compared with asTF-expressing MCF-7 cells. Our findings, as well as those in previous studies on this subject, illustrate that breast tumor growth is influenced by asTF-dependent proliferation and flTF/PAR2-dependent angiogenesis. ***In vitro*** studies showed that binding and co-localization of asTF to β1 integrins mediated proliferation, while blocking or silencing of β1 integrin diminished this effect [78]. However, asTF-dependent proliferation was not completely inhibited when β1 integrin was blocked, which does not exclude the idea that asTF might interact with other (transmembrane) proteins.

Although tumoral asTF secretion may influence thrombosis in cancer patients, the pro-coagulant activity of asTF is still under debate. Several studies using transfected cell systems were not able to pinpoint a role for asTF in coagulation [79, 85-86]. In these experimental setups i) no asTF secretion could be detected [86], or ii) the proper physiological milieu, i.e. lack of flTF expression, was not mimicked [79, 85]. In other studies, clotting time of platelet poor plasma was decreased in the presence of (recombinant) asTF and phospholipids [77, 87]. Stimulation of endothelial cells with IL-6 and tumor necrosis factor-alpha (TNF-α)

resulted in enhanced secretion of asTF to the media, which was pro-coagulant in the presence of phospholipids [87]. Whether or how asTF:FVIIa converts FX to FXa is still unclear, as asTF lacks a proper transmembrane domain and is not likely to associate with FX [87]. Nevertheless, asTF appears to be capable of converting FX into FXa when flTF is co-expressed on cells. In the pancreatic ductal adenocarcinoma cell line Pt45P1 overexpression of asTF increased coagulant activity on cells as well as on cell-derived MPs, compared to untransfected Pt45P1 cells. Similar effects were observed when Pt45P1 cells were stimulated with exogenous recombinant asTF. However, exogenously added asTF had no effect on procoagulant properties of MPs from control Pt45P1 cells, suggesting a key role for flTF-, rather than asTF-dependent coagulation. A significant increase of asTF surface expression on MPs was detected, while β1 and β3 integrin levels were not altered [88]. asTF has been observed at the growing edge of thrombi, suggesting a role for asTF in thrombosis [77]. Thus, the study of Unruh and colleagues supports the above mentioned theory, indicating a role for asTF and flTF synergy in the increased risk of thrombosis in pancreatic carcinoma patients [88].

Apart from asTF-integrin dependent angiogenesis and proliferation, this interaction can play a role in atherogenesis as this protein is also found in intraplaque macrophages [80, 85]. Binding of asTF to β1 integrins enhances interactions of endothelial cells with monocytes by upregulating VCAM, ICAM, E-selectin and P-selectin [89-90]. Under laminar flow asTF also increases the levels of CXC and CC chemokines, suggesting that asTF contributes to arterial thrombosis [87]. Of interest these effects are also observed in high asTF-secreting solid cancers [80, 91].

FX in cancer

The effects of FX on tumor progression in cancer are still unclear. However, increasing evidence points towards a role of FX in cancer-associated thrombosis. Studies show an association of FX presence in colon, gastric, non-small-cell lung cancer and breast cancer tumor cells [92-95]. In immunohistochemical studies FXa was frequently detected in cancer tissues such as lung small cell carcinoma, renal cell carcinoma and malignant melanoma [96]. Other studies have revealed that FXa is involved in migration and survival [21, 46]. Effector cell protease receptor-1 (EPR-2) was found on the cell membrane of cancer cells, and FXa might influence tumor progression via this receptor as well as PAR1 and PAR2 signaling [23, 97]. A recent study demonstrated FXa mRNA levels in colon cancer, which suggests constitutive expression of this coagulation factor. Interestingly, its naturally occurring inhibitor complex protein Z (PZ)/Z-dependent protease inhibitor (ZPI) was also transcribed and expressed, also co-localized with FXa in these tumor cells [92]. This complex inhibits

thrombin generation as it prevents the formation of the prothrombinase complex [98] (Fig. 2), although the PZ/ZPI complex was unable to completely prevent thrombin generation in colon cancer suggesting imbalanced expression of this complex [92]. A recent study showed that coagulation kinetics of circulating TF was regulated by high FX and FIX levels, albeit within normal physiological range. FX increased clotting rate and FIX increased enzyme generation rate [99]. This suggests that FX and FIX concentrations in blood could be a biomarker for cancer-associated thrombosis, although no effects of FX and FIX on tumor biology have been found yet.

Thrombin and cancer

Thrombin is a key effector of blood coagulation. However, when it interacts with cells it also affects a wide spectrum of cellular processes like proliferation, angiogenesis, inflammation, embryonic development and also tumor progression [100-102]. In tumor biology the effects of thrombin include enhanced tumor growth, angiogenesis, invasion and metastasis [102]. Tumor cells may activate thrombin directly by secretion of pro-coagulant factors or indirectly via secretion of cytokines, pro-inflammatory proteins, proteases and TF^+ MPs [103-104]. Thrombin on its turn activates platelets, fibrin formation, PAR1 signaling and leads to extracellular matrix ECM degradation [102]. These processes are specifically involved in angiogenesis, suggesting a key contribution from thrombin in tumor angiogenesis. Thrombin treatment of cancer cells resulted in a 3-fold induction of adhesion to human platelets [105]. Activation of PAR1 signaling by thrombin upregulates several pro-angiogenic proteins in endothelial cells and cancer cells like VEGF, angiopoietin-1, MMP1 and MMP2 [106-107]. Additionally, p38 MAP kinase-dependent thrombin expression in stroma of metastasized colon carcinoma facilitates PAR1 signaling in an autocrine fashion eventually leading to new vessel formation. Thrombin-dependent PAR1 activation also leads to upregulation of pro-oncogenic genes (e.g. Twist, GRO-α, MMP1 and MMP2) and subsequent metastasis in murine tumor cells [102]. Interestingly, this pro-metastatic phenotype requires the expression of TF on tumor cells ***in vitro*** and ***in vivo*** as melanoma cell line expressing low amounts of TF, but PAR1 overexpression did not support efficient metastasis [68].

Coagulation factors affect metastasis in distinct manners. First, coagulation activation protects metastatic tumor cells via fibrin formation. This shield of fibrin may be induced by TF expression on these tumor cells, disabling an immune attack mediated by natural killer cells. In fibrinogen-deficient mice TF expressing fibrosarcoma cells did not support lung metastasis [5]. Second, thrombin-mediated activation of platelets via PAR-1 induces adhe-

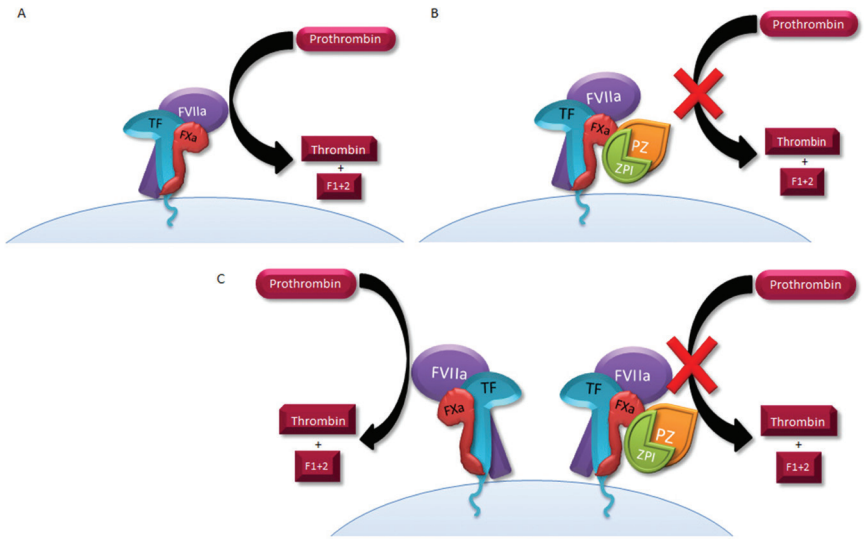


Figure 2 Schematic representation of the TF:FVIIa:FXa complex.

(**A**) Under normal circumstances, FX is generated into FXa leading to formation of thrombin and F1+2. (**B**) When the naturally occurring PZ/ZPI complex is present, binding of this complex to FXa prevents thrombin generation. (**C**) In cancer the balance between the TF:FVIIa:FXa and PZ/ZPI complex is disturbed, resulting in unchecked thrombin levels.

sion of tumor cell to platelets, contributing to thrombus formation around the metastatic cell, thus protecting against the immune system [105].

Hypoxic tumors express thrombin in their micromilieu by activating p38 MAPK and inducing the levels of thrombin even further [104]. In highly metastatic tumors PAR1 is upregulated by thrombin, which is associated with a poorer prognosis in breast cancer [108]. *In vivo* studies showed that specific inhibition of thrombin leads to decreased tumor growth and metastasis [109-110]. This identifies thrombin as a potential therapeutic target in order to suppress tumor progression.

Biomarkers in predicting cancer-associated thrombosis

As discussed above, increased coagulant activity in cancer patient results in poor prognosis and identification of such a procoagulant state could benefit diagnosis and treatment of

cancer patients. Thus, identification of biomarkers for a hypercoagulant state or increased risk for venous thrombosis is pivotal. Several studies investigated the suitability of specific biomarkers for the prediction of cancer-associated thrombosis [111]. The prognosis of cancer has been associated with TF$^+$ MPs [112-113], elevated platelet levels [3, 114] and D-Dimer levels [115]. An association has been found between increased risk for VTE and elevated D-Dimer levels in cancer patients [116-118]. Prothrombin factor 1+2 (F1+2) also shows promise as a valuable biomarker. A study of Ay and co-workers showed a 2-fold increase in the risk of VTE when F1+2 levels were high [119]. Although F1+2 showed an association with increased risk of VTE, this has not been validated in clinical studies yet. Thus, at present the best biomarker to predict VTE in cancer patients maybe D-Dimer levels [120].

The validity of TF$^+$ MPs as a biomarker to predict cancer-associated thrombosis is still under debate; studies into the association between TF$^+$ MPs and VTE provide contradicting results. Several preclinical and clinical studies have demonstrated a positive association of procoagulant TF$^+$ MPs with the risk of developing VTE, even with recurrent VTE [20, 38, 42]. Xenograft studies demonstrated the presence of TF$^+$ MPs within the developing thrombus [43]. In the plasma of breast and pancreatic cancer patients, high TF$^+$ MP activity associated with thrombotic events [41]. However, in a follow-up study from the same group, the numbers of MPs were not increased in cancer patients compared to healthy controls [42]. When TF$^+$ MP levels were investigated in multiple myeloma cancer patients before and after chemotherapy, no elevation of TF$^+$ MP levels were observed in patients who developed VTE compared to those that did not develop VTE during the follow-up. Nevertheless, coagulant activity of these MPs remained high in patients that developed VTE, even after chemotherapy [121]. Other studies were not able to detect significant differences in TF$^+$ MP levels in cancer patients versus healthy controls [122]; no statistically significant association was observed in patients suffering from gastric, colorectal and brain cancer [34]. These contradicting results could be explained by the different methodologies used; each method has specific advantages but also drawbacks thus limiting comparison of the studies. A recent study of Zwicker et al. showed a more sensitive method to measure TF$^+$ MP in cancer patients with advanced malignancy using impedance-based flow cytometry. Using this technique, the authors showed an association of TF$^+$ MP activity with VTE development in cancer patients with various histologies [38]. Thus, TF$^+$ MPs may be used as a biomarker for cancer-associated thrombosis, provided that the methodology is standardized and optimized.

Finally, increased thrombin generation has been linked to increased risk of developing VTE [123]. This approach could be used as a biomarker to determine the risk of VTE in cancer patients [4].

CONCLUSION

A picture is emerging in which ectopically expressed coagulation factors, either alone or in concert, influence cancer progression and thrombosis in cancer patients. We have discussed that, apart from TF, other blood coagulation factors can contribute to tumor progression. Tumor cells may shed TF^+ MPs, which may not only induce angiogenesis and metastasis, but may also mediate thrombotic complications. Furthermore, ectopic expression of FVII, which is expressed by various cancers mediates tumor growth, metastasis and invasion, at least in cellular models. The alternatively spliced isoform of TF, asTF, induces proliferation and angiogenesis via integrin ligation. FX is shown to be present in cancer tissue specimens, some tumor cells are capable of ectopically expressing FX. The effects of FX on tumor progression are not investigated yet, however new results are emerging. Thrombin mainly affects tumors by activating platelets and via PAR1 signaling and inhibition of thrombin leads to reduced tumor growth and angiogenesis. Finally, the absence of standardization and use of various methods hampers research into the validity of biomarkers as indicators for the risk of cancer-associated thrombosis.

Additional research is needed in order to unravel and fully understand the signaling pathways induced by the various coagulation factors and their effects on progression of tumors. In doing so, new therapeutic drugs may be designed in order to diminish the effects of coagulation factors on tumors and thromboembolism.

Authorship

BU wrote the manuscript and designed the figures, HHV supervised and edited the manuscript.

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Conflict of interest statement

The authors have no relevant conflicts to declare in relation to this paper.

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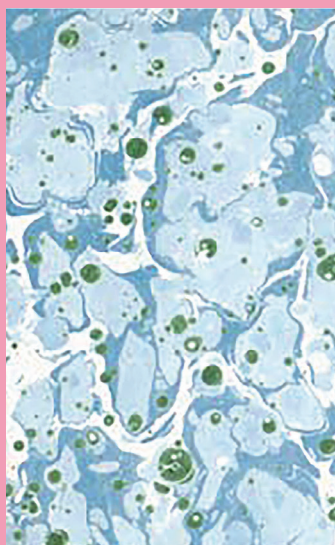
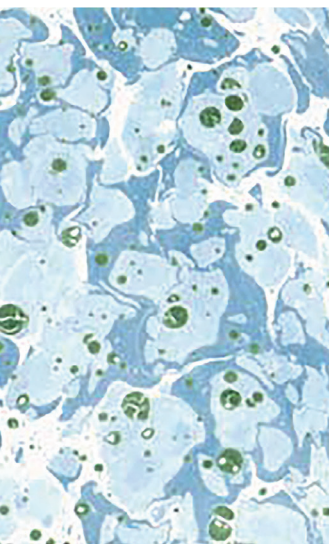


Chapter 4

Integrin regulation by Tissue Factor promotes cancer stemness and metastasis in breast cancer.

Betül Ünlü
Begüm Kocatürk
Araci M. da R. Rondon
Nathalie Swier
Rob F.P. van den Akker
Erik J. Blok
Wolfram Ruf
Peter J.K. Kuppen
Henri H. Versteeg

Manuscript under submission



One-sentence summary: Tissue Factor promotes early metastatic events in breast tumors by altering epithelial-to-mesenchymal transition and cancer stem cell programs through function modulation of β1 and β4 integrins.

ABSTRACT

Tissue Factor (TF) expression in breast cancer is associated with higher tumor grade, metastasis and poor survival. TF influences tumor growth through cellular signaling and that TF promotes metastasis via blood clotting. However, the role of TF signaling in metastasis has never been addressed. Here, an association between TF expression and metastasis was shown in our breast cancer cohort (N=574); and with a cancer stem cell (CSC) marker. Blockade of TF signaling inhibits metastasis 10-fold independent of primary tumor growth ***in vivo***. A reduction in epithelial-to-mesenchymal transition (EMT), CSCs and expression of the pro-metastatic markers Slug and SOX9 was observed ***in vitro***. Mechanistically, TF shifts from α3β1 integrin to α6β1 and α6β4 and dictates FAK recruitment, leading to reduced EMT and tumor cell differentiation. In conclusion, inhibition of TF signaling leads to a reduced EMT and CSC transcriptional programs, a reduction in primary tumor-resident CSCs and a subsequent reduction in metastasis.

INTRODUCTION

Breast cancer is the second leading cause of death in women. The lifetime-risk for breast cancer diagnosis among women is 1 in 8, with over 250.000 estimated new invasive breast cancer cases in 2017 [1]. Despite early diagnosis and improved treatment, breast cancer is still one of the leading causes of cancer-related death in women. Unfortunately, the 5-year relative survival dramatically declines to 35% in breast cancer patients with distant metastasis when compared to patients with a local tumor (98%). Nearly 40% of all breast cancer patients have regional or distant metastases, 30.8% and 6.2% respectively [1].

Prior to metastasis, tumor cells undergo epithelial-to-mesenchymal transition (EMT), in which cell-cell contact is lost and a spindle-shape morphology is acquired with enhanced migratory and invasive properties. This EMT program is accompanied by downregulation of epithelial markers (like E-cadherin) and upregulation of mesenchymal markers (e.g. Vimentin), that are under control of EMT-related transcription factors like members of the SNAIL, TWIST and ZEB families [2, 3]. After extravasation at distant organs, the reversed transition occurs, termed MET. However, it becomes more evident that there is an intimate relationship between EMT and cancer stem cells (CSCs) required for successful metastasis [4-6]. CSCs belong to the sub-population of the tumor with the ability to self-renew, seed new tumors and processing increased resistance towards chemotherapy [7]. CSCs in breast cancer may be identified based on the expression of surface markers CD133+, CD44+/CD24- and/or the intracellular protein aldehyde dehydrogenase 1 (ALDH1) [8]. The current hypotheses are either that i) cells that have undergone EMT de-differentiate into CSCs or ii) CSCs start expressing EMT-associated markers, after which they metastasize [9]. One protein that is suggested to drive cancer stemness is Tissue Factor (TF) [10].

TF is the initiator of the extrinsic coagulation pathway and is expressed on sub-endothelial cells. It binds and facilitates activation of its natural ligand factor VII (FVII) after vessel injury. This binary complex activates coagulation factor X (FX) that leads to prothrombin cleavage, activation of platelets and fibrin deposition in order to close the site of the wound. However, TF is also synthesized by breast tumor cells, and this expression has been found to associate with higher tumor grade, increased angiogenesis, reduced survival, as well as increased invasive and metastatic behavior [11].

Classically, it has been thought that TF influences tumor progression via two distinct processes. First, TF in complex with FVIIa induces i) direct cellular signaling via Protease activated Receptor-2 (PAR2) and ii) indirect TF signaling through crosstalk with integrins, to

promote tumor angiogenesis, tumor growth and migration, respectively. In addition, integrins also directly influence TF signaling by promoting TF-dependent PAR2 activation. TF signaling also appears to increase the invasive capacity through increased production of matrix metalloproteinases (MMPs). The latter degrade the extracellular matrix (ECM) allowing tumor cells to escape the local tumor environment. In addition, TF regulates cell adhesion and migration via integrins in a PAR2-independent manner [12, 13]. Upon entering the bloodstream, coagulant functions of TF becomes key, as it forms a fibrin/platelet-rich shield around circulating tumor cells. This prevents attack from the immune system, and thereby promotes survival of metastatic cells. PAR2-dependent signaling is facilitated by TF/β1 integrin complex assembly after binding of FVII to TF [14, 15]. So far, the role of TF signaling on the early facets of metastasis (***in vivo***) has not been addressed.

Here, we demonstrate that TF signaling, in addition to primary tumor growth, also impacts metastasis and the cellular processes underlying metastasis. Inhibition of TF signaling resulted in a downregulation of EMT-associated markers, decreased CSC activity and invasive capacity. Finally, a regulatory role for TF in integrin function and localization in cellular membrane compartments was observed, giving more insight in the mechanism underlying TF signaling-dependent metastasis.

METHODS

Reagents and cell culture

The breast cancer cell line MDA-MB-231-mfp were cultured in DMEM, supplemented with 10% fetal calf serum, 2 mM L-Glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco) in a 5% CO_2 incubator at 37 °C. To coat culture plates with ECM components vitronectin (Sigma-Aldrich) derived from human plasma and supernatant from 804G cells for laminin condition were used. For the antibody inhibition approach the flTF (Mab-10H10; mouse); β1 integrin (Mab-AIIB2; rat); PAR2 (Pab-pool7; mouse) were used.

Tissue Microarray Analysis

Tumor material from 574 non-metastasized breast cancer patients was collected who underwent tumor resection at the LUMC between 1985 and 1994 [16]. Approval was obtained from the Leiden University Medical Center (LUMC) Medical Ethics Committee. Age, tumor grade, histological type, tumor/node/metastasis status, median follow-up (17.9 years), lo-

co-regional or distant tumor recurrence and expression of ER, PgR and HER2 were known. Specimens were graded according to current pathological standards. Sections were cut and immunohistochemically stained for TF as described previously [17], including normal breast tissue of 266 patients (46%). The percentage of TF positive tumor cells was scored by two blinded observers. TF expression in breast specimens was compared to tumor recurrence and/or metastasis. Metastasis-free survival rates were calculated with the Kaplan-Meier method.

Orthotopic breast cancer cell injections into the mammary fat pad

All the animal experiments were approved by the animal welfare committee of the LUMC. All applicable institutional guidelines for the care and use of animals were followed. Orthotopic injections were performed as described previously [18]. In brief, $5x10^5$ MDA-MB-231-mfp cells were mixed with 500 µg Mab-10H10 or isotype matched mouse IgG1 (TIB115) and injected into inguinal fat pads of 6 week-old female NOD-SCID (n=4) or NOD-SCIDγ (n=5) mice (Charles River, Wilmington, MA); as an analgesic 0,05 mg/kg temgesic (Schering-Plough, Kenilworth, NJ) was injected. Sample size was selected on previous studies performed by our group [15, 17] and mice were randomly divided into two groups prior to xenograftment. The tumor dimensions were measured with a caliper and the volume was calculated with the formula $V=(LxW^2)/2$, by two independent observers. End points were when control tumors reached ~1000 mm^3 or for humane reasons defined by institutional guidelines. Primary tumors and organs were harvested and processed for further analysis. *Ex vivo* cells were isolated and cultured from tumors derived from NOD-SCID mice.

qPCR

For real time PCR analysis, total RNA was isolated using Trisure (Bioline; Bio-38033) and converted to cDNA using the Super Script II kit (Life Technologies). SYBR select (Life Technologies) was used to conduct qPCR. Primers are described in table S2. To quantify the presence of human tumor cells in mouse organs, a qPCR was performed with the housekeeping genes mouse β-actin and human GAPDH as a measure for metastasis.

Immunoprecipitation and western blotting

Prior to immunoprecipitation, cells were incubated with 50 µg/ml antibodies for 72 h. Cells were washed twice in ice-cold HBS and lysed in Brij35 buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Brij35, pH 7.4, and protease inhibitor) for 30 min on ice in order to collect non-raft fractions. Cells were scraped and centrifuged at 800 g for 10 min in order to pellet cell debris. Supernatant was centrifuged at 16.000 g for 30 min at 4°C. Pellet was

collected and lysed in Brij58 buffer for cholesterol-rich lipid raft fractions, spun at 16.000 g for additional 30 min. Supernatants of both Brij35- and Brij58 soluble fractions were subjected to immunoprecipitation O/N at 4°C in the presence of specific antibodies and protein A/G magnetic beads. After three washing steps with lysis buffer, the magnetic beads were resuspended in 2x sample buffer (Life Technologies).

For western blotting, cells were lysed in 2x sample buffer, sonicated for 10 sec and proteins were denatured at 95°C for 5 min. Lysates were loaded on 4-12% Bis-Tris PLUS Gels (Thermo Fischer Scientific) for 20 min at 200V and blotted on 0.2 µm pore size PVDF membranes and blocked in 5% milk/TBST for 1h. Blots were incubated with 1:1000 primary antibodies in blocking buffer O/N at 4°C, 3 TBST washing steps and incubated with HRP-conjugated secondary antibodies (Abcam) for 1h at RT. Antigens were visualized with Western Lightning Plus ECL (Perkin-Elmer) using the ChemiDoc imaging system (BioRad).

Matrigel invasion assay

To assess invasion, cells were serum starved for 24h, resuspended in serum-free DMEM in the presence of 50 µg/ml antibodies and seeded in the upper compartment of a 24-wel invasion chamber (BD Biosciences) at 5 x 10^4 cells/well. The lower compartment was filled with standard culture medium containing 10% serum. Cells were allowed to invade for 48h at 37°C, fixed in 2% formalin and stained with 1% crystal violet for 10 min at RT. Non-invaded cells were removed using a cotton swap; 5 randomly chosen pictures were taken per insert and invaded cells were counted.

Mammosphere and colony formation assay

A single cell suspension was seeded at 500 cells/well in a low-attachment 96 wells plate in mammosphere media (DMEM/F12 phenol-red free, 1% B27, 20 ng/ml hEGF, 20 ng/ml hFGF, 4 µg/ml heparin, 1% pen/strep). Cells were incubated for 14 days at 37°C. Spheroids with an area of >2000 μm^2 were considered as mammospheres and were counted. To calculate mammosphere forming efficiency (MFE), the number of mammospheres were divided by the number of originally seeded cells and expressed as a percentage.

For colony formation assays, 100 cells were seeded in a 6-wells plate in the presence of 50 µg/ml IgG or 10H10 antibody. Cells were incubated for 14 days at 37°C, media was refreshed twice weekly. After 2% formalin fixation and crystal violet visualization, holoclones were counted with a colony density of >50 cells.

Immunofluorescent staining

MDA-MB-231-mfp cells were grown on coverslips in the presence of 50 µg/ml antibody for 72h at 37°C. Cells were washed, fixed with 2% formalin and permeabilized with 0.1% Triton-X100 for 5 min at RT. After 1h in 5% BSA/PBS blocking buffer, primary antibody was applied at 50 µg/ml and incubated overnight at 4°C. After 3 wash steps, cells were incubated with goat anti-rabbit-Alexa-594 (Life Technologies) and phalloidin-FITC (Sigma-Aldrich) for 1h in the dark. Coverslips were mounted with DAPI in ProlongGold (Life Sciences) to stain the nuclei. Images of immunofluorescent labeled cells were captured using a Leica SP5 confocal microscope.

Statistical analysis

Data are represented as mean ± SD. Comparisons between data points were done with Student's *t* test for two conditions. With three or more data sets significance was calculated using one-way or two-way ANOVA analysis.

RESULTS

Tissue factor expression associates with metastasis in Estrogen Receptor negative tumors

We first investigated clinical associations between TF expression and metastasis-free survival of breast cancer patients. Tumor specimens from 574 breast cancer patients were stained for TF and stratified for Estrogen Receptor (ER) expression. Expression of TF in ER-positive tumors did not associate with metastasis-free survival (Fig. 1A). However, a significant association between high TF levels and metastasis in ER-negative tumors was observed that was especially evident in the first five years after diagnosis (Fig. 1B). Cancer stem cells play a fundamental role in metastasis, thus we also determined associations between TF expression and the CSC marker ALDH1; a strong association between high TF expression and ALDH1, was observed (Fig. 1C).

Inhibition of TF signaling reduces metastasis *in vivo*

Our own work in patient material and that in ***in vitro*** models [10] indicate a role for TF signaling in CSCs and suggest that metastasis may be critically dependent on TF signaling. To further investigate the role of TF signaling in metastasis, the highly aggressive and triple-negative MDA-MB-231-mfp cell line [19] was grafted orthotopically in the mammary

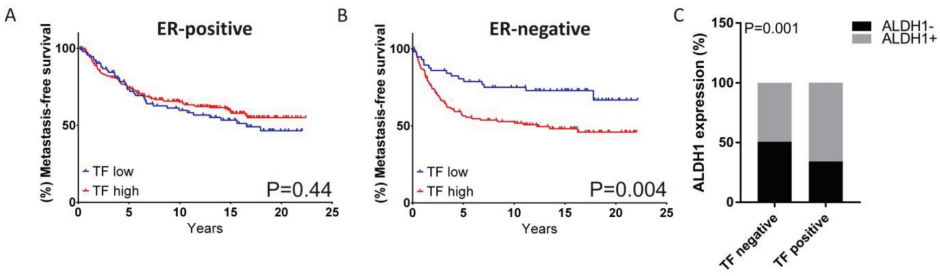


Figure 1 TF expression associates with metastasis-free survival in patients with ER-negative breast tumors, and ALDH1.

(**A**) Kaplan-Meier analysis of metastasis-free survival in breast cancer patients with ER-positive tumors, stratified for TF expression. (**B**) Kaplan-Meier analysis of metastasis-free survival in breast cancer patients with ER-negative tumors, stratified for TF expression. (**C**) Association between the breast cancer stem cell marker ALDH1 and TF expression. Assessments of associations were performed using SPSS and Stata.

gland. In the presence of Mab-10H10 – a specific inhibitor of TF signaling - a 5-fold decrease of tumor growth was observed in comparison to IgG control (Fig. 2A). After mice were sacrificed, lungs were processed to study the presence of human cells using qPCR. Strikingly, the presence of Mab-10H10 reduced metastasis 100-fold (Fig. 2B). The immune system plays a key role in TF-mediated metastasis, as TF deficient cells can only efficiently metastasize in a mouse model that lacks natural killer (NK) cells [20]. To further evaluate the contribution of TF signaling to metastasis, we repeated the ***in vivo*** experiment in NK cell deficient mice. Tumor volumes were equal after 7 weeks of inoculation (Fig. 2C). In contrast, there was a significant 10-fold reduction of metastasis in the lungs when tumor cells were grafted orthotopically in the presence of Mab-10H10 (Fig. 2D). These data suggest that TF signaling is directly responsible for pro-metastatic events in the primary tumor. Indeed, qPCR analysis of the tumors for EMT-associated markers showed a significant reduction in SNAIL (*SNAI1*) and SLUG (*SNAI2*) mRNA levels, in a NOD-Scid setting (Fig. 2E). While a significant downregulation of *SNAI2* was observed in tumors grown in NK cell deficient mice, *SNAI1* expression remained unaffected and a trend towards decreased *SOX9* expression was present (Fig. 2F). This suggests that inhibition of TF signaling decreases EMT transcription programs ***in vivo,*** regardless of primary tumor volume.

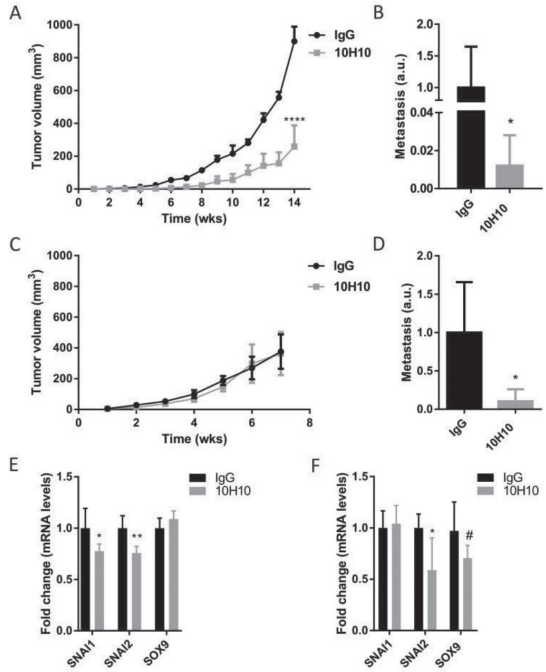


Figure 2 TF signaling inhibition reduces metastasis *in vivo*.

(**A**) MDA-MB-231-mfp cells were orthotopically injected in the presence of 500 ug IgG control or Mab-10H10 antibody in NOD-SCID mice (n=4). Tumor growth was monitored until week 14. Mean and SD are shown. (**B**) Lungs from NOD-SCID mice were analyzed by qPCR to assess metastasis by determining human GAPDH levels corrected for mouse β-actin levels. (**C**) MDA-MB-231-mfp cells were orthotopically injected in the presence of 500 ug IgG control or Mab-10H10 antibody in NOD-SCIDγ mice (n=5). (**D**) Lungs from NOD-SCIDγ mice were analyzed by qPCR to assess metastasis by determining human GAPDH levels corrected for mouse β-actin levels. (**E-F**) Tumors were analyzed by qPCR for the mRNA expression of *SNAI1*, *SNAI2* and *SOX9* from NOD-SCID (**E**) and NOD-SCIDγ mice (**F**). # P <0.10, * P<0.05, **** P <0.0001

Blockade of TF signaling reduces cancer stem cell program *in vitro*

To investigate if blockade of TF signaling resulted in a decreased EMT transcription program *in vitro*, MDA-MB-231-mfp cells were pre-treated with antibodies for 72 hours to inhibit TF signaling. As expected, well-established downstream targets of TF signaling, *CXCL8*, *CXCL1* and *VEGF*, were significantly decreased (Fig. 3A). Although treatment of MDA-MB-231-mfp cells with Mab-10H10 had no effect on *SNAI1* expression, *SNAI2*, *SOX9* and *MMP9* were significantly downregulated by 2-fold at mRNA levels (Fig. 3B, C). Furthermore, similar results

were obtained at antigen levels, with lowered Snail and SOX9 protein levels (Fig. 3D). This attenuated EMT program coincided with a change in morphology, i.e. cells displayed an increased epithelial phenotype compared to cells treated with control IgG (Fig. 3E). Matrix metalloproteinase 9 (MMP9) is a protein involved in the degradation of the extracellular matrix (ECM), allowing tumor cells to escape the primary tumor. In line with reduced ***MMP9*** mRNA expression levels upon Mab-10H10 treatment, a 2-fold reduction in invasive capacity was observed using matrigel invasion assays compared to control IgG (Fig. 3F). Since SOX9 is considered a driver of cancer stem cell genesis [21] and expression showed a significant decrease in Mab-10H10 treated MDA-MB-231-mfp cells, we hypothesized that these cells would possess diminished CSC properties. Cells were seeded on low attachment plates in order to evaluate mammosphere formation. As expected, the number of tumor colonies were decreased by 2-fold in the Mab-10H10 condition (Fig. 3G). Additionally, Mab-10H10 treated cells showed a similar reduction of holoclone formation (Fig. 3H), being the most aggressive clone-type, with highly proliferative properties and self-renewal capacity [22]. Previously, Endothelial Protein C Receptor, EPCR (encoded by ***PROCR***) was described as a CSC marker in triple-negative breast cancer [23]. Likewise, a reduction of ***PROCR*** was observed when TF signaling was inhibited (Fig. 3I), although no significance could be reached. Thus, inhibition of TF signaling ***in vitro*** decreases both EMT-associated expression profile and cancer stem cell behavior.

Mab-10H10-treated tumors are less tumorigenic *ex vivo*

To investigate if blockade of TF signaling resulted in a permanent change of malignant tumor cell phenotype, tumors were collected and cultured ***ex vivo.*** Similar to what was observed with cells treated ***in vitro***, tumor cells isolated from Mab-10H10-treated mice showed a morphological change towards an epithelial-like phenotype (Fig. 4A). Ingenuity pathway profiling after gene array analysis indicated alterations in expression profiles that associate with morphology, cellular development, function and cell-to-cell interactions (table S1). This epithelial-like phenotype was supported by an increased expression of the adherens junctions and desmosomes associated genes ***JUP*** (γ-catenin), ***PLEKHA7*** (pleckstrin) and ***DSP*** (desmoplakin) - all contributing to epithelial homeostasis (Fig. 4B). Furthermore, expression of EMT markers ***SNAI1, SOX9*** and ***VIM*** was decreased in Mab-10H10 ***ex vivo*** cells (Fig. 4C). Similarly, Snail antigen levels were significantly decreased by 2-fold in Mab-10H10 ***ex vivo*** cells, while β1 integrin and TF expression remained unchanged (Fig. 4D). Mab-10H10 ***ex vivo*** cells showed diminished ***MMP9*** mRNA expression (Fig. 4E). As expected, a significant 8-fold reduction of cell invasion was observed compared to control IgG ***ex vivo*** cells (Fig. 4F, G). Furthermore, Mab-10H10 ***ex vivo*** cells had reduced CSC activity, since formation of

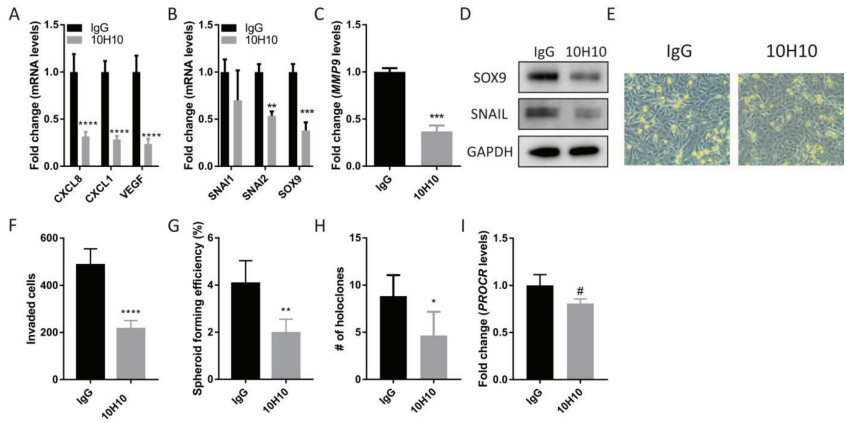


Figure 3 TF signaling inhibition reduces EMT and CSC program *in vitro*.

(**A**) Treatment of MDA-MB-231-mfp cells with 50 µg/ml Mab-10H10 for 72 hours reduces mRNA expression of *CXCL8*, *CXCL1* and *VEGF*. (**B**) Decreased mRNA expression of EMT-associated markers *SNAI1*, *SNAI2* and *SOX9* after 72h treatment with Mab-10H10. (**C**) Mab-10H10 treatment down-regulates *MMP9* expression. (**D**) Expression of SOX9 and Snail antigen levels after 72 h antibody treatment. (**E**) Morphological changes were observed during cell culture when cells were treated with 50 µg/ml Mab-10H10. (**F**) Invasion assay of MDA-MB-231-mfp cells in the presence of 50 µg/ml IgG control or Mab-10H10 antibody. (**G**) 500 MDA-MB-31-mfp cells were plated into ultra low-attachment 96-well plates in the presence of 50 µg/ml IgG control or Mab-10H10 antibody and cultured in tumor sphere medium for 14 days. Tumor spheres with a surface larger than 2000 µm² were counted. (**H**) Suppressive effect of TF signaling inhibition on colony formation capacity of MDA-MB-231-mfp cells. Colonies consisting over 50 cells with a holo-type phenotype were counted. (**I**) *PROCR* expression after 72 h Mab-10H10 treatment. # P<0.10, *P<0.05, **P<0.01, ***P<0.001, **** P <0.0001

spheroids and holoclones was significantly hampered (Fig. 4H, I). In line with these results, mRNA expression of *PROCR* was also reduced in 10H10 cells (Fig. 4J). Thus, ***ex vivo*** cells of tumors that had been treated with the TF signaling inhibitor Mab-10H10 display changes in tumor cell phenotype, with decreased EMT and CSC features, that persist during culture *in vitro*.

TF regulates the location of integrins at the plasma membrane

Thus far, we have shown that TF signaling is involved in EMT/CSC programs and in pro-metastatic events. TF influences migration and cell adhesion via the regulation of integrins,

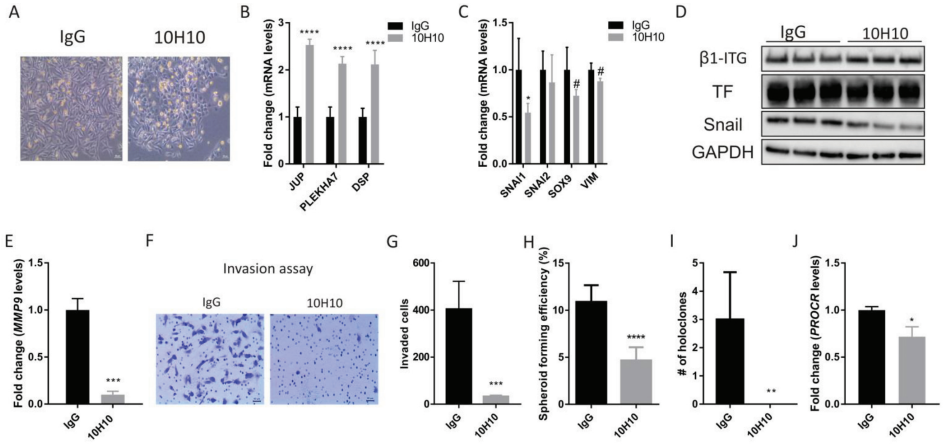


Figure 4 Blockade of TF signaling reduces EMT and CSC program in *ex vivo* cells.
(**A**) Morphological changes were observed during cell culture of *ex vivo* cells that were isolated from tumors. Scale bar = 50 µm. (**B–C**) Transcription levels of adherens junction associated markers *JUP*, *PLEKHA7, DSP* (**B**), and EMT-associated markers *SNAI1*, *SNAI2*, *SOX9* and *VIM* (**C**) in IgG or 10H10 *ex vivo* cells measured using qPCR. (**D**) Protein levels in *ex vivo* cells derived from control or Mab-10H10 tumors. (**E**) mRNA level of *MMP9* in IgG and 10H10 *ex vivo* cells. (**F**) Matrigel invasion assay with *ex vivo* IgG and 10H10 cells. Crystal violet staining of invaded cells are shown. (**G**) Data are represented as mean ± SD of cell numbers that invaded in total per well in 4-plo. (**H**) 500 *ex vivo* cells were plated into ultra low-attachment 96-well plates and cultured in tumor sphere medium for 14 days. Tumor sphere numbers were counted with a surface larger than 2000 µm^2. (**I**) Colonies (>50 cells) with a holoclone phenotype were counted. (**J**) mRNA transcription levels of *PROCR* in IgG or 10H10 *ex vivo* cells. # P <0.15, ***P<0.001, **** P <0.0001

that is independent of PAR2-mediated signaling [13]. Therefore, to unravel the mechanism as to how TF signaling affects CSCs we focused on the interaction with integrins. Since integrins enable the cells to bind ECM, mediate migration and regulate CSCs [24], we addressed whether inhibition of TF signaling influences integrin behavior. First, we investigated the effects of integrin activation on EMT programs using different extracellular matrix (ECM) components, i.e. vitronectin that binds αvβ3 integrin [25] and laminin, a ligand for many β1 integrin dimers and α6β4 integrin [26]. Cells were seeded on vitronectin or laminin-coated plates and treated with Mab-10H10 for 72 hours, followed by mRNA analysis of EMT-associated factors. A 2-fold reduction of *SNAI1* and *SNAI2* expression was found when TF signaling was inhibited in cells on vitronectin coated plates, while expression remained equal

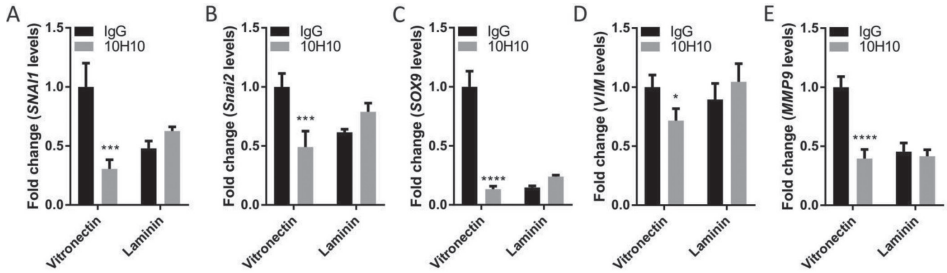


Figure 5 Vitronectin and laminin contribute to different EMT and CSC program in *in vitro* cells.

Transcription levels of *SNAI1* (**A**), *SNAI2* (**B**), *SOX9* (**C**), *VIM* (**D**) and *MMP9* (**E**) in MDA-MB-231-mfp cells in the presence of IgG control or 10H10 antibodies for 72 hours on vitronectin or laminin coated plates. *P<0.05, ***P<0.001, **** P <0.0001

when cells were seeded on laminin (Fig. 5A, B). The effects of different ECM components on *SOX9* mRNA expression were even more pronounced, with a 90% reduction, on vitronectin, (Fig. 5C). Similar trends were observed with respect to *VIM* and *MMP9* expression after treatment with 10H10 on vitronectin but not on laminin as an ECM component (Fig. 5D, E).

Next, we further investigated how integrins influence TF signaling mediated EMT- and CSC-associated behavior. Cells were treated with Mab-10H10 for 72 hours, subsequently lysed in Brij35-buffer that dissolves non-raft, cholesterol-poor cell membrane fractions [27]. β1 integrins were immunoprecipitated with various β1 integrin conformation-recognizing antibodies, after which precipitates were analyzed for β1 integrin and TF. Inhibition of TF signaling resulted in an approximately 2-fold reduction of TF/β1 integrin complexes in the Brij35-soluble fraction (Fig. 6A), while β1 integrin expression remained equal. Interestingly, pull down assays with AIIB2 (total β1 integrin) and HUTS21 (active β1 integrin) co-immunoprecipitated FAK and Src when MDA-MB-231-mfp cells were treated with Mab-10H10, suggesting an active β1 integrin conformation. The β1 integrin antibody TS2/16, that activates β1 on intact cells and immunoprecipitates the TF-FVIIa complex [28], did not immunoprecipitate FAK/Src in the presence of Mab-10H10,indicating that FVIIa is not involved in the antibody effect. In addition, immunofluorescent staining for FAK in MDA-MB-231-mfp cells treated with Mab-10H10 demonstrated the formation of focal adhesions at the plasma membrane (Fig. 6B), confirming FAK activation in the presence of Mab-10H10. Inhibition of FAK resulted in a significant increase of *SNAI1/2* expression, while *SOX9* remained unaffected (Fig. 6C). Mab-10H10 treatment decreased α2 and α3 integrin heterodimers with β1

integrin and co-precipitating TF in the Brij35-soluble fraction (Fig. 6D). In contrast, precipitation of TF/β1 integrin was increased markedly by Mab-10H10 treatment in the α6 integrin pull down from cholesterol-rich membrane fraction – Brij-58 soluble – and less so in the non-raft fractions. Total expression of alpha-integrin subunits did not change upon Mab-10H10 treatment (Fig. 6E). These data suggest that blockade of TF signaling causes a shift of TF towards α6β4 integrin into the cholesterol-rich membrane fractions. These data also indicate that integrin activation is induced by Mab-10H10 treatment to suppress the CSC phenotypes. Consistently, inhibition of β1 integrin – but not PAR2 – increased mammosphere formation in cells treated with Mab-10H10 (Fig. 6F). Treatment of MDA-MB-231-mfp cells with the PAR2 antagonist GB83 showed similar results as those observed after antibody-mediated inhibition (Fig. 6G). Additionally, mRNA expression profiles of EMT-related genes were examined after inhibition of β1 integrin and PAR2. Inhibition of either receptor resulted in increased *SNAI2* expression. A trend towards increased *SOX9* expression was observed when β1 integrin was inhibited with Mab-AIIB2, while inhibition of PAR2 had no effect on *SOX9* transcription levels (fig. 6H). These data suggest that TF signaling mediates the localization and function of β1 integrin on the cell membrane. Furthermore, disruption of the TF/β1 integrin complex results in an epithelial-like morphology with less tumorigenic properties.

TF signaling keeps cells in a mesenchymal state via suppression of β4 integrin expression

How integrins contribute to cancer stem cell behavior is as yet obscure. Recently, Bierie and co-workers showed that α6β4 integrin distinguishes subpopulations in mesenchymal triple negative breast cancer [29]. Cells that are β4 integrin positive in MDA-MB-231 associated with a more epithelial morphology and decreased tumorigenic properties. We found that treatment with Mab 10H10 influenced β4 integrin expression in our highly aggressive MDA-MB-231-mfp cell line. After 72 hours Mab-10H10 treatment, magnetic activated cell sorting (MACS) isolated the same number of β1 integrin positive cells (Fig. 7A), but TF signaling inhibition increased the number of cells that express β4 integrin 15-fold (Fig. 7B). After MACS sorting these cells were cultured for 1 week. Whereas the cells displaying low integrin β4 expression from control IgG treated cells had mesenchymal-like morphology, an epithelial-like morphology was observed in the β4 integrin^{high} population isolated from Mab-10H10-treated cells (Fig. 7C). Immunoprecipitation of β4 integrin confirmed the increased association of α6 integrin and TF in Mab-10H10 treated cells. Furthermore, increased FAK and Src antigen were co-precipitated with integrin β4 after Mab-10H10 treatment (Fig. 7D). Diminished TF signaling caused a morphological change into less tumorigenic cells via in-

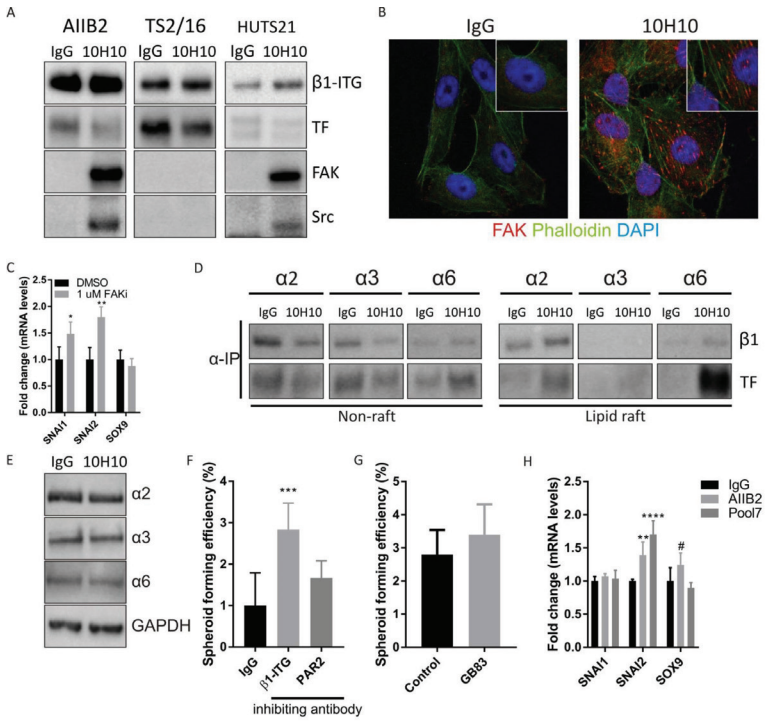


Figure 6 TF determines β1 integrin localization on the cell membrane.

(**A**) Pull down assay of β1 integrins with AIIB2, TS2/16 and HUTS21 after 72h treatment with IgG or Mab-10H10. Co-immunoprecipitation was analyzed with Western Blot for β1 integrin, TF, FAK and Src. (**B**) Blockade of TF signaling increases focal adhesion complex formation after 72 h Mab-10H10 treatment. MDA-MB-231-mfp cells were permeabilized and stained for FAK. (**C**) mRNA expression of *SNAI1, SNAI2* and *SOX9* in MDA-MB-231-mfp cells after 72h treatment with control (DMSO) or 1 uM FAK II inhibitor. (**D**) Localization of α2, α3 and α6 integrin was studied in non-raft and lipid raft fractions after 72h 10H10 treatment. Precipitates were analyzed for the presence of β1 integrin and TF on Western Blot. (**E**) Western blotting for α-integrin subunits on total cell lysates of MDA-MB-231-mfp cells showed no changes in antigen levels after blockade of TF signaling. (**F**) 500 MDA-MB-231-mfp cells were plated into ultra low-attachment 96-well plates in the presence of 50 µg/ml control IgG, Mab-AIIB2 (β1 integrin) or Pab-pool7 (PAR2) antibody and cultured in tumor sphere medium for 14 days. Tumor spheres with a surface larger than 2000 µm^2 were counted. (**G**) 500 cells were plated into ultra low-attachment 96-well plates in the presence of control (DMSO) or 10 µM PAR2 antagonist (GB83) and cultured in tumor sphere medium for 14 days. Tumor sphere numbers were counted with a surface larger than 2000 um^2. (**H**) Transcription levels of *SNAI1, SNAI2* and *SOX9* in MDA-MB-231-mfp cells in the presence of control IgG, Mab-AIIB2 (β1 integrin) or Pab-pool7 (PAR2) antibody for 72 hours.

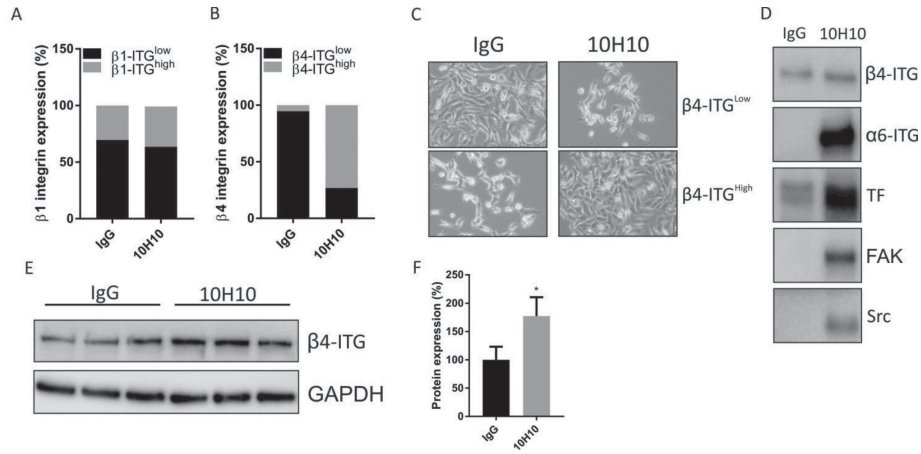


Figure 7 Inhibition of TF signaling increases expression and crosstalk with α6β4 integrin. (A-B)

Cells were treated with IgG or 10H10 antibody for 72 h and sorted with MACS antibodies recognizing β1 (**A)** or β4 integrin (**B)** expression. Table represents relative percentage of β1 and β4 integrin low and high expression. (**C**) One week of cell culture after MACS sorting show morphological changes in β4 integrin^{High} cells. (**D**) Pull down assay for β4 integrin after Mab-10H10 confirms complex formation with α6 integrin subunit, TF, FAK and Src in MDA-MB-231-mfp cells. (**E**) Protein expression analysis of β4 integrin in IgG and 10H10 ***ex vivo*** cells. (**F**) Band intensity was quantified using ImageJ software. *P<0.05

creased α6β4 integrin expression and activation at the cell surface. Interestingly, β4 integrin expression was twice as high in 10H10 ***ex vivo*** cells (Fig. 7E, F), whereas TF and β1 integrin expression remained unchanged (Fig. 4D).

DISCUSSION

Here, we show that TF expression negatively associates with metastasis-free survival in ER-negative breast tumors. We also demonstrate in orthotopic xenograft mouse models that TF signaling influences epithelial to mesenchymal transition, cancer stem cell programs and metastasis that is independent of NK cells. Finally, we show that TF regulates integrin function, focal adhesion complexes and adherens junction components resulting in an epithelial cell morphology. Based on these data we conclude that TF signaling maintains breast cancer cells in a mesenchymal state by suppressing epithelial features, thus promoting CSC generation and a pro-metastatic state (Fig. 8).

TF expression has been linked to decreased metastasis-free survival in lung, gastric, pancreatic and colorectal cancer [30-33], but associations between TF and metastasis in breast cancer patients have remained obscure [34-36]. In this study an association could be found when breast tumors were stratified for ER status, with a significant association in ER-negative tumors. Surprisingly, such an association was not found in tumors from breast cancer patients with ER-positive tumors, possibly reflecting the different biological and clinical characteristics of these breast tumor types. In support, a recent study demonstrates increased TF expression in triple-negative breast cancer [37], a highly invasive subtype of breast cancer that is associated with poor survival [38]. Of note, in our work we used MDA-MB-231-mfp cells constituting a triple-negative breast cancer cell line and in these cells TF supports invasion and metastasis in an ER^- setting.

When MDA-MB-231-mfp cells were orthotopically grafted in the presence of the TF signaling inhibitor Mab-10H10 a dramatic decrease in lung metastasis was observed. As this antibody does not inhibit coagulant properties of TF, our results demonstrate that in an orthotopic setting, TF signaling impacts metastasis. It should also be noted that TF signaling promoted metastasis both in murine models harboring NK cells and models lacking NK cells. This is of importance as previous work making use of experimental metastasis models - relying on injection of cancer cells into the bloodstream - suggested that in this setting evasion of NK cells was dependent on coagulant function of TF. Nevertheless, experimental metastasis does not fully recapitulate the metastatic process as primary tumor growth, degradation of the basement membrane, local invasion and intravasation into the bloodstream are circumvented [39]. One other outcome of our ***in vivo*** work is that primary tumor growth was unaffected by Mab-10H10 treatment in the absence of NK cells. This raises the possibility that Mab-10H10 treatment in NK proficient mouse models primarily mediates antibody-dependent cellular cytotoxicity (ADCC) [15, 40].

We next questioned why inhibition of TF signaling had such an impact on metastasis and reasoned that Mab-10H10 treatment influenced metastatic potential of the primary tumor cells. Our experiments using ***in vitro*** and ***ex vivo*** approaches demonstrated that TF signaling affects early metastatic events such as maintenance of an EMT-program in tumors. Mab-10H10 treatment resulted in a downregulation of EMT transcription factors Snail and/or Slug. As TF appears to influence EMT, which is dynamically linked to cancer stem cells [10], and as expression of the cancer stem cell effector SOX9 was significantly reduced in our ***in vitro*** model after Mab-10H10 treatment, the question raised whether TF expression is associated with CSCs. Indeed, our clinical data demonstrates an association between high

TF levels and ALDH1, a marker for CSCs. In support, Mab-10H10 treatment resulted in decreased mammosphere formation and holoclone formation, suggesting that specifically TF signaling influences cancer stem cells. Shaker et al. have previously demonstrated that TF expression promotes CSCs in breast cancer, nevertheless, our study is the first to show that the involvement of TF in CSC maintenance is dependent on its signaling properties. Collectively, these data show that TF signaling is directly responsible for NK-independent metastasis via modulation of the EMT and CSC program.

Unfortunately, insights into effects of Mab-10H10 on CSC-associated markers remained limited in this study. $CD44^+/CD24^-$ is classically regarded as a CSC signature, but MDA-MB-231-mfp cells are enriched in $CD44^+/CD24^-$ (>90%) and ALDH1, another proposed marker for CSCs is hardly detected in MDA-MB-231-mfp cells [23]. Schaffner et al. previously demonstrated that EPCR may serve as a marker for CSCs in MDA-MB-231-mfp cells [23]. In our model, ***PROCR*** transcript levels were significantly downregulated in Mab-10H10 ***ex vivo*** cells, whereas only a trend was observed in Mab-10H10 treated ***in vitro*** cells. Unfortunately, no differences on EPCR surface expression could be observed using flow cytometry (data not shown). Hwang-Verslues et al. previously suggested that EPCR as a CSC marker on itself is a weak biomarker in breast cancer cell lines [41]. Rather we found that β4 integrin expression, which was recently shown by Bierie et al. to be a negative marker for CSCs, discriminated between aggressive and less aggressive Mab-10H10-treated cells [29]. It should be noted that the study by Bierie et al., like ours, did not find differences in ***PROCR*** expression when comparing integrin $\beta4^{high}$ MDA-MB-231 cells with integrin $\beta4^{low}$ cells.

An interesting observation in this study was the change in morphology of 10H10-treated cells from a mesenchymal to an epithelial-like phenotype both ***in vitro*** and ***ex vivo*** concomitant with elevated β4 integrin expression. Furthermore, our 10H10-treated ***ex vivo*** cell model demonstrated increased expression profiles of adherens junction and desmosome components again reflective of an epithelial-like state. Despite these apparent changes in morphology, we were unable to show significant changes in EMT-related transcriptional programs in our 10H10-treated ***ex vivo*** cells as opposed to those observed in our ***in vitro*** model, where only ***SNAI1*** and ***SOX9*** expression were affected. Although these data appear to be in conflict, we hypothesize that the tumor microenvironment may have influenced the EMT state of our ex vivo cells. A recent study shows that cells undergo several intermediate stages during EMT and that these stages are determined by the presence of stromal cells [42]. Indeed, Magnus et al. reported previously that tumor cell TF expression and the microenvironment dynamically regulate each other [43]. Therefore, we hypothesize that

in vitro Mab-10H10 treatment skews tumor cells towards a transition state to MET (mesenchymal-to-epithelial transformation), while *ex vivo* cells derived from 10H10 tumors, influenced by the tumor microenvironment, have fully undergone MET. Thus, TF signaling appears to be required for the initial transformation and the microenvironment further directs a full MET transition.

TF signaling may occur via multiple receptors. Classically, TF mediates FVIIa-dependent activation of PAR2, however, inhibition of PAR2 did not impact spheroid forming efficiency. In addition, we performed our experiments in the absence of FVIIa making it unlikely that TF-dependent effects on EMT/CSC are FVIIa- or PAR2-mediated, although we have not investigated this extensively. Rather, we postulate that TF, in the context of EMT and CSC biology, regulates integrin function. Indeed, we observed that EMT/CSC-associated markers were downregulated when TF/integrin crosstalk was inhibited with 10H10 on vitronectin, an activator of β3 and β5 integrins [44]. In contrast, expression of these markers was already low when cells were seeded on laminin a ligand for β1 integrin [45], and expression was not further downregulated when TF signaling was inhibited. In addition, inhibition of β1 integrin resulted in increased *SNAI2* and *SOX9* expression, whereas inhibition of PAR2 only affected transcription levels of *SNAI2*. These data suggest that β1 integrin is involved in TF signaling-dependent EMT and CSC transcriptional programs. Altogether, we hypothesize that TF/PAR-mediated signaling is required for angiogenesis and proliferation, while the TF/integrin axis is responsible for EMT and CSC.

To further unravel the nature of the β1 integrin/TF signaling pathway, β1 integrin immunoprecipitates were investigated. Mab-10H10 led to uncoupling of TF/β1 integrin complexes, as shown in this study and previous studies. Furthermore, in the presence of 10H10 increased binding of focal adhesion kinase to β1 integrin was observed, as well as increases in focal adhesion complexes and presence of actin fibers. This may be initiated by Mab-10H10 that shifts TF in a complex with α6β1 integrin – which may not be recognized by TS2/16 – and recruits filamin and FAK through the cytoplasmic domain [46]. Further, this Mab-10H10 treatment resulted in an increase of the active β1 integrin conformation and a decrease in cancer stemness. Although α6β1 integrin has been implicated in CSC phenotypes [47], our data show that TF dictates FAK/Src recruitment to any α6 integrin heterodimer – including α6β4 integrin – that promotes differentiation. Thus, we postulate that 10H10 reverses TF-dependent inhibition of β1 integrin, leading to focal adhesion assembly and an epithelial state.

The epithelial morphology may be further influenced by β4 integrin [48], and others have shown that an interaction of α6β4 integrin with CD151 increases cell adhesion and formation of hemidesmosomes, that mediates a stable cell attachment to the ECM [49]. Indeed, we observed a significant increase in cellular β4 integrin expression upon inhibition of TF signaling. Furthermore, we have observed a physical interaction between TF and β4 integrin. It is tempting to speculate that the TF/β4 integrin complex is actively involved in the transition to an epithelial state and CSC differentiation. Therefore, it would be of interest to further elucidate the nature of the TF/β4 integrin signaling axis and its impact on hemidesmosome assembly.

In conclusion, uncoupling of TF/β1 integrin signaling pathways, increases integrin β4 expression, decreases EMT/CSC programs, resulting in diminished lung metastases. This study shows that TF signaling may be an important target for the treatment of triple negative breast cancer, and TF/β1 interactions may be targeted to prevent relapse and increase overall survival.

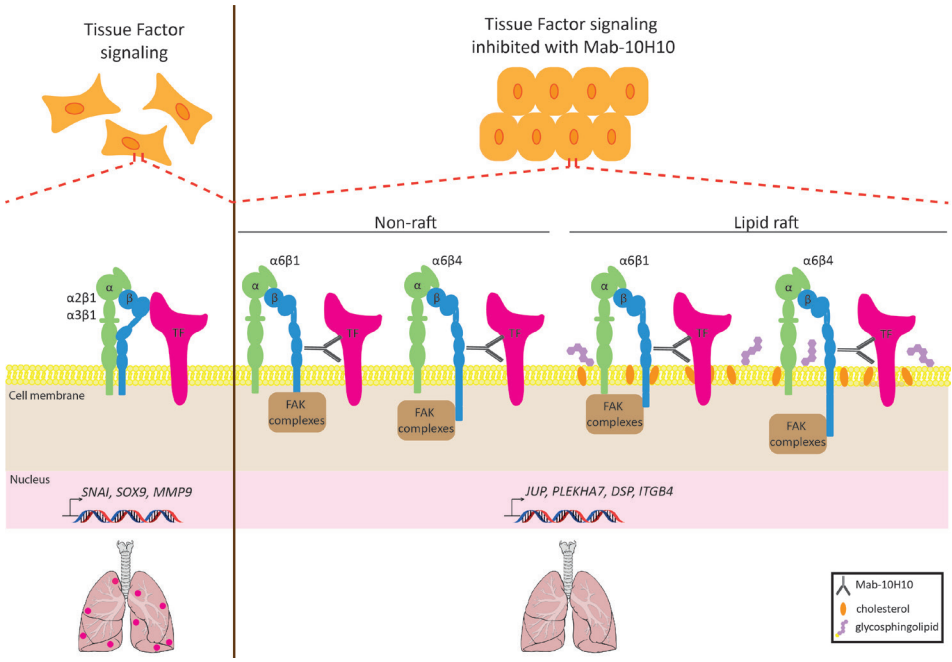


Figure 8 Schematic overview.

Inhibition of TF signaling with Mab-10H10 results in an epithelial morphology, with less CSC behavior and metastasis to the lungs. The proposed mechanism comprises disruption of TF/β1 integrin complexes, an increase in β4 integrin expression and formation of focal adhesion complexes by α6β1 and α6β4 integrins.

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SUPPLEMENTARY MATERIALS

Table S1

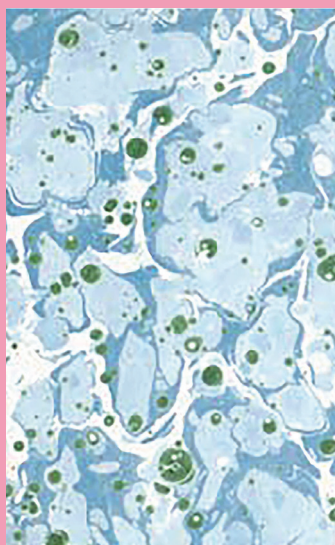
Summary of Ingenuity pathway analysis on 10H10 *ex vivo* cells.

10H10 *ex vivo* cells	
Top canonical pathways	
	p-value
ERK5 signaling	8,99E-06
Protein ubiquitination pathway	5,22E-05
Antigen presentation pathway	1,23E-04
Virus entry via endocytic pathways	1,29E-04
Fcy receptor-mediated phagocytosis in macrophages and monocytes	1,32E-04
Molecular and cellular functions	
	p-value range
Cellular growth and proliferation	8,91E-03 - 7,00E-19
Cell death and survival	9,81E-03 - 6,74E-11
Protein synthesis	9,80E-03 - 5,76E09
Cellular movement	9,45E-03 - 5,83E-09
Cellular development	8,91E-03 - 3,40E-07
Top Upstream regulators	
	p-value of overlap
EZH2	4,72E-14
TNF	1,52E-12
SND1	5,58E-10
TREM1	1,60E-09
TP63	2,24E-09
Top networks	
Cell morphology, cellular assembly and organization, cellular function and maintenance	
Dermatological diseases and conditions, organismal injury and abnormalities, cellular function and maintenance	
Post-translational modification, cell-to-cell signaling and interaction, hair and skin development and function	
Cell death and survival, organismal injury and abnormalities, protein synthesis	
Organismal development, cell death and survival, cellular development	

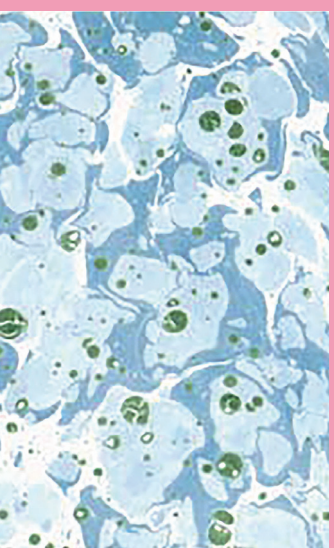
Table S2

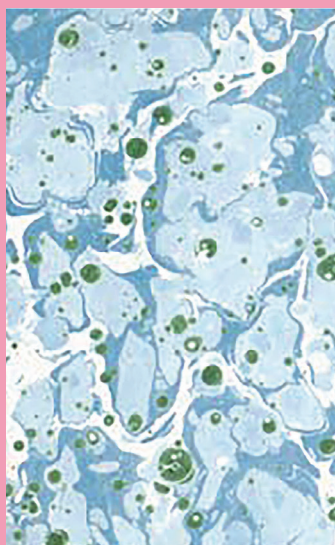
qPCR primer sequences.

Gene	Primer sequence		
SNAI1	Forward 5'-	CCCCAATCGGAAGCCTAACT	-3'
	Reverse 5'-	GCTGGAAGGTAAACTCTGGATTAGA	-3'
SNAI2	Forward 5'-	TGTTGCAGTGAGGGCAAGAA	-3'
	Reverse 5'-	GACCCTGGTTGCTTCAAGGA	-3'
SOX9	Forward 5'-	CTCTGGAGACTTCTGAACG	-3'
	Reverse 5'-	AGATGTGCGTCTGCTC	-3'
CXCL8	Forward 5'-	AGGTGCAGTTTTGCCAAGGA	-3'
	Reverse 5'-	TTTCTGTGTTGGCGCAGTGT	-3'
CXCL1	Forward 5'-	AGTCATAGCCACACTCAAGAATGG	-3'
	Reverse 5'-	GATGCAGGATTGAGGCAAGC	-3'
VEGF	Forward 5'-	CTCCACCATGCCAAGTGGTC	-3'
	Reverse 5'-	CTCGATTGGATGGCAGTAGCT	-3'
JUP	Forward 5'-	CAAGAACAACCCCAAGTTC	-3'
	Reverse 5'-	ATGATCAGCTTGCTCTCC	-3'
PLEKHA7	Forward 5'-	AAGACCAGCTAGAATCTGTG	-3'
	Reverse 5'-	CACATCATTCTCCAACTTCA	-3'
DSP	Forward 5'-	GAGATGGAATACAACTGAC	-3'
	Reverse 5'-	CCTTTTCTGGTAAGCATCAC	-3'
VIM	Forward 5'-	GGAAACTAATCTGGATTCACTC	-3'
	Reverse 5'-	CATCTCTAGTTTCAACCGTC	-3'
m β-ACTIN	Forward 5'-	AGGTCATCACTATTGGCAACGA	-3'
	Reverse 5'-	CCAAGAAGGAAGGCTGGAAAA	-3'
hGAPDH	Forward 5'-	TTCCAGGAGCGAGATCCCT	-3'
	Reverse 5'-	CACCCATGACGAACATGGG	-3'



Part III



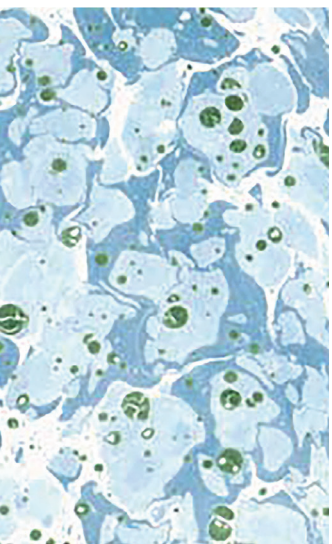


Chapter 5

Development of a spontaneous model for cancer-associated thrombosis

Betül Ünlü
Marco Heestermans
El Houari Laghmani
Jeroen T. Buijs
Bart van Vlijmen
Henri Versteeg

Manuscript in preparation



INTRODUCTION

One in eight women will be diagnosed with breast cancer in their life-time. In addition, it is estimated that 250.000 new invasive cases will be reported in 2017 [1]. Despite early detection and improved treatment over the last decade, breast cancer is still ranked among the cancer types with high mortality rates [1]. Survival of breast cancer patients is further reduced when these patients present with cancer-associated thrombosis (CAT). The risk of venous thromboembolism (VTE) in patients with breast cancer is increased by 3-4-fold when compared to women that are not diagnosed with breast cancer [2-4]. In breast cancer patients the relative incidence of CAT is around 1-2% of all breast cancer patients and is therefore generally categorized as a cancer type with a low risk of CAT [5]. However, the absolute incidence of VTE event in breast cancer is high as i) metastasis dramatically increases the risk of CAT and ii) breast cancer is one of the most frequently diagnosed types of cancer. For these reasons, up to 17% of all CAT cases are breast cancer-related [2, 6]. While studies have revealed that most cancer patients have a procoagulant state [7-9] this does not necessarily lead to formation of a thrombus, the reasons for which are unknown. This poor understanding of the mechanism underlying CAT makes it is a challenge to predict which cancer patient will develop CAT and which patient might benefit from prophylactic anticoagulant treatment.

In our laboratory we have developed a preclinical spontaneous thrombosis model relying on siRNA-dependent downregulation of antithrombin (*Serpinc1*) expression in the liver [10]. This imbalance of coagulation causes occlusive venous thrombi and hemorrhages in the head of these mice within days, with consumption of platelets, fibrin deposition in the liver and thrombus formation in the head. However, fibrinogen levels in the plasma are not affected [10, 11]. This study examines the impact of an aggressive breast cancer on thrombus formation in mice using the spontaneous thrombosis model. We demonstrate that the presence of breast cancer alleviates the thrombotic phenotype in mice, when tumor compared to cancer-free mice.

METHODS

Animal experiments

All the animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (LUMC). Orthotopic injections were performed as described previously [12]. In brief, $5x10^5$ MDA-MB-231-pcDNA-GFP-lung cells or 50 µl serum free media (Sham) were injected into inguinal fat pads of 6 week-old female NOD-SCID mice (Charles River, Wilmington, MA, USA); as an analgesic 0.1 mg/kg temgesic (Schering-Plough, Kenilworth, NJ, USA) was injected. The tumor dimensions were measured with a caliper and the volume was calculated with the formula $V=(LxW^2)/2$. When tumors reached ~400 mm^3, the experiment siRNA-mediated silencing was started as previously described [10]. In short, siRNAs targeting antithrombin (***Serpinc1***; cat. #S62673; Ambion, Carlsbad, CA, USA) or control (***NEG***; cat #4404020; Ambion, Carlsbad, CA) were complexed with Invivofectamine 3.0 (Invitrogen, Carlsbad, CA, USA) and 1.2 mg/kg of body weight siRNA was injected intravenously. Citrated blood was collected 1 day prior to xenograftment and siRNA treatment and at the end point (sacrifice).

Four days after tail vein injections, before mice were sacrificed, citrated blood was collected for blood analyses using a hematology analyzer (Sysmex XP-300; Sysmex Corporation, Kobe, JPN). To collect circulating tumor cells [13], 450 µl blood was drawn from the right atrium via heart puncture, after red blood cell lysis, cells were grown ***ex vivo*** for 1 week in 10 cm culture dishes. Furthermore, tumors, lungs and livers were collected, a part was snap-frozen in liquid nitrogen or fixed in 4% formaldehyde. Mouse heads were collected and fixed in 4% formaldehyde.

ELISA

Plasma antithrombin and fibrinogen protein levels were investigated using a commercial murine ELISA kit, according to manufacturer's protocol (Affinity Biologicals). Pooled plasma from mice was set as a reference.

To determine presence of metastasis in organs, a qPCR was performed with primers against the housekeeping genes human GAPDH (Fw: 5'-TTCCAGGAGCGAGATCCCT-3'; Rv: 5'-CACCCATGACGAACATGGG-3') and mouse β-actin (Fw: 5'-AGGTCATCACTATTGGCAACGA-3'; Rv: 5'-CCAAGAAGGAAGGCTGGAAAA-3'). According to manufacturer's protocol total RNA was isolated using Trisure (Bioline; Bio-38033; London, UK) and converted to cDNA using the Super script II kit (Life Technologies, Waltham, MA, USA). SYBR Select (Life Tech-

nologies, Waltham, MA, USA) was used to conduct qPCR on a CFX384 Touch real-time PCR detection system (BioRad, Veenendaal, the Netherlands.

Western blotting

Fibrin deposits in tumor and organs were determined using western blotting as described previously [14]. In brief, fibrin was extracted from tissue specimens, equal protein concentrations were loaded onto 4-12% Bis-Tris Plus Gels (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min at 200V and blotted on 0.2 µm pore size PVDF membranes and blocked in 5% milk in TBST (Tris-buffered saline with Tween-20) for 1 h at room temperature. Blots were incubated with mAb 59D8 (a kind gift from C. Esmon, Oklahoma City, OK, USA) O/N at 4°C, 3 TBST washing steps and incubated with a horseradish peroxidase secondary antibody (Abcam, Cambridge, UK) at room temperature for 1 h. Antigens were visualized with Western Lightning Plus ECL (Perkin-Elmer, Waltham, MA, USA) using the ChemiDoc imaging system (BioRad, Veenendaal, The Netherlands). Band intensity was quantified via ImageJ software.

Statistical analysis

Data are represented as median and range. Non-parametric testing was performed, comparisons between data points were done with Student's *t* test for two conditions, or with 1way or 2way ANOVA for three or more data sets.

RESULTS

To investigate if the presence of an aggressive breast tumor contributes to timing and morphology of thrombus formation, mice were Sham operated or xenografted with the highly aggressive subclone MDA-231-pcDNA-lung, which is a cell line that was previously re-isolated from metastatic lung foci. All mice were sacrificed four days after tail vein injections with siAT (*Serpinc1*) or siNEG (control) siRNAs. To verify knockdown, antithrombin plasma levels were analyzed. Indeed, a >95% reduction of antithrombin levels in plasma was confirmed 4 days post-injection, whereas control siRNA had no effect on plasma levels after tail vein injections (Fig. 1A). The presence of an aggressive breast tumor had no effect on plasma antithrombin concentration. As reported earlier [10], no plasma fibrinogen consumption was detected in this thrombosis model where only *Serpinc1* was targeted, although the presence of a tumor significantly increased overall fibrinogen levels 3-fold (Fig. 1B). Interestingly, white blood cell counts were elevated by 4-fold in mice bearing a

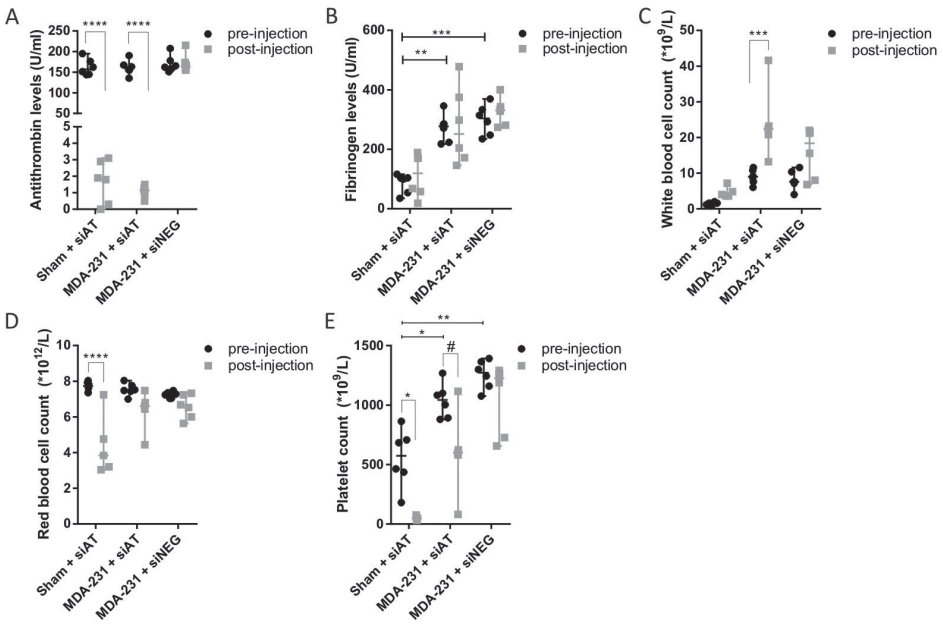


Figure 1 Plasma analysis of NOD-SCID mice before and after siRNA treatment.

(**A**) At 4 days post injection mouse plasma analysis for antithrombin knockdown and (**B**) fibrinogen levels. (**C**) White and (**D**) red blood cell counts in plasma before and after siRNA treatment. (**E**) Platelet levels at baseline (black) and consumed (grey) levels. #P<0.15, * P<0.05, *** P<0.001, **** P<0.0001

tumor when compared to the Sham group. In addition, a significant increase in white blood cells was only observed in tumor-bearing mice after siAT treatment, while in Sham- and control siRNA-treated mice the increase in white blood cells did not reach significance (Fig. 1C). Furthermore, a 2-fold decrease of red blood cells was observed only in mice with siAT injections in the absence of a tumor (Fig. 1D). Platelets were completely consumed in the Sham group, while a trend towards only moderate platelet consumption was shown in mice with a tumor (Fig. 1E). Interestingly, baseline platelet levels were twice as high in mice with a tumor compared to those without cancer. We conclude that an efficient knockdown of antithrombin resulted in platelet consumption while fibrinogen levels remained unaffected.

To investigate whether siRNA treatment had any short-term effects on malignancy, tumor characteristics were studied. No effects of siRNA treatment on tumor volume and weight were observed (Fig. 2A and B). Furthermore, similar numbers of circulating tumor cells were

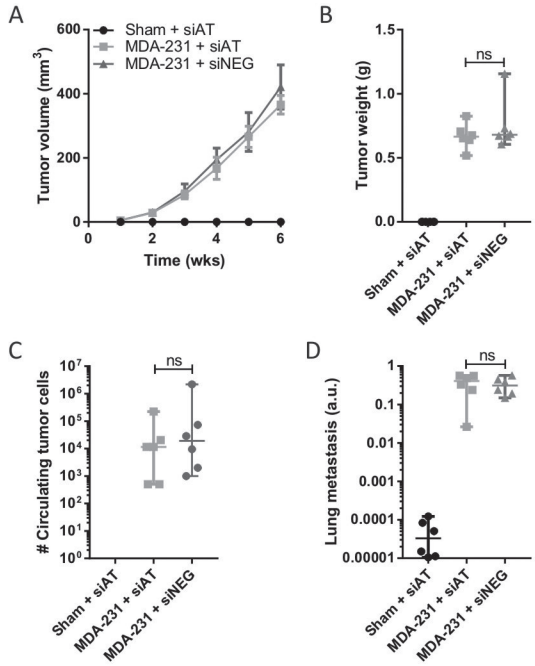


Figure 2 Tumor characteristics of NOD-Scid mice 4 days after siRNA tail vein injections.

(**A**) Sham or tumor cells were orthotopically injected and tumor volumes were monitored until week 6. (**B**) Tumor weight after mice were sacrificed. (**C**) The total amount of outgrown circulating cells *ex vivo* were counted. (**D**) Lungs were collected and metastasis was assessed via qPCR, where human GAPDH expression was normalized to mouse β-actin levels. NS = not significant.

present in mice with breast tumors (Fig. 2C). As expected the Sham group had no circulating tumor cells. As for metastasis to the lungs, no differences were observed in mice treated with siAT or siNEG (Fig. 2D). These data show that siRNA treatment and targeted knock-down of antithrombin in the liver has no short-term effects on tumor progression *in vivo*.

We investigated whether the presence of an aggressive tumor affected the formation of thrombosis *in vivo.* The typical clinical features of the thrombotic coagulopathy after siAT injection were detected in the head with swellings in the head (Fig. 3A). Five out of 6 mice presented clinical signs in the Sham+siAT group, whereas only 3 out of 6 mice had hemorrhages in the MDA-231+siAT condition (Fig. 3B), albeit not statistically significant. As expected, none of the control mice (MDA-231+siNEG) showed signs of bleeding. We finally

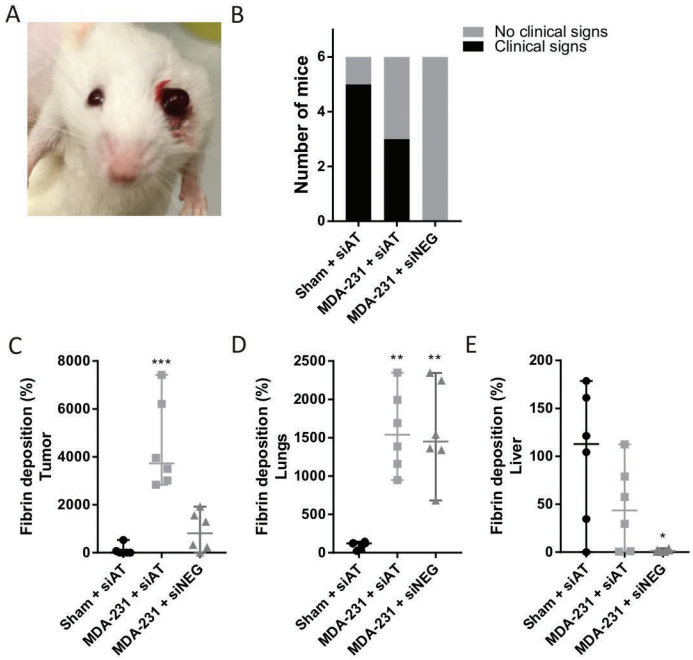


Figure 3 The presence of an aggressive breast cancer tumor improves CAT score. (**A**) Clinical sign of a bleeding phenotype in the left eye of a NOD-Scid mouse 4 days post siAT injection. (**B**) Total number of mice that presented the clinical signs after 4 days of siRNA treatment. (**C-E**) Fibrin deposition in the (**C**) tumor in tumor-bearing mice or mammary fat pad in the Sham group, (**D**) lungs and (**E**) liver were analyzed western blot antigen levels using ImageJ. * P<0.05, ** P<0.01, *** P<0.001

investigated fibrin deposition in various tissues. In Sham mice treated with siAT low levels of fibrin deposition were present in the mammary fat pad (Fig. 3C). However, a dramatic increase in fibrin deposition was found in tumors, 45-fold and 9-fold in siAT and siNEG treated mice respectively. Remarkably, fibrin deposition in the lungs was significantly increased 15-fold in tumor bearing mice when compared to Sham mice, independently of siRNA injection (Fig. 3D). As these mice had lung metastasis, this suggests fibrin formation by the tumor and not siRNA injections. Furthermore, as reported previously [10], high fibrin deposits were present in the liver in Sham+siAT mice and this was reduced in the MDA-231+siAT group (Fig. 3E). Together, these data suggest that the presence of a tumor in combination with antithrombin knockdown has synergistic effects on fibrin deposition in the mammary fat pad, with reduced clinical signs of hemorrhage.

DISCUSSION

Development of thrombosis in cancer patients correlates with poor survival. Unfortunately, the underlying mechanisms remain poorly understood. In this study, we investigated the presence of an aggressive breast tumor on thrombus formation in a spontaneous mouse model for thrombosis. Surprisingly, this study reveals that the presence of an aggressive breast cancer rescues mice from hemostatic abnormalities upon lowering antithrombin levels. In addition, platelet counts were elevated in tumor bearing mice. siRNA treatment on itself had no effect on tumor characteristics. Increased white blood cell counts suggested a pro-inflammatory status in mice with breast tumors that was further elevated after siRNA injections.

Although this ***in vivo*** model is described previously as a spontaneous thrombosis model, based on the data presented it suggests a consumptive coagulopathy that might also reflect a state of disseminated intravascular coagulation (DIC). DIC is one of the most extreme forms of dysregulated hemostasis and is characterized by hemorrhages and/or thrombosis concomitant with decreased platelet count, low fibrinogen levels, prolonged prothrombin time and increased fibrin deposition [15]. This extreme form of hypercoagulation presents in 5% of all breast cancer patients and especially adenocarcinomas of the breast are frequently associated with increased risk of DIC [16]. Although the mechanism behind cancer-associated DIC has remained elusive, it is believed that it may have the same triggers as cancer-associated thrombosis, such as elevated Tissue Factor either on primary tumor cells or extracellular vesicles, and a pro-inflammatory status in patients [17].

In contrast to our hypothesis, in the current model, the presence of a tumor appeared to protect from severe bleeding in the head, albeit not statistically significant. Plasma analysis revealed high platelet counts upon establishment of a tumor before the induction of spontaneous VTE. The platelet counts were above $1000x10^9$ counts/L, which would mimic thrombocytosis in a human setting. Interestingly, thrombocytosis is associated with inflammatory breast cancer [18] and metastasis [19], both being present in our mouse model. As platelets in our model are not completely consumed upon tumor growth, it is tempting to speculate that thrombocytosis produces a phenotype that rescues mice from hemorrhagic bleedings in our preclinical model.

Besides elevated platelet counts, mice with MDA-231 tumors showed upregulated plasma fibrinogen levels, that remained unaltered after siRNA treatment. Several studies have indicated an association of elevated plasma fibrinogen with higher tumor grade and poor

survival [20-23]. In fact, increased fibrinogen levels are considered a marker of systemic inflammation [24]. This is in line with our results, as mice showed 10-fold higher white blood cell counts prior to siRNA treatment. These cell counts were further increased after siRNA injections.

Fibrin deposits in the liver after antithrombin knockdown were increased, as previously reported [25]. Interestingly, in the presence of a tumor less fibrin deposition products were detected in the liver after knockdown of antithrombin, although no significance could be reached. In contrast, a tumor and decreased plasma antithrombin synergistically increased fibrin levels in the mammary glands of mice when compared to Sham+siAT or tumor+siNEG conditions. The reason for this drop in liver fibrin deposition and increase in tumor fibrin deposits remain unclear. Although speculative, this increased fibrin deposition may be formed by the tumor cells as these cells are known to activate platelets and thereby mediate fibrin formation. This latter may - on its turn - prevent attack by natural killer and promote survival of cancer cells [26]. Furthermore, fibrin deposits in the lungs were low in Sham mice, while the lungs of tumor-bearing mice displayed equal fibrin deposits, irrespective of siAT treatment. This might be explained by the selection of the breast cancer cell line for this study. To ensure an aggressive breast cancer phenotype in mice, MDA-MB-231-pcDNA-GFP-lung cells were selected. This cell line was originally derived from MDA-MB-231 cells that stably express GFP and was isolated from lung metastatic foci. Therefore, it has a high preference of metastasis to the lungs, which may directly result in tumor-induced fibrin deposition in the lung.

Tumor expressed TF or TF$^+$ extracellular vesicles are considered to be major players in cancer-associated thrombosis – including DIC. In our study we have used an aggressive breast cancer cell line that overexpresses TF. Based on literature, it was expected that plasma from mice with a tumor would be hypercoagulant, with increased FXa generation rate. Despite high TF content on our tumor cells, plasma analysis showed no differences in FXa presence (data not shown). In contrast with our findings, Hisada et al. reported increased FXa generation in mice bearing orthotopic pancreas tumors [27], although this is the only cancer type that is reported to show an association between TF$^+$ EVs and venous thromboembolism [28]. Therefore, it is unlikely that high amounts of circulating coagulant TF (extracellular vesicles) were present in this mouse model with an aggressive breast tumor.

An increased inflammatory status was observed in mice bearing a tumor, and after siRNA treatment. Unfortunately, the hematology analyzer used in this study did not discriminate

between various types of white blood cells. Therefore, it would be of interest to determine the nature of the immune cells involved in our model.

According to Virchow's triangle three components are of importance in the risk of VTE , i.e.: i) damaged endothelium, ii) disrupted blood flow and iii) changes in blood composition [29]. In this spontaneous model only blood composition is affected; although it might be argued that a tumor, which secretes various (pro-inflammatory) cytokines and has leaky vessels, might contribute to damaged or disrupted endothelium [30]. To reduce blood flow in mice, the stenosis model - partial ligation of the vena cava - might be a sophisticated model to further test our hypothesis.

To our knowledge, this is the first-ever study that investigated the role of a tumor in DIC in preclinical models. This showed that the presence of a tumor in mice with downregulated plasma antithrombin levels has a protective phenotype against DIC. The tumor introduces systemic changes in the blood leading to thrombocytosis, increased fibrinogen levels and white blood cells, all contributing to less formation of clinical bleedings. Unfortunately, the underlying mechanism behind DIC in aggressive breast cancer is still not unraveled. Therefore, we propose further experiments in order to investigate and unravel the pathophysiological mechanism of breast cancer on DIC.

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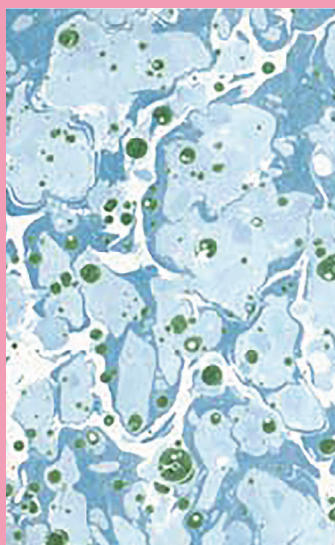

Chapter 6

Genes associated with venous thromboembolism in colorectal cancer patients

Betül Ünlü
Nick van Es
Wibowo Arindrarto
Szymon M. Kiełbasa
Hailiang Mei
Johan Westerga
Saskia Middeldorp
Peter J.K. Kuppen
Hans-Martin Otten
Suzanne Cannegieter
Henri H. Versteeg

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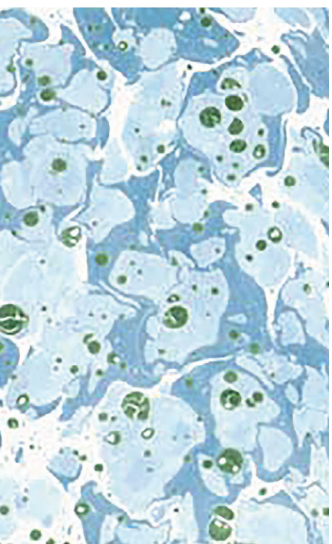

Essentials

- The underlying pathophysiological mechanisms behind cancer-associated thrombosis are unknown.
- We compared expression profiles in tumor cells from patients with and without thrombosis.
- Tumors from patients with thrombosis showed significant differential gene expression profiles.
- Patients with thrombosis had a pro-inflammatory status and increased fibrin levels in the tumor.

ABSTRACT

Background: Venous thromboembolism (VTE) is a frequent complication in patients with cancer and is associated with significant morbidity and mortality. However, the mechanisms behind cancer-associated thrombosis are still incompletely understood.

Objectives: The aim is to identify novel genes that associate with VTE in patients with colorectal cancer (CRC).

Methods: Twelve CRC patients with VTE were age- and sex-matched to twelve CRC patients without VTE. Tumor cells were isolated from surgical samples using Laser Capture Microdissection approaches and mRNA profiles were measured with next generation RNA sequencing.

Results: This approach led to the identification of new genes and pathways that might contribute to VTE in CRC patients. Ingenuity Pathway Analysis indicated significant links with inflammation, methionine degradation pathway and increased platelet function, which are all key processes in thrombus formation. Tumor samples of patients with VTE had a pro-inflammatory status and contained increased fibrin and fibrin degradation products compared to samples of those without VTE.

Conclusion: This case-control study provides proof-of-principle that tumor gene expression can discriminate between cancer patients with low and high risk of VTE. These findings may help further unravel the pathogenesis of cancer-related VTE. The identified genes could potentially be used as candidate biomarkers to select high risk colorectal cancer patients for thromboprophylaxis.

Keywords: case-control studies, colorectal cancer, homocysteine, inflammation, RNA sequencing

INTRODUCTION

Venous thromboembolism (VTE) is a frequent complication in patients with cancer and is associated with substantial morbidity and mortality. Cancer patients have a 7-fold higher risk of VTE compared to those without cancer. It has been estimated that 20% of all VTE events are related to cancer [1, 2]. Although the relation between cancer and thrombosis is well established, the underlying pathophysiological mechanisms are still incompletely understood. Many clinical and patient-related factors are known to contribute to the risk of VTE in cancer patients, such as high age, genetic disposition, immobility and prior history of VTE [3, 4]. However, the VTE risk in these patients appears to be mainly driven by cancer-related factors, such as high tumor grade, advanced disease stage, antineoplastic therapies and tumor type. Tumor types are often classified into high (pancreas, brain), moderate (colon, lung) and low (breast, prostate) VTE risk groups [5]. Furthermore, cancer treatment affects the risk of VTE after surgery and chemotherapy, by 2-fold and 6-fold, respectively [3, 6].

Biomarkers hold promise for risk stratification. Knowledge about the pathophysiological mechanism underlying the pro-thrombotic state in cancer patients could identify new candidate biomarkers. For example, high procoagulant activity of circulating tumor cells and tumor-derived extracellular vesicles (EVs) that expose tissue factor (TF) – the primary initiator of blood coagulation - has been associated with VTE, although this finding has been questioned by others [7, 8]. Other coagulation factors, such as factor VII – the protease that binds TF to start coagulation - can also be upregulated in tumors [8], potentially contributing to development of cancer-associated thrombosis. Finally, neutrophil extracellular traps (NETs) have also been proposed to contribute to cancer-associated thrombosis (CAT) [9], although, such a role for NETs in cancer patients has not yet been firmly established. Taken together, this indicates that, the biological factors that contribute to cancer-associated thrombosis remain largely unidentified.

On average, cancer patients with VTE have a 5-fold increased risk of death compared to those without VTE [4]. After cancer itself, thrombosis is the second cause of death in these patients [10]. Despite the high risk of VTE in cancer patients, routine thromboprophylaxis in outpatients is not recommended, since it increases the risk of (fatal) major bleeding leading to an unfavorable risk-benefit ratio when applied in all ambulant cancer patients. Selection of high risk patients may guide decisions about thromboprophylaxis, but current prediction scores appear to perform poorly and are therefore infrequently used. Hence, it is essential

to be able to predict which patients will develop VTE and who will benefit from prophylactic anticoagulants.

The aim of this study was to identify tumor-expressed genes that associate with VTE in cancer patients and may be used as novel biomarkers for cancer-associated thrombosis. This study focused on colorectal cancer (CRC), because the prevalence of CRC is high, while it associated with a moderate to high risk of VTE. We isolated mRNA of tumor cells with Laser-Capture Microdissection (LCM) method after which a gene expression profile was determined via Next Generation RNAsequencing. This enabled us to study gene expression exclusively in tumor cells, but not the stromal compartment. Finally, we identified biological processes associated with CAT using Ingenuity Pathway analysis.

METHODS

Patient cohort

With a cohort of unselected patients who underwent curative or palliative surgery for CRC at the Slotervaartziekenhuis (Amsterdam, The Netherlands) between January 2008 and August 2013, a total of 206 patients were identified, of whom 19 (9.2%) patients were diagnosed with objectively confirmed VTE. As we defined cancer-associated thrombosis as VTE occurring within one year before or after CRC diagnosis, and based on the availability of snap-frozen colorectal tumor specimens, we excluded 5 patients. A case-control study was performed on the remaining twelve patients with confirmed VTE and patients without VTE that were individually matched on sex, age and tumor type. Additionally, sub-group analyses were performed based on the timing of diagnosis of VTE; VTE prior to CRC diagnosis (defined as VTE max. 12 months before; 4 patient couples) and VTE around the time of CRC diagnosis (defined as VTE max. 3 months before or 3 months after cancer diagnosis; 5 patient couples) (Fig. 1). Of note, because of these criteria patient couple 1 was included in both groups. Also, based on these criteria, patient couples 9-12 were excluded from these subgroup analyses. Furthermore, in this cohort patients were included with a first VTE which was at least five years before the second (cancer-associated) VTE.

RNAsequencing

Tumor cells were isolated from tumor specimens based on morphological differences between tumor cells and stromal cells, using the Laser-Capture Microdissection (LCM) meth-

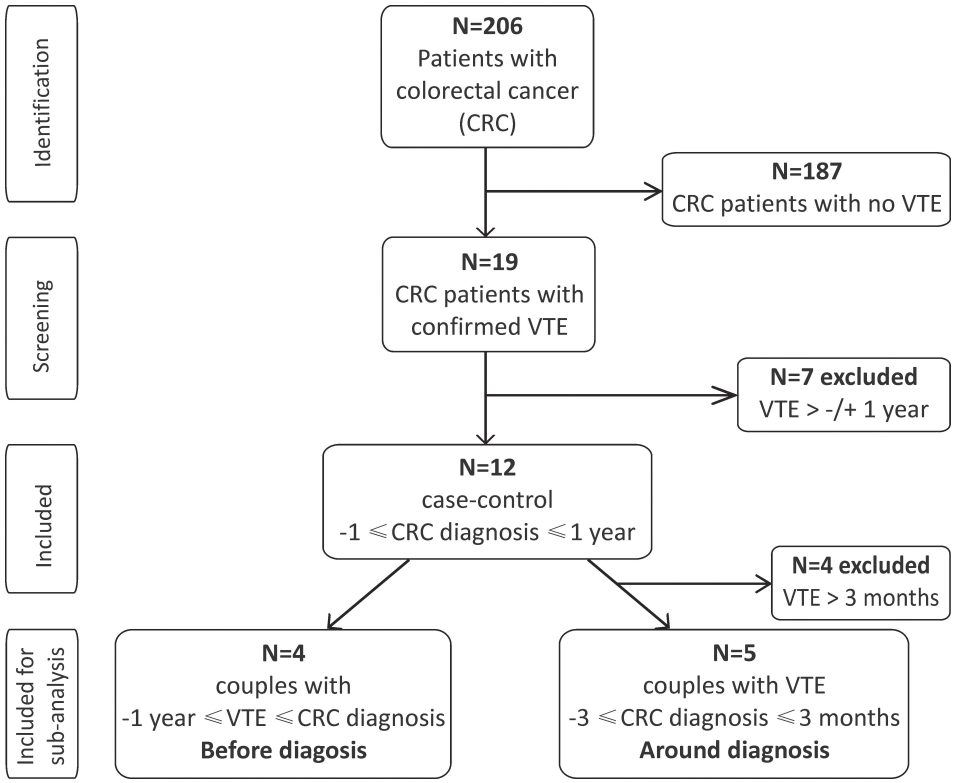

Figure 1 Design and inclusion flowchart of the study.

CRC, colorectal; VTE, venous thromboembolism.

od. Briefly, 7 µm sections were mounted on Membrane Slide NF 1.0 PEN glasses, rehydrated, hematoxylin treated to stain nuclei, washed in 1% ammonia and finally dehydrated. Sections were processed on the PALM MicroBeam (Zeiss) with a UV laser and tumor cells were collected into adhesive clear cap tubes (Zeiss). To prevent RNA degradation, tumor slices were subjected to LCM within 60 min after thawing as our pilot experiments showed that RNA is stable within a 60 min time frame-, and isolated tissue was snap-frozen. LCM dissected tissue from each patient was pooled and RNA was isolated with the NucleoSpin RNA XS kit (Magery-Nagel) according to manufacturer's instructions.

A Fragment Analyzer was used to assess the quality of the mRNA samples before RNAseq was started. The NEBNext Ultra Directional RNA library Prep Kit for Illumina was used to process the isolated RNA, after which clustering and RNA sequencing with the Illumina and

NextSeq 2500 was performed according to manufacturer's protocols at GenomeScan B.V. (Leiden, The Netherlands).

Analysis of RNA sequencing data

All RNA sequence files were processed using the BIOPET Gentrap pipeline version 0.5 developed at the LUMC (http://biopet-docs.readthedocs.io/en/latest/releasenotes/release_notes_0.5.0/). The BIOPET Gentrap pipeline consists of FASTQ preprocessing (including quality control, quality trimming and adapter clipping), read alignment, read and base quantification, and optionally transcript assembly. FastQC version 0.11.2 was used for raw read quality control. Low quality read trimming was done using sickle version 1.33 with default settings. Cutadapt version 1.9.1 with default settings was used for adapter clipping based on the detected adapter sequences by FastQC toolkit. The reads were aligned against the human reference genome GRCh38 using RNAseq aligner GSNAP version 2014-12-23 with settings "--npaths 1 --quiet-if-excessive". GENCODE genome annotation version 20 was used for raw read counting. This gene read quantification step was performed using htseq-count version 0.6.1p1 with settings of "--stranded=no". After TMM normalization, the differential gene expression analysis was performed with edgeR 3.14.0 using a model with the VTE effect and effects of matched patient-control pairs (http://bioconductor.org/packages/release/bioc/html/edgeR.html) [11]. IPA core analysis (Ingenuity Systems, Inc) was performed on the top 10 genes to identify the most significant pathways and (upstream) regulators.

Quantitative real-time PCR

Selected up- or down-regulated gene expression levels were validated by real-time PCR. In brief, RNA from snap-frozen tissues was isolated with Machery-Nagel kits. Reverse transcription was performed with Superscript II (Thermo Fisher scientific) and gene expression was performed using SYBR Select (Life Technologies) on a CFX284 Touch real-time PCR detection system (BioRad). See supplementary table 2 for primer sequences.

Western Blotting

For detection of fibrin deposition in tumors, samples were processed as described elsewhere [12]. Briefly, equal concentration of protein lysates were loaded on 4-12% Bis-Tris Plus Gels (Thermo Fischer Scientific) and separated for 18 min at 200V; blotted on 0.2 µm pore PVDF membranes and blocked for 1h in 5% milk-TBST. Fibrin was detected with mAb 59D8 antibody (a kind gift from prof. dr. Charles Esmon; Oklahoma, USA) and the primary antibody was incubated O/N at 4°C. After multiple TBST washing steps, membranes were incubated

Table 1

Characteristics of the twelve CRC patients with VTE and their individually matched patient controls on sex and age.

Couple	Sex	Tumor site	TNM stage	Age	VTE pre/ post diagnosis	days VTE diagnosis	VTE pre/ post surgery	days VTE surgery	VTE type	Type of therapy	days start chemo-therapy	History VTE	Years VTE before diagnosis	Hb levels	Leukocyte counts	Platelet counts	D-dimer
1	M	ascending colon	T3N0M0	76.35						-	-	no	-	9.7	30.1	583	-
			T3N0M0	76.41	pre	-13	pre	-104	PE	-	-	no	-	6.0	16.3	388	1032
2	M	ascending colon	T3N1M0	57.87						chemo	72	no	-	6.9	7.5	243	-
			T3N2aM0	55.76	pre	-319	pre	-349	PE	chemo	77	yes	13	5.8	6.9	291	618
3	M	rectosigmoid	T2N0M0	67.41						-	-	no	-	9.6	11.1	311	-
			T3N0M0	65.76	pre	-143	pre	-198	PE	-	-	yes	5	8.8	8.1	288	-
4	F	ascending colon	T3N0M0	83.34						-	-	no	-	9.1	6.2	184	-
			T3N0M0	83.04	pre	-263	pre	-288	DVT	-	-	no	-	6.9	4.8	319	-
5	M	rectum	T3N1M0	72.47						chemo	84	no	-	9.9	-	-	-
			T3N0M0	72.76	post	48	pre	-84	DVT	pre-surgery chemoradiation	-10	no	-	5.9	6.0	-	-
6	M	rectosigmoid	T3N2M0	66.32						chemo	62	no	-	9.4	10.5	-	-
			T1N0M0	62.36	post	45	post	4	Fatal PE	pre-surgery chemoradiation	unknown	no	-	10.2	-	223	>10000
7	M	descending colon	T2N1M0	60.89						chemo	54	no	-	9.1	7.9	253	-
			T3N0M0	58.93	post	12	post	4	PE	chemo	49	no	-	8.1	13.4	286	-
8	F	ascending colon	T3N0M0	77.54						-	-	no	-	8.3	11.5	355	-
			T3N1bM0	77.36	post	35	post	3	PE	-	-	no	-	6.8	8.0	220	-
9	M	ascending colon	T3N0M0	51.96						pre-surgery chemoradiation	unknown	no	-	5.2	-	275	-
			T3N2bM1	55.18	post	799	post	763	DVT	chemo	77	no	-	7.0	10.1	303	>10000
10	M	rectosigmoid	T3-4N0M0	78.11						-	-	no	-	7.6	-	-	-
			T3N2bM1	77.59	post	415	post	391	DVT	chemo	269	yes	45	7.9	5.4	145	6431
11	F	ascending colon	T3N0M0	85.32						-	-	yes	18	7.9	9.2	499	-
			T3N2bM1	93.19	post	208	post	209	DVT	-	-	yes	7	9.0	16.1	133	-
12	F	transvering colon	T4N0M1	68.61						chemo	196	no	-	7.1	11.1	432	-
			T3N0M0	68.37	post	320	post	305	DVT	chemo	264	yes	7	8.1	5.8	227	-

M, male; F, female; VTE, venous thromboembolism; T = tumor, N = lymph node, M = metastasis; Hb, hemoglobin is in mmol/L; Leukocyte and platelet counts are $*10^9$/L; D-dimer levels are in ng/mL; -, unknown values.

with an HRP-conjugated secondary antibody (Abcam) for 1h at RT. Antigens were visualized with Western lightning Plus ECL (Perkin-Elmer) using the ChemiDoc imaging system (BioRad).

RESULTS

Mean age of the selected cohort with twelve patient couples was 71 years and 66 % of the patients were male (Table 1). The distribution of DVT and PE was similar within this case-control study. No clear trends were observed in pre-surgery measured hemoglobin levels, leukocyte and platelet counts in patients with VTE compared to their control patients.

Next-generation RNA-sequencing analysis of tumor cells showed no significant differential gene expression in tumors of the selected twelve patients with VTE compared to CRC patients without VTE (data not shown). However, when the group was separated according to the timing of the VTE in relation to the cancer diagnosis - VTE before, and VTE around the time of CRC diagnosis (maximum -/+ 3 months) - different gene profiles were observed. Analysis of the first group revealed 20 genes that were significantly up- or down-regulated in cancer cells from patients with VTE compared to their controls (Table 2, left panel). After correction for multiple testing, four genes remained significantly differently expressed. Differential gene expression analysis of patients with VTE around CRC diagnosis (-/+3 months) showed a panel of 30 significant differently-expressed genes, which remained after adjustment for multiple testing (Table 2 right panel, and Supplementary Table 1).

Differentially expressed genes within these two selected patient groups are associated with a broad and diffuse set of biological pathways. Therefore, to narrow down relevant pathways and upstream regulators, we selected the top 10 differently expressed genes and performed core analysis using Ingenuity Pathway Analysis (IPA) Software. IPA is a web-based set of algorithms that predict upstream and downstream cellular processes, diseases and signaling pathways associated with the observed gene expression profile. The predictions are based on comparisons with previously performed gene expression analyses. A summary of the results are listed in Table 3. Multiple top canonical pathways and upstream regulators were identified, such as the coagulation pathway, methionine degradation and inflammation pathways via liver- and retinoid X receptor (LXR/RXR) pathway and interferons.

Significant upregulation of the genes ***SPINK4, SERPINA1*** and ***REG4*** in samples derived from patients with VTE pre-CRC diagnosis was confirmed using quantitative-PCR, except for patient couple 4 (Fig. 2A-C). ***SPINK4*** and ***SERPINA1*** (Fig. 2A, B) were also increased in at least 60% of the patients with a VTE around CRC diagnosis. ***REG4*** transcript levels were also elevated in the 'VTE around diagnosis' patient couples (Fig. 2C), except for couple 6. ***XKR9,*** a gene identified in the 'VTE around CRC diagnosis' group, was indeed increased in couples 1, 6 and 7 by at least 3-fold (Fig. 2D), while down regulated expression was found in patients with VTE prior to CRC diagnosis. Both ***SORBS1*** and ***NES*** were at least 2-fold downregulated in all patient couples (Fig. 2E, F). Additionally, expression of the tissue factor gene (***F3)*** was increased in most patients who developed VTE before cancer diagnosis (Fig. 3A), however, whether ***F3*** was also upregulated in the patients with VTE around CRC diagnosis remained inconclusive.

Table 2

Top 10 differently-expressed genes in tumor cells from patients with VTE before CRC diagnosis (left) and VTE around CRC diagnosis (right), compared to their controls. Genes, average log2^fold change, p-values and adjusted p-values are listed.

Before diagnosis				Around diagnosis			
Genes	*AvgLog2FC*	*p-value*	*adjusted p-value*	*Genes*	*AvgLog2FC*	*p-value*	*adjusted p-value*
REG4	7,32	7,02E-14	1,18E-09	*GBP4*	3,88	1,92E-11	3,07E-07
SPINK4	6,67	1,93E-09	1,63E-05	*XKR9*	6,21	1,35E-10	1,08E-06
SERPINA1	6,84	9,71E-08	5,45E-04	*CTSE*	7,21	3,50E-10	1,87E-06
SLITRK6	4,05	1,53E-06	6,44E-03	*AHCYL2*	2,77	6,37E-09	2,55E-05
SBSPON	4,17	2,52E-05	8,49E-02	*GRM8*	-5,05	8,63E-09	2,77E-05
DEFA5	4,32	4,02E-05	1,13E-01	*REG4*	5,50	5,56E-08	1,49E-04
KLHL32	3,04	4,87E-05	1,13E-01	*PTPRR*	4,75	2,21E-07	5,07E-04
LPL	3,83	5,37E-05	1,13E-01	*PIGR*	7,34	1,04E-06	1,94E-03
NTRK2	-4,31	8,52E-05	1,53E-01	*SORBS1*	-2,31	1,09E-06	1,94E-03
RNF217	4,89	9,59E-05	1,53E-01	*SAMD9*	2,37	1,44E-06	2,30E-03

Since inflammation was one of the top canonical pathways identified by IPA, we questioned if a pro-inflammatory status was present in these tumor specimens. Therefore we investigated the levels of CCL2, a key chemokine that regulates migration and infiltration

Table 3

Summary of IPA analysis on the list of top 10 differentially expressed genes in patients with VTE before and around CRC diagnosis. Table shows top IPA canonical pathways, diseases, molecular functions and upstream regulators.

Before diagnosis		Around diagnosis	
Top canonical pathways		***Top canonical pathways***	
	p-value		p-value
LXR/RXR activation	1,39E-03	Methionine degradation I (to Homocysteine)	9,79E-03
FXR/RXR activation	1,51E-03	Cysteine biosynthesis III	1,07E-02
Atherosclerosis signaling	1,53E-03	Superpathway of Methionine degradation	1,67E-02
Coagulation system	1,63E-02	Glutamate receptor signaling	2,64E-02
Thyroid cancer signaling	1,86E-02	Autophagy	2,86E-02
Diseases and disorders		***Diseases and disorders***	
	p-value range		p-value range
Auditory disease	1,63E-2 - 2,95E-6	Cancer	4,95E-2 - 1,64E-5
Neurological disease	4,67E-2 - 2,95E-6	Dermatological diseases and conditions	4,90E-2 - 1,64E-4
Organismal Injury and abnormalities	4,85E-2 - 2,95E-6	Organismal injury and abnormalities	1,95E-2 - 1,64E-4
Cancer	4,76E-2 - 3,94E-4	Connective tissue disorders	4,68E-4 - 4,68E-4
Developmental disorder	3,77E-2 - 4,68E-4	Developmental disorder	4,76E-2 - 4,68E-4
Molecular and cellular functions		***Molecular and cellular functions***	
	p-value range		p-value range
Cell morphology	4,04E-2 - 1,08E-5	Cell-to-cell signaling and interaction	4,85E-2 - 4,68E-4
cellular movement	4,80E-2 - 1,08E-5	Cellular function and maintenance	3,37E-2 - 4,68E-4
cell death and survival	4,67E-2 - 4,68E-4	Cellular movement	4,63E-2 - 4,68E-4
Cell-to-cell signaling and interaction	3,86E-2 - 4,68E-4	Protein synthesis	4,00E-2 - 8,96E-4
Cellular assembly and organization	4,45E-2 - 4,68E-4	Cell signaling	1,87E-3 - 1,87E-3
Top Upstream regulators		***Top Upstream regulators***	
	p-value of overlap		p-value of overlap
TCF	7,84E-04	*IFNG*	2,25E-03
SLC9A6	9,32E-04	*ADAM9*	2,33E-03
RRAD	9,32E-04	*BICC1*	2,79E-03
miR-185-3p	9,32E-04	Metribolone	4,04E-03
Oleylamide	9,32E-04	*IFNA2*	5,03E-03

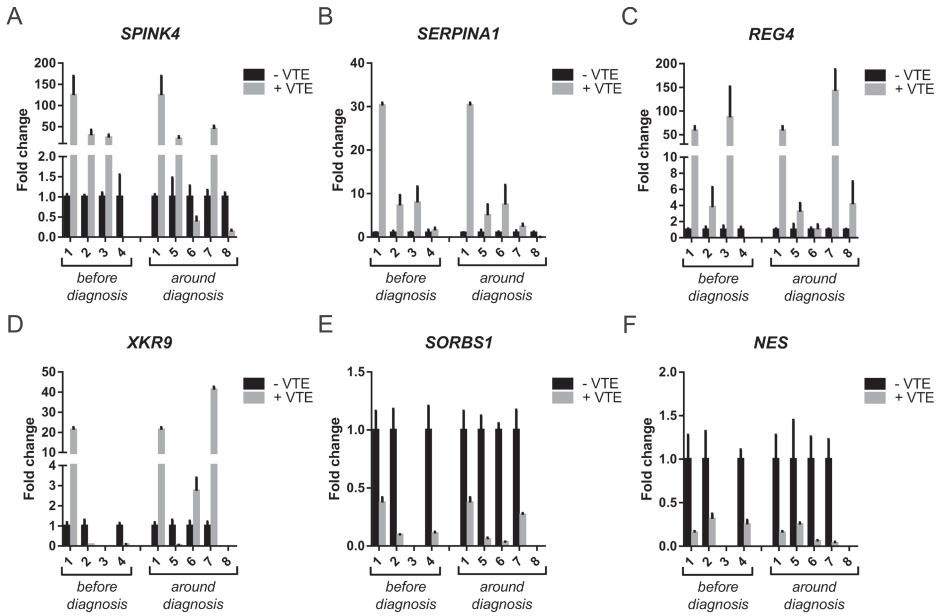

Figure 2 Validation of differentially expressed genes with a quantitative-PCR.

Fold change of mRNA expression of *SPINK4* (**A**), *SERPINA1* (**B**), *REG4* (**C**), *XKR9* (**D**), *SORBS1* (**E**), and *NES* (**F**) from patients with VTE (grey bars) normalized to their control patients (black bars). Data are shown as the mean ± SD.

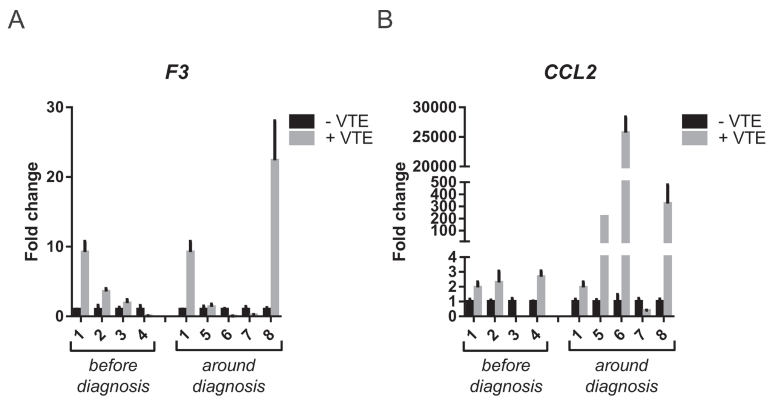

Figure 3 Assessment of tissue factor and *CCL2* expression in CRC patients with VTE.

Fold change expression of *F3* (**A**) and *CCL2* (**B**) in tumors from patients with VTE (grey bars) normalized to their control patients (black bars). Data are shown as the mean ± SD.

of monocytes [13]. Increased *CCL2* mRNA levels were observed in most patients with VTE compared to their controls, except for couples 3 and 7 (Fig. 3B).

Furthermore, we hypothesized that tumors from patients with VTE contain more fibrin deposition due to the hypercoagulant state. Therefore fibrin levels in tumor specimens were studied with an antibody that recognizes the amino-terminus of the beta-chain of fibrin [12]. All tumors from patients with VTE had higher fibrin antigen levels compared to their individual control patients, except for couple 6 (Fig. 4). This antibody also recognized fibrin degradation products in tumors from patients 3, 5 and 8 with VTE.

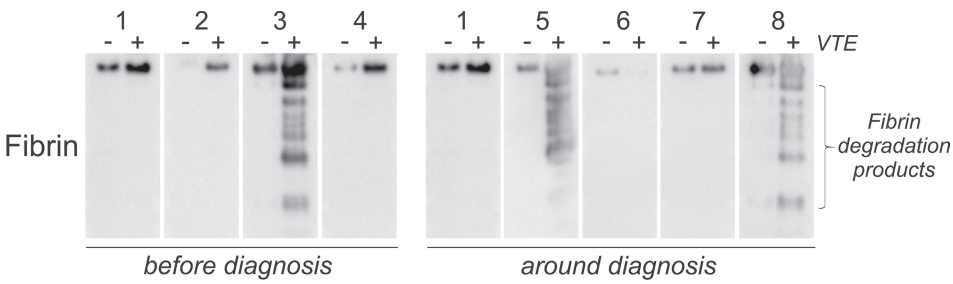

Figure 4 Expression of β-fibrin deposition in tumor specimens from CRC patients with and without VTE.

Also, fibrin degradation products were visualized. Equal protein lysates were loaded.

DISCUSSION

In this study, genes were identified that associate with development of VTE in colorectal cancer patients. RNAseq results were validated using qPCR on RNA isolated from whole tumors. IPA analysis was performed on the top 10 genes of the RNAseq analysis, showing that multiple pathways are affected in tumor cells from patients with VTE compared to controls, confirming a complex relationship. An increased pro-inflammatory state was shown in tumors from patients with VTE. Additionally, fibrin and fibrin degradation products were elevated, which suggests that a procoagulant phenotype was present in tumors of patients with VTE. Overall, these findings indicate a differential expression profile in cancer cells from patients diagnosed with VTE compared to those without VTE.

Multiple genes associated with inflammation were significantly up- and down-regulated in both VTE pre-CRC diagnosis and around CRC diagnosis (Table 2). Of note, ***REG4*** and ***SPINK4*** are tightly regulated in inflammatory bowel disease (IBD), thus co-expression of these two genes in cancer may indicate a pro-inflammatory status of the tumors. However, none of the patients in our cohort had a history of (known) IBD. Eventually, one in five IBD patients develop colitis-associated cancer over time [14]. Apart from the association of ***REG4*** with inflammation, overexpression in colorectal cancer has been linked to increased tumor progression [15, 16], suggesting that REG4 might affect both cancer and VTE.

CCL2 is a strong chemoattractant for inflammatory monocytes, which are involved in the early onset of thrombus formation [13, 17]. Indeed, elevated ***CCL2*** mRNA transcript (Fig. 3B) was detected in tumor specimens from cancer patients with thrombosis.

Our study also implicates involvement of the LXR/RXR and FXR/RXR pathway. Some studies have suggested that ligands of RXR negatively regulate VTE, as increased antithrombin production and decreased platelet activation and aggregation were observed ***in vitro*** and ***in vivo.*** [18, 19]. However, it must be mentioned that there are 2 types of platelet populations during thrombosis formation: 1) activation and aggregation of platelets which support thrombus growth; 2) loosely bound phosphatidylserine (PS)-exposing platelets with procoagulant properties [20]. Of note, ligands of LXR and FXR, such as cholesterol derivatives, positively affect the coagulant state of platelets by increased PS-exposure, EV shedding and volume size [21]. Membrane incorporated cholesterol positively regulates TF coagulant activity and hypercoagulability [22-24]. These considerations make it likely that activation of LXR/RXR and/or FXR/RXR pathways contribute to thrombosis in colorectal cancer patients. A hypercoagulant state could be further induced by apoptosis of tumor cells and increased ***XKR9*** expression, leading to elevated EV shedding and phosphatidylserine-exposure [25].

One other pathway identified by IPA was methionine degradation into homocysteine. Altered methionine metabolism by tumor cells could result in increased shedding of homocysteine into tumormilieu and/or plasma, which could result in systemic increased homocysteine levels. Multiple (epidemiological) studies have shown that increased homocysteine levels in plasma are mildly associated with VTE [26-28]. Homocysteine could elevate the risk of thrombosis by influencing TF and factor V function via inhibition of activated protein C (reviewed by Undas [27]). However, in mouse models displaying elevated homocysteine levels, no pro-thrombotic phenotype was observed [29]. This suggests that dysfunction of

methionine metabolism might be a contributor to VTE, and elevated homocysteine plasma levels, as a caudal consequence, could serve as a potential biomarker for patients at risk.

One remarkable finding was that tumors from CRC patients with a VTE diagnosis before cancer showed a different gene profile than those with a VTE diagnosis around the time of cancer diagnosis. Two possible explanations for these disparate results can be put forward. Firsts of all, patients with a VTE diagnosis around the time of cancer diagnosis have undergone cancer treatment. Surgery increases the risk of VTE by 2-fold and chemotherapy even by a 6-fold, thus in these patients chemotherapy and/or surgery could have contributed to the VTE risk, and thus this group may be biologically different from the group with a VTE diagnosis before cancer diagnosis. Secondly, during analysis of RNAseq data not all RNA reads could be aligned to the human RNA library. This might have resulted in an underestimation of significantly differentially expressed genes in patients with VTE compared to controls. Indeed, qPCR analysis (Fig. 2) demonstrated that selected genes that were identified in our RNAseq approach were similarly up- or downregulated in both groups.

Our study has a number of other limitations. We realize that the number of patients analyzed in our study was relatively small. Nevertheless, we were able to show significant expression profiles in tumors from patients with VTE compared to non-VTE controls, providing proof-of-concept that certain tumor-expressed genes associate and maybe even dictate a pro-thrombotic state in cancer patients. Increased patient numbers would perhaps give more detailed insights into the pathways involved. However, in general, it may reveal similar contributors to increased VTE risk such as inflammation, cellular metabolism and modulators of the coagulation system. Furthermore, an external validation is needed to validate these results in another CRC cohort. Additionally, we were able to detect some variation in biological processes involved in cancer patients with VTE-events at different time-points, suggesting that a more pro-inflammatory status was present in patients with VTE before diagnosis. Patients who developed VTE around CRC diagnosis might have deregulated cellular metabolism and increased apoptosis. Unfortunately, no plasma samples of patients were available, so potential plasma biomarkers could not be evaluated in this cohort.

Our cohort contains five patients with a prior history of VTE. The timing of a first VTE was at least five years before the second VTE, which makes it unlikely that the first VTE is cancer-related. However, VTE is a multifactorial disease, and even when a predisposing (genetic) factor is present, cancer is likely to be a contributing factor in causing the second VTE, especially as cancer is the largest risk factor for VTE [30]. Unfortunately, we could not

experimentally address whether our results would have been different if the patients with a prior history of VTE were to be excluded, as this would have undermined the power of our analysis.

Although literature suggests increased hemoglobin levels, leukocyte and/or platelet counts as a predictive biomarker(s) of VTE in cancer patients [31], associations between these parameters and VTE were rather weak or absent in our cohort, but this may be the result of the relative small cohort size. Therefore, we postulate that differential gene expression profiles show better associations with VTE in colorectal cancer patients than blood cell counts or Hb levels, even in a small cohort.

This study focused on CRC, and therefore our results cannot be extrapolated to other types of cancer. Thus, it would be interesting to investigate if similar, or alternatively, unique tumor genes and pathways are associated with VTE in other cancer types.

In conclusion, tumor specimens of colorectal cancer patients with VTE show a different gene profile, presence of fibrin deposition and increased inflammation compared to that of patients without VTE. Distinct pathways might be detected and discriminated into biological pathways affecting cancer-associated thrombosis or cancer related treatment. The newly identified genes from patients with VTE around cancer diagnosis could potentially be used as candidate biomarkers in order to predict thrombosis in colorectal cancer patients who might benefit from prophylactic anticoagulants.

Addendum

Conceived and designed the project: B. Ünlü, N. van Es, H.M. Otten, S. Middeldorp, P.J.K. Kuppen, S.C. Cannegieter and H.H. Versteeg. Provided study material and/or patients: J. Westerga and H.M. Otten. Performed the experiments: B. Ünlü. Bioinformatics and statistical analyses: W. Arindrarto, S.M. Kiełbasa and H. Mei. Analyzed the data: B. Ünlü, H.H. Versteeg. Wrote the paper: B. Ünlü, H.H. Versteeg. Reviewed and approved the paper: all authors.

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

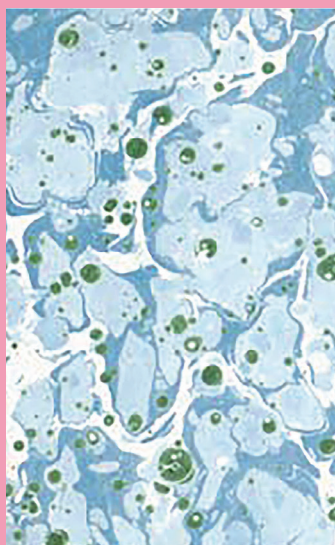
List of all differently expressed genes after RNAseq analysis in tumor cells from patients with VTE before CRC diagnosis (left) and VTE around CRC diagnosis (right).

Before diagnosis				Around diagnosis			
Genes	*AvgLog2FC*	*p-value*	*adjusted p-value*	*Genes*	*AvgLog2FC*	*p-value*	*adjusted p-value*
REG4	7,32	7,02E-14	1,18E-09	*GBP4*	3,88	1,92E-11	3,07E-07
SPINK4	6,67	1,93E-09	1,63E-05	*XKR9*	6,21	1,35E-10	1,08E-06
SERPINA1	6,84	9,71E-08	5,45E-04	*CTSE*	7,21	3,50E-10	1,87E-06
SLITRK6	4,05	1,53E-06	6,44E-03	*AHCYL2*	2,77	6,37E-09	2,55E-05
SBSPON	4,17	2,52E-05	8,49E-02	*GRM8*	-5,05	8,63E-09	2,77E-05
DEFA5	4,32	4,02E-05	1,13E-01	*REG4*	5,50	5,56E-08	1,49E-04
KLHL32	3,04	4,87E-05	1,13E-01	*PTPRR*	4,75	2,21E-07	5,07E-04
LPL	3,83	5,37E-05	1,13E-01	*PIGR*	7,34	1,04E-06	1,94E-03
NTRK2	-4,31	8,52E-05	1,53E-01	*SORBS1*	-2,31	1,09E-06	1,94E-03
RNF217	4,89	9,59E-05	1,53E-01	*SAMD9*	2,37	1,44E-06	2,30E-03
LINC00261	6,30	9,99E-05	1,53E-01	*REEP1*	-4,46	1,63E-06	2,37E-03
B3GNT6	6,02	1,24E-04	1,73E-01	*NOTUM*	-5,95	1,81E-06	2,41E-03
AKR1B10	4,66	2,17E-04	2,74E-01	*FRMD3*	3,95	2,30E-06	2,78E-03
HSPA8P1	2,13	2,51E-04	2,74E-01	*MYOM3*	-3,49	2,43E-06	2,78E-03
THRB	-3,32	2,61E-04	2,74E-01	*C2orf72*	3,80	3,19E-06	3,41E-03
PDIA2	-3,25	2,73E-04	2,74E-01	*HIF3A*	-4,75	4,33E-06	4,34E-03
SPDEF	4,20	2,76E-04	2,74E-01	*B3GALT5*	3,37	4,68E-06	4,40E-03
IGHG4	3,37	4,31E-04	4,03E-01	*RP11-401P9.6*	-4,06	4,94E-06	4,40E-03
RAP1GAP	5,73	4,58E-04	4,06E-01	*CD274*	4,72	5,66E-06	4,77E-03
COL4A1	7,50	6,39E-04	5,38E-01	*TNFSF13B*	5,24	7,28E-06	5,83E-03
				PTP4A3	-2,51	7,91E-06	6,04E-03
				CD74	2,08	1,02E-05	7,15E-03
				ST6GALNAC1	3,61	1,03E-05	7,15E-03
				LAP3	2,13	1,20E-05	8,02E-03
				PTK7	-2,21	1,76E-05	1,13E-02
				MYEF2	-4,25	2,63E-05	1,62E-02
				UGT2A3	-5,51	2,78E-05	1,65E-02
				GALNT7	1,76	3,20E-05	1,83E-02
				SYT7	-1,88	4,89E-05	2,55E-02
				PGM1	3,85	4,92E-05	2,55E-02

Supplementary Table 2

Primer sequences used for the qPCR.

Gene	Primer sequence		
SPINK4	Forward 5'-	CAGTGGGTAATCGCCCTGG	-3'
	Reverse 5'-	CACAGATGGGCATTCTTGAGAAA	-3'
SERPINA1	Forward 5'-	ATGCTGCCCAGAAGACAGATA	-3'
	Reverse 5'-	CTGAAGGCGAACTCAGCCA	-3'
REG4	Forward 5'-	CTGCTCCTATTGCTGAGCTG	-3'
	Reverse 5'-	GGACTTGTGGTAAAACCATCCAG	-3'
XKR9	Forward 5'-	ACTTGTGGCTCAGTGTTTTAGTT	-3'
	Reverse 5'-	AGCTGCATGGTAACCCCTTTT	-3'
SORBS1	Forward 5'-	ATTCCCAAGCCTTTCCATCAG	-3'
	Reverse 5'-	TTTTGCTGTTCTCGATTGTGTTG	-3'
NES	Forward 5'-	CTGCTACCCTTGAGACACCTG	-3'
	Reverse 5'-	GGGCTCTGATCTCTGCATCTAC	-3'
F3	Forward 5'-	CTG CTC GGC TGG GTC TTC	-3'
	Reverse 5'-	AATTATATGCTGCCACAGTATTTGTAGTG	-3'
CCL2	Forward 5'-	AAGATCTCAGTGCAGAGGCTCG	-3'
	Reverse 5'-	TTGCTTGTCCAGGTGGTCCAT	-3'
GAPDH	Forward 5'-	TTCCAGGAGCGAGATCCCT	-3'
	Reverse 5'-	CACCCATGACGAACATGGG	-3'

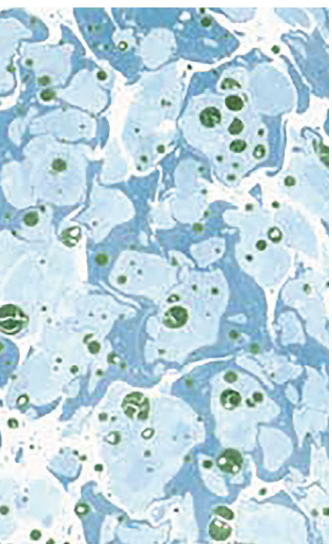


Chapter 7

Cancer-associated thrombosis: the search for the Holy Grail continues

Betül Ünlü
Henri H. Versteeg

Res Pract Thromb Haemost, **2018** *Oct*; **2(4):622-629**



Essentials:

- The mechanisms behind cancer-associated thrombosis are poorly understood.
- The link between mutations and risk of cancer-associated thrombosis is discussed.
- Genetic profiling of tumors from patients may elucidate the underlying mechanisms.
- An unbiased molecular profiling could form a diagnostic tool to predict thrombosis in cancer.

SUMMARY

Cancer patients have an increased risk of developing venous thromboembolism (VTE), a condition that is associated with increased morbidity and mortality. Although risk assessment tools have been developed, it is still very challenging to predict which cancer patients will suffer from VTE. The scope of this review is to summarize and discuss studies focusing on the link between genetic alterations and risk of cancer-associated thrombosis (CAT). Thus far, classical risk factors that contribute to VTE have been tried as risk factors of CAT, with low success. In support, hypercoagulant plasma profiles in patients with CAT differ from those with only VTE, indicating other risk factors that contribute to VTE in cancer. As germline mutations do not significantly contribute to elevated risk of VTE, somatic mutations in tumors may significantly associate with and contribute to CAT. As it is very time-consuming to investigate each and every mutation, an unbiased approach is warranted. In this light we discuss our own recent unbiased proof-of-principle study using RNA sequencing in isolated colorectal cancer cells. Our work has uncovered candidate genes that associate with VTE in colorectal cancer, and these gene profiles associated with VTE more significantly than classical parameters such as platelet counts, D-dimer and P-selectin levels. Genes associated with VTE could be linked to pathways being involved in coagulation, inflammation and methionine degradation. We conclude that tumor cell-specific gene expression profiles and/or mutational status have superior potential as predictors of VTE in cancer patients.

Keywords: cancer, germline mutation, risk factors, RNA sequence analysis, venous thromboembolism

INTRODUCTION

The relationship between cancer and venous thromboembolism (VTE) is well established, however the underlying pathogenic mechanism has remained elusive. Among all patients with VTE, approximately one in five is diagnosed with cancer, whereas cancer patients have a 4-7-fold increased risk for a VTE-event [1-3]. Furthermore, cancer-associated thrombosis (CAT) contributes to high morbidity and mortality, with VTE being the second cause of death – after cancer itself [4, 5]. Besides the 'classical' patient-related factors like age, ethnicity and prior history of VTE, several other risk factors intrinsic to cancer that may contribute to CAT have been addressed, such as higher tumor grade, metastatic disease and cancer type. Cancer types may be classified into those that confer a high risk (pancreas, brain), moderate risk (lung, colon) and low risk (prostate, breast) of VTE [6, 7]. In addition, cancer treatment such as surgery and chemotherapy further increases the risk of VTE [8, 9].

In order to select those patients that are at (high) risk for VTE and those who might benefit from thromboprophylaxis, development of an accurate prediction models is key. These models will undoubtedly become more accurate as we learn more on the mechanisms underlying CAT. Although extensive research has been performed on finding biomarkers that predict VTE in cancer patients, the focus in most studies was on coagulation factors – either in terms of expression or genetic variants - and mediators. In this review we will discuss some of these risk factors, focusing mainly on potential tumor-derived biomarkers. Furthermore, we will present future directions that may be taken to increase the accuracy of CAT prediction models.

Risk assessment tools

Over the years several risk assessment tools have been developed to estimate the risk of CAT [6, 10-12] but unfortunately, the accuracy of such tools is very low [9, 13, 14]. The main limitations of these risk assessment tools are; i) while performing better in large cohort studies these tools are unable to predict CAT at the individual level, ii) these models are not developed for specific cancer types, iii) they underperform when used to predict risk of recurrence VTE and iv) they poorly predict increased risk of mortality. Inclusion of variables classically associated with VTE, such as platelet counts, D-Dimer and P-selectin levels, moderately improves power of such models. At the same time, it should be noted that these plasma-derived biomarkers are sensitive to circumstances like inflammation, surgery and chemotherapy, and therefore introduce a wide variability in their plasma concentrations.

The most recently developed risk score, TiC-Onco - that also includes genetic risk factors - showed a positive predictive value of up to 37%, which is only an incremental increase over the predictive values obtained after using the Khorana score that correctly predicted VTE in only 22% of the CAT patients [15].

The main reason why progress in understanding and predicting CAT is slow is the fact that many investigators extrapolate classical VTE risk factors to CAT patients, with addition of a few extra risk factors related to cancer. However, a recent publication indicates that cancer patients with VTE have different plasma profiles compared to patients with VTE only [16]. In this study the authors measured concentrations of 31 plasma proteins using multiplexed targeted proteomics. Here, the authors were able to identify and cluster 17 out of 25 cancer patients with VTE compared to healthy controls and patients with VTE only, based on their plasma protein levels. This research indicates that a 'unique fingerprint' protein profile in CAT patients, and a combination of coagulation factors that differs from those in patients with VTE only, should be considered. Unfortunately, the authors do not explain what this unique barcode in their plasma is. Yet, while these findings need to be validated in other cohorts this approach holds promise for the future.

Tissue Factor

A protein that is considered the center of cancer-associated thrombosis is Tissue Factor (TF) as it plays a role in both tumor progression and VTE. Since the association and putative role of TF in CAT is extensively investigated and reviewed [17-21] we will only briefly summarize the most important findings. TF is the activator of the extrinsic coagulation pathway, ultimately resulting in fibrin degradation and platelet activation. TF overexpression has been associated with reduced survival, increased angiogenesis, migration and invasive capacity of tumor cells in a number of cancer types (previously reviewed in [19, 20]). At present only a hand full of studies have investigated the clinical association between tumor-expressed TF and the incidence of VTE. In pancreatic cancer the risk of VTE was increased 4-fold in patients with high tumor TF expression when compared to those with low TF levels [22]. Furthermore, in a relatively small cohort, consisting of 32 ovarian cancer patients, TF expression showed a correlation with the incidence of thrombosis and D-dimer levels [23]. However, not all studies confirm a link between TF and VTE. In a prospective study on non-small cell lung carcinoma (n=39), TF expression did not associate with increased risk of VTE [24]. Similarly, in a study by Thaler et al. TF expression in brain tumors did not associate with increased VTE events [25]. Thus, high TF expression in tumors does

not lead to VTE in cancer patients per se, while associations between TF and VTE risks may very well be cancer type specific.

Stubborn as scientists may be – including ourselves -, the search for a 'black-and-white' association between TF and VTE in cancer patients continued. The majority of research attention then focused on associations between VTE and TF-positive extracellular vesicles (TF^+ EVs). Tumor cells may shed EVs into the bloodstream as a consequence of cellular activation or cell death. As EV's typically contain similar membrane-bound proteins as their mother cell, EVs can possess procoagulant activity that may contribute to VTE. Preclinical mouse models have demonstrated that TF^+ EVs are being shed from pancreatic cancer cells into the bloodstream, mediating platelet activation and thrombus formation [26-28]. Unfortunately, a relationship between circulating TF^+ EVs and VTE in a clinical setting was only established in pancreas cancer patients, while no correlation was found in other moderate-to-high risk groups such as brain, colorectal or lung cancer patients [29, 30]. Although we would have wished to consider TF the center of cancer-associated thrombosis, no evidence has been found to consider TF (EVs) as the one and only risk factor or biomarker.

Host-specific genetics

Mutations in coagulation related genes are known contributors of VTE in non-cancer patients. Therefore, initial studies investigating CAT have focused on these 'classical' targets. Factor V Leiden (FVL) – a genetic variant that is resistant to inactivation by activated protein C - confers an increased risk of VTE with an odds ratio of 3.49 in the healthy population [31]. While some studies suggest a 2-5-fold increased risk of VTE in cancer patients with FVL [32-34], other cohort studies were unable to confirm this association [35-37]. Similarly, polymorphisms in other coagulation-related genes, such as FII G20210A, FIII -603A/G, FIII +5466A>G, FXIII Val34Leu and methylenetetrahydrofolate reductase (MTHFR) C667T, showed no effect on VTE incidence in patients with and without cancer [34-36, 38, 39]. Altogether, these studies suggest that host-specific mutations and SNPs in coagulation factors are not main contributors of VTE in cancer patients, and therefore should not be considered as potential biomarkers.

In recent years studies have also addressed involvement of unsuspected gene variants as contributors to VTE in cancer patients. One example is a study in which colorectal cancer (CRC) patients with a β3 integrin rs3809865 A/A genotype were shown to have an increased risk of VTE compared to CRC patients with an A/T or T/T genotype [40]. Although the causality between this gene variant and CAT remains unknown, the authors speculate that this

variant might lead to an increased expression of β3 integrin, as this genotype is less susceptible to microRNA-mediated downregulation. To our knowledge, rs3809865 A/A–dependent β3 integrin expression on endothelial cells and platelets has not been investigated. Moreover, the risk of VTE in non-cancer patients with this genotype is unknown. Thus, while it is tempting to speculate on a link between β3 integrin rs3809865 A/A and an increased risk of VTE in patients with cancer, this hypothesis cannot be validated.

Others have reported synergistic effects of germline polymorphisms and chemotherapy - an anticancer strategy that increases the risk of VTE 6-fold - on the incidence of VTE in cancer patients [8]. Specifically, patients with a polymorphism in the promoter region of vascular endothelial growth factor (VEGFA), at location -1154, appear to have a 4-fold reduced risk of VTE (OR=0.26) while treated with standard chemotherapies, like fluorouracil, irinotecan or platinum-based drugs [41]. Gastrointestinal cancer patients carrying the tumor necrosis factor alpha (TNFα) -857 C/T polymorphism or a five-loci CTGGG haplotype (-863C/-857T/-376G/-308G/-238G) are at increased risk of VTE during fluorouracil-based chemotherapy [42].

Tumor-specific genetics

Tumor cells contain an abundance of mutations and show different gene expression profiles when compared to their untransformed counterparts. It is now believed that both somatic mutations and tumor cell-specific gene profiles might contribute to increased risk of CAT.

A number of studies have shown that mutational status associates with TF expression. For instance, in colorectal cancer TF expression is upregulated via MAPK and PI3K signaling pathways due to mutations in K-ras and loss of the tumor suppressor p53 [19, 20]. In glioblastoma, TF expression is regulated in an EGFR-dependent manner together with loss of PTEN [21]. In support, the link between elevated TF levels and mutations in K-ras, PTEN and p53 were confirmed in tumor specimens derived from patients with non-small cell lung cancer [22, 23]. Although TF expression does not necessarily associate with a high risk of VTE, as discussed above, it may very well be that K-ras, p53, EGFR and PTEN mutations have an impact on VTE (summarized in Table 1).

In a multicenter retrospective study cohort (activating) mutations in K-ras – specifically in codons 12 and 13 –associated with a 2-fold increased risk of VTE in metastatic colorectal cancer patients when compared to those patients bearing a wild-type K-ras in colon tumors (OR=2.21). Interestingly, when VTE was separated into patients with DVT or PE

the odds ratio changed to 2.62 and 1.36, respectively. Although, investigation of the seven most common K-ras mutation types did not reveal a specific variant that associates with VTE, suggesting that hyperactivation of K-ras ***in general*** contributes to VTE in metastatic colorectal cancer patients [43]. A retrospective case-control study in lung cancer confirmed the association between K-ras mutation and increased VTE risk (OR=2.67) [44]. Mechanistic studies have given more insight in the consequences of K-ras activation on tumor progression. K-ras promotes several signaling pathways, resulting in increased angiogenesis, inflammation and invasion [45, 46]. Moreover, elevated levels of inflammatory mediators, e.g. IL-6 and IL-8, may be found in tumor cells harboring a K-ras mutation [45]. Interestingly, increased IL-6 and IL-8 levels in plasma are associated with increased risk of VTE in non-cancer patients [47]. It should, however, be noted that no correlations were found between interleukins and VTE in the Vienna Cancer and Thrombosis Study (CATS) cohort [48], except for patients with pancreatic cancer. This might be attributed to the relatively low incidence of VTE in the cohort (7.2%) and that plasma was collected prior to cancer-related therapy, ruling out contributions of surgery and/or chemotherapy. Overall, this suggests that mutational status of K-ras might serve as a potential biomarker, and might serve as an upstream regulator of CAT.

Unfortunately, associations between EGFR and VTE in cancer are less obvious. Although tumor specimens of high-grade astrocytoma (a specific type of brain cancer) showed a strong correlation between TF and EGFR expression coinciding with an increase in intravascular thrombosis in the tumor, it was not examined if these patients indeed had (a)symptomatic VTE [49]. In contrast with these data, a retrospective study showed a decreased hazard risk of VTE in EGFR-mutation bearing lung adenocarcinoma patients [50]. Yet, in another retrospective case-control study, no association of VTE events in EGFR mutated patients was found when compared to those without [44]. This latter group included all types of non-small cell lung carcinoma, with lung carcinoma consisting in 72% and 57% of case and control patients, respectively. The majority of VTE events in lung cancer is associated with non-small cell lung carcinoma [51].

Another mutation found in 5% of the tumors from non-small cell lung carcinoma patients is chromosomal rearrangement of anaplastic lymphoma kinase (ALK). The first study on this mutation and its link with CAT showed an increased risk of VTE [52]. In a cohort of Canadian lung adenocarcinoma patients VTE was diagnosed in over 40% of patients with ALK rearrangements, and in an Israeli validation cohort 28% of the patients with ALK rearrangements had VTE. In this latter cohort patients were not screened for asymptomatic

VTE diagnosis, which could explain the lower incidence rate. In contrast, in a retrospective study that consisted of a similar group size a trend of decreased VTE risk in patients with ALK rearrangement in lung adenocarcinoma was determined [50]. Thus, ALK mutational status as a marker or even a driver for increased VTE risk in non-small cell lung carcinoma remains controversial.

In brain cancer, aggressive glioblastoma frequently harbor the wild-type variant of isocitrate dehydrogenase 1 or 2 (IDH1/2) [53], while somatic point mutations in IDH1/2 are associated with less aggressive behavior and less necrosis [54, 55]. A recent study has investigated whether patients with wild-type IDH1/2 glioblastoma are more likely to develop VTE. Interestingly, patients harboring IDH1/2 mutations did not develop VTE neither in a discovery nor in a validation cohort, both consisting of approximately 150 patients [56]. Furthermore, only 2% of the tumors with IDH1/2 mutation showed intratumoral microthrombi versus 86% in wild-type IDH1/2 tumors. This association could be linked to reduced TF expression in the tumors and circulating procoagulant active TF^+ EVs. Therefore, IDH1/2 mutation might be an interesting biomarker to predict which cancer patients have a decreased risk of VTE.

Unbiased screen for risk factors in CAT

It is a time-consuming effort to identify all mutations in tumors and to link them individually to risks of VTE in cancer. Therefore, we have previously proposed to screen – in an unbiased manner – tumor gene expression profiles and/or mutations that associate with VTE in cancer patients. In a proof-of-principle study we showed that it is feasible to link tumor-specific gene expression profiles with VTE in colorectal cancer patients [57]. In this study RNA from isolated tumor cells was subjected to next generation RNAsequencing, making it possible to compare expression profiles in tumor cells from colorectal cancer patients with VTE compared to colorectal cancer patients without VTE. Tumors from CAT patients had different expression profiles that involved pathways related to coagulation, inflammation, homocysteine production and liver- and retinoid X receptor (LXR/RXR) function (Table 2). In addition, tumor specimens from CAT patients displayed a pro-inflammatory state and elevated fibrin deposition levels. Stratification of patients for timing of VTE (i.e. VTE before CRC diagnosis or VTE around the time of CRC diagnosis), suggested that time of a VTE event influenced the set of observed gene expression profiles. Particularly, gene expression profiles suggested a pro-inflammatory status in patients with VTE prior to CRC diagnosis, and altered cellular metabolism in patients included in the group that experienced VTE around the time of CRC diagnosis. This may suggest that altered expression

profiles within the tumor are affected by cancer treatment like surgery or chemotherapy. Hence, we assume that treatment-related CAT and CAT in the absence of such treatment have different etiologies, and this warrants further investigation.

This study opens up new possibilities in improving our understanding of the pathophysiological mechanism of CAT, to better treat CAT, and to improve CAT prediction models. It would be of interest to further investigate whether single or co-expression of the top 3 genes as identified in the patient group experiencing VTE before CRC diagnosis (***REG4***, ***SPINK4*** and ***SERPINA1***) could serve as a strong predictor of VTE in CRC patients. Additionally, these 3 genes encode secretable proteins and therefore future work is required to study if plasma levels could also serve as prognostic biomarkers [58-60]. Furthermore, it would be interesting to investigate if there is a relationship and/or synergism with mutational status of K-ras, as this is already associated with CAT in colon cancer [43]. Finally, future work may demonstrate that there is a link between the expression profiles in CAT and different subtypes of colon cancer [61], as Magnus et al. previously reported glioblastoma subtype-specific phenotypes and altered coagulation-related genes. Such identification may allow for personalized treatment of CRC patients to prevent CAT.

Significant upregulation of ***REG4*** was detected both in patients with VTE before, as well as around CRC diagnosis. Overexpression of Reg4 is associated with tumor progression, metastasis and reduced survival [62-64]. As mentioned before, risk of CAT increases dramatically in patients experiencing metastasis compared to non-metastatic cancer patients [65-67]. Tumor cells must gain cancer stem cell (CSC) properties and should undergo epithelial-to-mesenchymal transition (EMT), for successful metastasis [68, 69]. In the bloodstream procoagulant functions rescue the circulating tumor cell (CTC) from immune attack and shear stress, which additionally supports metastasis [70-72]. Of note, REG4 is associated with cancer stemness and metastasis [73, 74], suggesting that Reg4-dependent metastasis may be another mechanism leading to CAT. Unfortunately, thus far, no (genetic) reports have been published on the mechanism linking metastasis to increased risk of VTE. We believe that rather than the metastatic lesion itself, CTCs contribute to VTE, as they possess i) procoagulant activity, ii) may consist of large clumps of multiple cells and iii) are found in thrombi [75, 76]. Although, studies on this particular topic are rather inconclusive, Mego et al. recently reported in a US-based retrospective study that 9% of (metastatic) breast cancer patients experienced CAT with CTCs detectable, whereas patients without detectable CTCs had no VTE [77]. Discrimination of CTCs into epithelial or mesenchymal-like CTCs in a Slovakian cohort with 116 early breast cancer patients showed no differences, with

only 1 patient with mesenchymal-like CTCs eventually developing VTE [78]. Therefore, future research directions may include genetic profiling, using RNAseq, of CTCs, primary and metastatic tumors in patients with and without CAT.

CONCLUSION

Despite over 150 years of effort to elucidate mechanisms behind CAT, or to accurately predict which cancer patients have an increased risk of CAT, research has made only incremental steps forward. With the most recently developed risk assessment tools only 37% cases of CAT can be predicted, which is – in our opinion – not accurate enough. Therefore, scientists should change their view on the mechanisms behind VTE in cancer patients. Classical risk factors of VTE cannot be extrapolated to cancer patients, nor do (mutations in) coagulation-related genes significantly contribute to CAT. So far, germline variants have only been shown to affect VTE risk during chemotherapy. Thus, we believe that understanding the mechanism behind CAT comes from genetic profiling of tumors. At present, only mutations in K-ras in colon and lung cancer show an association with increased risk of VTE, while IDH1/2 mutations are associated with a decrease in VTE risk in glioblastoma patients. As it is time consuming to investigate the role of every gene in CAT one by one searching for genes that associate with CAT an unbiased manner may be more appropriate. This should ultimately lead to the discovery of novel biomarkers that potentially serve as a diagnostic tool. Furthermore, it will also give more insight in the upstream biological processes that provoke a hypercoagulant state, leading to VTE. We furthermore recommend assessing genetic profiles in each cancer (sub)type separately, since different genetic events that associate with CAT may be dependent on processes that are cancer type-specific.

Author contributions

BU wrote the manuscript, HHV supervised and edited the manuscript.

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Conflict of interest statement

The authors have no relevant conflicts to declare in relation to this paper.

Table 1

Clinical studies on mutational status and cancer-associated thrombosis

Cancer	cohort type	cohort size	tumor type	Gene of interest	Outcome	Remarks	Ref
Colorectal	Retrospective	172	metastatic colorectal cancer	K-ras	OR=2.21	Bevacizumab independent; Multicenter	Ades et al. 2015
Lung	Retrospective case-control	159	non-small cell lung carcinoma	K-ras	OR=2.67		Corrales-Rodriguez et al. 2014
				EGFR	OR=0.99		
	Retrospective	293	lung adenocarcinoma	EGFR	HR=0.46	Tyrosine kinase inhibitor (TKI) treatment reduces VTE risk	Davidsson et al. 2017
				ALK	HR=0.61 (ns)		
	Discovery	55	lung adenocarcinoma	ALK	41.8% VTE	All patients had ALK rearrangement; included patients with VTE history and thromboprofylaxis	Zer et al. 2017
	Validation	43	non-small cell lung carcinoma	ALK	27.9% VTE	All patients had ALK rearrangement; included patients with VTE history and thromboprofylaxis	
Brain	Discovery	169	glioma	IDH1/2	0% VTE	wild-type: 25.5% VTE; microthrombi in 85.5% WT vs 1.9% in mutant	Unruh et al. 2016
	Validation	148	glioma	IDH1/2	0% VTE	wild-type: 29.5% VTE; microthrombi in 90.4% WT vs 5.9% in mutant	

Table 2

Expression profile and associated canonical pathways in CRC patients with VTE before or around diagnosis. Table adjusted from Ünlü et al. [57].

−1 year ≤ CRC diagnosis			−3 ≤ CRC diagnosis ≤ +3 months		
Top canonical pathways					
		p-value			p-value
LXR/RXR activation		1,39E-03	Methionine degradation I		9,79E-03
FXR/RXR activation		1,51E-03	Cysteine biosynthesis III		1,07E-02
Atherosclerosis signaling		1,53E-03	Superpathway of Methionine degradation		1,67E-02
Coagulation system		1,63E-02	Glutamate receptor signaling		2,64E-02
Thyroid cancer signaling		1,86E-02	Autophagy		2,86E-02
Gene expression profile					
Genes	*AvgLog2FC*	Adjusted p-value	Genes	*AvgLog2FC*	Adjusted p-value
REG4	7,3	1,18E-09	*GBP4*	3,9	3,07E-07
SPINK4	6,7	1,63E-05	*XKR9*	6,2	1,08E-06
SERPINA1	6,8	5,45E-04	*CTSE*	7,2	1,87E-06
SLITRK6	4,0	6,44E-03	*AHCYL2*	2,8	2,55E-05
SBSPON	4,2	8,49E-02	*GRM8*	-5,1	2,77E-05
DEFA	4,3	1,13E-01	*REG4*	5,5	1,49E-04

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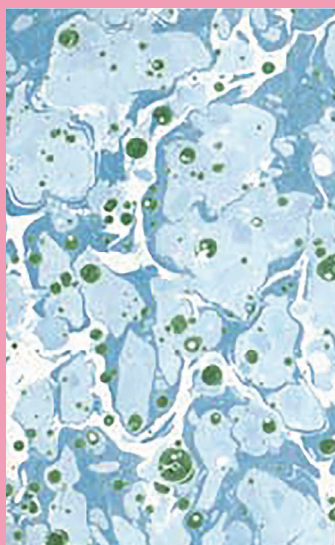
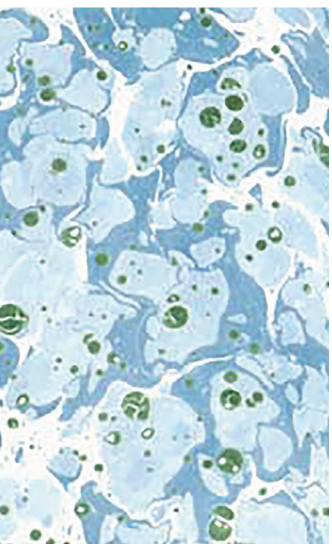


Chapter 8

General discussion and summary



BACKGROUND

The human body is a complex system and regulates various processes, from synthesis of tiny molecules to complete organ function. The vascular system allows the blood to circulate through the body, deliver nutrients, oxygen and immune cells to the tissue and transport metabolic degradation products away from the cells. The hemostatic system keeps the vascular system in equilibrium between liquid (bleeding) and solid (clotting) phase. Hemostasis consists of a number of independent biochemical and cellular processes. Upon wounding, primary hemostasis i.e. platelet binding, activation and aggregation is initiated [1]. In addition, secondary hemostasis, i.e. the blood coagulation cascade is initiated by the principal activator of coagulation, tissue factor (TF). According to current cellular models, TF – expressed on subendothelium – activates extrinsic coagulation after it comes in contact with the blood. There, it binds and activates its natural ligand VII (FVII) to generate activated factor X (FXa), that on its turn generates low levels of thrombin. Via feedback loops, previously known as the intrinsic coagulation cascade, these levels of thrombin lead to activation of clotting factors IX, XI, FV, FVIII and FX on the surface of activated platelets. As this is a relative slow process, it contributes to clot stability [1]. Deficiencies in components of this pathway results in increased bleeding risk [2]. Finally, FX in complex with FV generates thrombin levels that are sufficient to convert soluble fibrinogen into fibrin. The fibrin-rich blood clot further stabilizes the previously formed platelet plug [1]. In disease settings, the balance of blood coagulation is disturbed. This may lead to excessive bleeding e.g. in case of coagulation deficiency such as in hemophilia or to venous thromboembolism (VTE) in case of a hypercoagulant state.

In 2003 an alternatively spliced isoform of TF (asTF) has been described, that lacks a transmembrane domain [3]. Therefore, asTF is a soluble protein and has a unique C-terminal tail as a consequence of a frameshift. It has been shown that asTF induces angiogenesis by binding to endothelium-expressed αvβ3 integrins to promote endothelial cell migration and α6β1 integrins to form capillaries [4]. In a cancerous setting, asTF promotes tumor growth after ligation to β1 integrin [5]. Additionally, in breast cancer it synergizes with the estrogen receptor pathway to further promote breast cancer progression [6]. Interestingly, in pancreatic ductal adenocarcinoma asTF increases coagulant activity on cells and microvesicles (MVs) [7], however, whether asTF has coagulant properties has been a matter of debate. Although the N-terminal part of the protein is identical to full-length TF (flTF) and has a binding site for FVII, asTF lacks the binding site for FIX and FX. Nevertheless, asTF is present in occlusive thrombi [8]. To further elucidate whether asTF has coagulant properties,

the first part of this thesis is dedicated to the interplay between asTF and flTF in in the initiation of coagulation on endothelial cells.

In patients with cancer a hypercoagulant state is often observed and accordingly these patients are at increased risk of cancer-associated thrombosis (CAT). CAT is the second leading cause of death in cancer patients, after cancer itself, but diagnosis of VTE in these patients is also associated with increased morbidity and mortality that cannot be explained by the occurrence of VTE itself. This reduced survival may be influenced by increased activation of cellular and circulating coagulation factors that activate transmembrane receptors. In addition, while circulating coagulation factors are usually synthesized by the liver, tumor cells are also able to ectopically express procoagulant factors that promote tumor-associated processes like tumor growth, angiogenesis and metastasis [9, 10]. The second part of the thesis deals with the contribution of coagulation factors to tumor progression. Specific focus is placed on the role of TF signaling in metastasis in breast cancer, since TF is considered the linking pin in cancer and thrombosis. The final part of the thesis addresses genomic and non-genomic key players that may contribute to CAT. We describe a novel mouse model to investigate the effects of tumor development on thrombus formation. Furthermore, a proof-of-principle study is presented that elucidates molecular mechanisms and biological pathways behind CAT in an unbiased manner.

1. Alternatively spliced Tissue Factor has no function in hemostasis

Since its discovery 15 years ago, the contribution of asTF to blood coagulation has remained unclear. Despite the lack of a functional FX binding site, asTF has minimal coagulant activity in the presence of phosphatidylserine [3]. Yet, asTF has been found at the edge of growing thrombi in mouse models [8], which suggests a role for asTF in blood clotting. However, studies that had been performed so far were lacking, as no asTF protein could be detected in endothelial cells or supernatant after cytokine stimulation [11] or experiments were performed in the absence of flTF [12, 13], while asTF expression does not occur in the absence of flTF [14]. Therefore, conclusions from these studies focusing on direct or indirect asTF coagulant activity should be taken with caution [11, 12]. Hence, to address the interplay between asTF and flTF in hemostasis we co-expressed these proteins in endothelial cells and studied changes in coagulant activity of flTF in **chapter 2**. With relative low concentrations of asTF no alterations on flTF-dependent clotting was observed, neither on cells nor on MVs. In fact, when asTF and flTF were co-expressed, these isoforms did not co-localize and were found in different cellular compartments.

Based on the results in **chapter 2** it may be concluded that asTF has a limited role in coagulation activation, with minimal coagulant activity and no regulatory activity towards flTF. Nevertheless, it is possible that asTF plays a role in hemostasis. Thus far, asTF was detected in thrombi [8, 15], and associates with platelets, where it may increase coagulant potential. asTF ligation to β1 integrins also induces interactions between endothelial cells and monocytes [3, 16], the latter being one of the known players involved in initiation and propagation of a thrombus [17]. In support of the view that asTF may modulate monocyte function, treatment of microvascular endothelial cells (MVECs) with asTF increases expression and activation of cell adhesion molecules like E-selectin, VCAM-1 and ICAM-1 to further support monocyte binding [18]. Although extremely speculative, asTF on endothelial cell membranes and/or MVs might further support thrombus formation acting as a kind of 'Velcro'. Collier et al. previously reported that endothelial cells can internalize TF^+ MVs and recycle TF to the cell membrane thus increasing coagulant activity on these cells [19]. asTF might facilitate fusion of TF^+ MVs to endothelial cells via ligation to integrins. Alternatively, asTF may mediate integrin-dependent TF^+ MV shedding. Although no increase in TF^+ MVs was observed in our study, asTF did increase procoagulant MVs shedding in a pancreatic cancer model in a β1 integrin-dependent manner, while flTF levels remained unchanged [7]. Furthermore, β1 integrin on TF^+ MVs increases coagulant activity as it preserves the FVII binding site [19], a similar mode-of-action may apply to asTF. Therefore, it would be of interest to test effects of an asTF blocking antibody, RabMab1, in VTE ***in vivo*** models to understand the contribution of asTF to thrombus formation. Finally, determining associations between asTF plasma levels in VTE patients and controls appears warranted.

One other aspect of our study was that excessive asTF expression led to decreased flTF expression in the non-raft membrane. This reduction of flTF at the cell surface resulted in decreased coagulant activity as measured with FXa generation assays. It has to be noted that supraphysiological levels of asTF induced endoplasmic reticulum stress, as reflected by increased BiP levels, and asTF was subsequently degraded in a proteasome-dependent manner. Thus, excessive asTF expression might also result in recognition of 'incorrect' folded TF and as a result, degradation of flTF.

2. Coagulation factors contribute to tumor progression

The contribution of coagulation factors to tumor progression has been extensively investigated. Not only do coagulation factors facilitate tumor growth via diffusion from the blood into the tumor milieu due to leaky vessels, it has also been reported that tumors cells can express clotting factors ectopically. Roles of blood-derived and tumor-derived clot-

ting factors in tumor progression are extensively reviewed in **chapter 3**. It becomes clear that coagulation factors noticeably contribute to two specific hallmarks of cancer: angiogenesis and metastasis. As mentioned before, asTF induces angiogenesis in a β1 integrin dependent manner. Angiogenesis is also promoted via intracellular signaling triggered by flTF/FVIIa and thrombin that activate members of the Protease-activated Receptor (PAR) family. Activation typically leads to increased expression and secretion of IL8, VEGF and angiopoietin-1, and thereby supports tumor growth [20]. Upon entering the bloodstream, tumor cell survival becomes critically dependent on flTF-dependent coagulation activation to form a fibrin/platelet-rich shield around the tumor cell that provides protection against the immune system and shear stress. Recently, the role of von Willebrand factor (VWF) was demonstrated to further support successful metastasis. Tumor cells can activate endothelial cells in order to secrete VWF strings. Even under shear stress, these VWF strings can activate and bind platelets, which creates a docking-site for the tumor cell to escape the bloodstream and invade in the tissue at a metastatic site [21, 22].

Tissue Factor signaling supports metastasis

For successful metastasis, tumor cells must undergo epithelial-to-mesenchymal transition (EMT) and gain cancer stem cell (CSC) properties. As others already had reported that TF influences migration of tumor cells and influences cancer stemness [23, 24], we decided to study for the first time the nature of the mechanism linking TF to these processes in **chapter 4**. We show that the antibody Mab-10H10, which inhibits TF signaling, but does not influence the coagulant properties of TF, decreases breast cancer metastasis by i) regulation of EMT-associated markers to maintain an epithelial phenotype, ii) decreasing tumor-initiating capacity through maintenance of CSCs and iii) regulating integrin function to further suppress a mesenchymal state. Furthermore, treatment of breast cancer cells with the antibody Mab-10H10 shifted TF complex formation with α3β1 integrin to α6β1 and α6β4, that dictates focal adhesion kinase activation and recruitment. This finally induced an epithelial morphology with decreased tumorigenic properties and metastasis.

In our research, we used the breast cancer cell line MDA-MB-231-mfp, which expresses the asTF isoform, albeit at relative low levels. We did not assess asTF function in metastasis in **chapter 4**, nor its potential synergy with flTF. Thus far, abundant expression of asTF has been found in pancreatic and breast cancer, where it – in a β1 integrin-dependent manner - promotes angiogenesis, proliferation in both tumor types, and metastasis in pancreatic cancer [7, 25, 26]. In breast cancer asTF is mainly expressed in ER^+ tumors, and a synergy with the ER signaling pathway led to induced cell proliferation [5, 6]. Interestingly, when

β1 integrin was blocked with an antibody, asTF-dependent proliferation could not be completely inhibited [5]. This would suggest another integrin binding partner for asTF in breast cancer progression. As we have shown elevated α6β4 integrin expression after treatment with Mab-10H10 in **chapter 4**, it is plausible that asTF can ligate to and signal via α6β4 integrins to further contribute to the epithelial-like morphology in cancer, as increased expression and secretion of cell adhesion molecules was observed in MVECs in the presence of asTF [18]. Thus far, interactions between asTF and β4 integrin to influence cell adhesion, capillary formation and migration of endothelial cells in a physiological context has been ruled out [4], but β4 integrin and asTF in cancer has not been addressed yet.

It is unlikely that in the breast cancer model used in this thesis asTF plays a dominant role in cancer-associated angiogenesis; we have previously shown that asTF affects proliferation, but not angiogenesis, in the MDA-MB-231 breast cancer model [5]. In contrast, asTF induces angiogenesis - in the absence of flTF - in MCF-7 breast cancer cells, with similar results in pancreatic cancer [7, 13, 27]. In pancreatic cancer it was shown that asTF increases angiogenesis in an integrin-dependent manner, i.e. via ligation to α6β1. A number of explanations for this apparent contradiction may be postulated: i) asTF expression levels in MDA-MB-231-mfp cells are low in comparison to the rather artificial overexpression cell lines used in other studies; ii) asTF might be involved in physiological angiogenesis rather than pathological angiogenesis in the presence of flTF and/or iii) flTF sequesters all β1 integrin molecules at the cell membrane, thus preventing asTF from binding integrins and induce intracellular signaling. It would be of interest to further study the above-mentioned possibilities in order to elucidate the potential interplay between asTF and flTF in tumor-associated angiogenesis.

Thus far, the focus of this thesis was mainly on changes within the tumor cell prior to metastasis. One other contributor to metastasis that has not been studied in the context of this thesis is MVs. According to Stephen Paget's 'seed-and-soil' hypothesis in which he compared metastatic cells to plant seeds, tumor cells can only grow at metastatic sites that constitute a favorable microenvironment to these tumor cells [28]. In recent years it has been shown that MVs can dictate organ-specific metastasis by preparing the pre-metastatic niche. Of note, MV-directed pre-metastatic niche formation is dependent on the MV integrin expression profile [29, 30]. αvβ5 integrin positive MVs predominantly mediate liver-specific metastasis, while α6β1 and α6β4 integrin positive MVs direct homing of circulating tumor cells to the lung [29]. Extensive research has been performed on TF^+ MVs with respect to tumor progression [31, 32]. Da Rocha Rondon et al. recently showed by using

CRISPR/Cas9 approaches, that knockout of TF in MDA-MB-231 cells decreased MV shedding [31]. Furthermore, phosphorylation of TF at Ser253 and Ser258 has been shown to be an 'on-off switch' for the incorporation of TF into MVs in endothelial cells [33]. We propose that TF signaling inhibition with Mab-10H10 may lead to a similar reduction in MV shedding, especially as Mab-10H10 also prevents cytoplasmic phosphorylation of TF by PAR2 [34, 35]. In addition, it would be interesting to study if TF influences integrin incorporation into MVs, and thereby influences organotropic metastasis.

3. Molecular mechanism underlying cancer-associated thrombosis

Cancer-associated thrombosis is a frequent complication in cancer patients and contributes to high morbidity and mortality. Unfortunately, as the mechanisms underlying CAT are incompletely understood, it is a challenge to predict those patients with elevated risk and those who might benefit from thromboprophylaxis. Therefore, the third part of this thesis focusses on CAT. In **chapter 5** a mouse model is used to investigate the interactions between cancer and spontaneous thrombosis. Thus far, established VTE ***in vivo*** models are invasive as the vessels needs to be exposed, or do not mimic the pathological nature of thrombosis. The ferric chloride model relies on the induction of oxidative stress to the vessels. Although a relative simple procedure, it does not reflect the clinical setting of thrombus initiation [36]. In the inferior vena cava (IVC) stenosis model the vena cava is partially ligated while preventing vessel injury. In this stenosis model, development of a VTE is dependent on immune cells like leukocytes, neutrophils, which can form neutrophil extracellular traps, thus better mimicking a DVT as in a human setting [36, 37]. Unfortunately, the incidence of thrombus formation is variable and the thrombus propagates in a direction opposite to the blood flow. Therefore, we have recently developed a new non-invasive spontaneous VTE model in which antithrombin is silenced in the liver, via siRNA injections into the tail vein. Downregulation of antithrombin leads to an imbalance in coagulation factors resulting in venous thrombosis and hemorrhages in the head of mice [38, 39]. We observed that mice with breast tumors were partially rescued from these hemostatic abnormalities, while this treatment had no effect on short-term tumor characteristics, such as tumor growth and metastasis. Furthermore, the presence of a tumor induced elevated platelets counts, fibrinogen levels and a systemic pro-inflammatory status, all of which were unaffected by antithrombin knockdown. Closer examination of the organs showed increased fibrin deposition in the livers of non-tumor-bearing mice, while tumor-bearing mice presented with high fibrin deposits in the tumor. Interestingly, macrovascular thrombosis in the large

veins of the head of these mice was less frequently observed, the latter being a phenotype that is typically associated with antithrombin knockdown in mice.

Thus, in contrast to the hypothesis, a protective phenotype was observed in tumor-bearing mice. This might be explained by elevated platelet counts in the plasma of these mice. We hypothesize that the protective phenotype is caused by lack of a complete consumption of platelets in mice with breast cancer. The elevated platelet counts found in these mice would represent thrombocytosis in patients. In support, thrombocytosis is associated with metastasis and inflammatory breast cancer [40, 41]. Furthermore, the phenotype presented in mice with tumors would suggest disseminated intravascular coagulation (DIC), which is defined by e.g. low antithrombin levels, fibrin products, platelet consumption, venous thrombosis and/or hemorrhages [42]. DIC occurs in 5% of breast cancer patients and is an extreme form of hypercoagulation [10]. As this extreme form was presented, it would be of great interest to combine MDA-MB-231 breast cancer model with the classical IVC stenosis model. In murine pancreatic cancer models, larger venous clots were observed in tumor-bearing mice when compared to tumor-free mice, and that this clot formation was dependent on TF^+ MVs [43]. However, in our breast cancer model, no TF-dependent hypercoagulant plasma was observed, which warrants studying CAT in a TF^+ MV independent manner.

A different approach to study involvement of key factors and biological pathways in CAT is to investigate tumor gene expression and to link these expression profiles to VTE. The study described in **chapter 6** is the first ever study to report differential gene expression profiles in tumors from patients with both colorectal cancer (CRC) and VTE compared to those from patients with CRC only. A pro-inflammatory status was shown in the tumors of patients with CAT, and elevated levels of fibrin deposits were present in the tumors from patients with VTE. Increased fibrin products could serve as a matrix for tumor vessel formation, or promote metastasis, which may contribute to the decreased survival in CAT patients [44-46]. When patients with VTE were subdivided into two groups of VTE before (max 1 year) and around (max 3 months) diagnosis, altered expression profiles were observed. This might be induced by cancer-related treatment, as metabolism related pathways were up-regulated in the 'VTE around diagnosis' group.

In order to fully understand the pathophysiologic mechanism of CAT, irrespective of cancer treatment, the top 3 genes that were found to be differentially expressed in CRC patients with VTE before cancer diagnosis are of interest for further investigation, especially since

links can be found with coagulation-related factors. Reg4 can bind to the naturally occurring anticoagulant heparin in the absence of calcium [47] and Spink4 is a serine protease inhibitor of the Kazal type [48] and might inhibit coagulation factors. Furthermore, co-expression of Reg4 and Spink4 are tightly regulated in inflammatory bowel disease [49], indicating a (chronic) pro-inflammatory status that might increase TF expression and thereby further contribute to VTE [1, 50]. One in five patients with inflammatory bowel disease get diagnosed with cancer, furthermore they are 2-3 fold at increased risk of VTE [51-54]. In addition, elevated Reg4 expression is associated with tumor progression, metastasis and a poorer survival [55-57]. A more direct link with coagulation regulation is constituted by ***SERPINA1***, which encodes α1-antitrypsin (A1AT). A1AT can directly and indirectly stimulate thrombus formation by inactivation of several coagulation factors such as FXa, FXII and activated protein C; and complex formation of A1AT with elastase may increase systemic neutrophil activation that triggers VTE via neutrophil extracellular traps [58-60]. Additionally, A1AT prolongs clot-clearance as it inhibits neutrophil elastase-mediated fibrinolysis [58, 61, 62]. Furthermore, A1AT expression was found in fibrin-rich blood clots, suggesting a direct role for this protein in VTE [58]. In relation to cancer, A1AT overexpression has been found in several tumor types such as brain [63], colorectal [64] and gastric cancer [65], or elevated A1AT plasma levels were observed in patients with breast, malignant melanoma and gastric cancer [66]. In the latter study, cancer patients with elevated risk of VTE were included, although no remarks have been made on confirmed VTE in these patients. Therefore, no direct associations between A1AT plasma levels and VTE could be established.

Unfortunately, the majority of studies that have attempted to find biomarkers for CAT have focused on coagulation factors, with TF being in the center of cancer-associated thrombosis. More importantly, scientists and clinicians have, perhaps unjustified, attempted to extrapolate classical VTE risk factors to CAT, resulting in a low success-rate of biomarkers that accurately predict CAT. Several risk assessment tools have been developed to predict which cancer patients are at elevated risk of VTE, unfortunately with low prediction accuracy. In **chapter 7** we plea to stop using classical VTE risk factors as predictors for CAT, especially as a recent study by Mohammed et al. showed different plasma protein profiles in patients with CAT, when compared to plasma form those diagnosed with either cancer or VTE [67]. Furthermore, we discussed the link between mutations and elevated risk of CAT. As it is a very biased and time-consuming approach to investigate all and every gene on CAT, therefore, we propose an unbiased approach by performing molecular profiling in patients with CAT, as was described in **chapter 6**.

Conclusion and further directions

This thesis describes i) the function of asTF in hemostasis, ii) the contribution of coagulation factors on cancer progression, and iii) expands our view on cancer-associated thrombosis. Inhibition of TF signaling with Mab-10H10 resulted in decreased EMT- and CSC associated transcription program, tumor initiating capacity and metastasis in a triple negative breast cancer (TNBC) cell line. Since this is a tumor type that is difficult to treat, and has high relapse-rates, it would be of interest to target TF signaling. Dual treatment of TNBC with conventional chemotherapy and Mab-10H10 could result in a positive treatment strategy as both highly proliferative and cancer stem cells are targeted.

Furthermore, we provided a proof-of-principle study to search for novel biomarkers in CAT patients in an unbiased manner. Expansion of this study to validation cohorts and other tumor types will give insights in the underlying molecular mechanism of cancer-associated thrombosis. Eventually, this will aid a better prediction model to select those cancer patients with high risk of VTE and those who might benefit from thromboprophylaxis.

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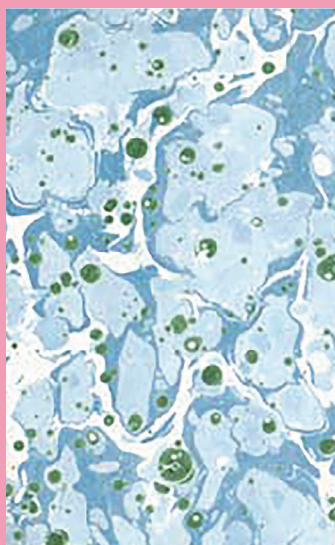
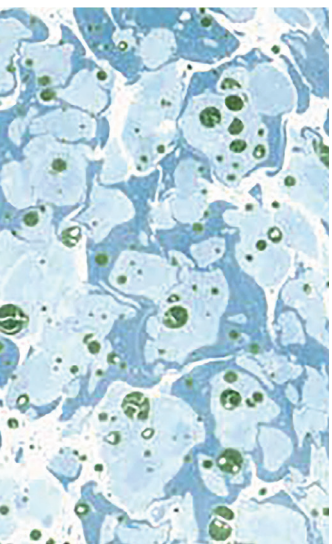


Chapter 9

Nederlandse discussie en samenvatting



ACHTERGROND

Het menselijk lichaam is een complex systeem en reguleert verschillende processen, van het synthetiseren van kleine moleculen tot de werking van complete organen. Het vasculaire systeem zorgt ervoor dat het bloed door het lichaam kan circuleren, het levert voedingstoffen, zuurstof en immuuncellen af aan weefsels en het voert afbraakproducten uit de cellen af. De hemostase zorgt voor de balans van het vasculaire systeem. Het bloed moet vloeibaar blijven in de afwezigheid van schade aan bloedvaten, en stollen na beschadiging van bloedvaten. Na een verwonding, wordt de primaire hemostase gestart, waarin bloedplaatjes geactiveerd worden en gaan samen klonten om het gat in het bloedvat af te dekken. Vervolgens, wordt de secundaire hemostase, ofwel de bloedstollingscascade, geïnitieerd door cellulair transmembraan eiwit genaamd weefselfactor (Tissue Factor; TF). Volgens de huidige cellulaire modellen, activeert TF dat tot expressie komt in het subendotheel de extrinsieke bloedstolling nadat het in contact komt met het bloed. Daar bindt en activeert het zijn natuurlijke ligand factor VII (FVII) om vervolgens actief factor X (FXa) te genereren, wat op zijn beurt lage concentraties trombine genereert. Via feedback-loops, ook wel bekend als de intrinsieke bloedstolling, zorgen deze trombine niveaus voor activatie van de stollingsfactoren IX, XI, V, VIII en X op de oppervlakte van geactiveerde bloedplaatjes. Doordat het een relatief langzaam proces is, draagt dit bij aan een stabilisatie van het stolsel. Deficiënties in componenten van deze signaleringscascade resulteren in verhoogd risico op bloedingen. Tenslotte, genereert FX in complex met FV dergelijk hoge concentraties trombine die voldoende zijn om oplosbaar fibrinogeen om te zetten in fibrine. Dit fibrine-rijke bloedstolsel stabiliseert de eerder door bloedplaatjes gevormde stolsel. In geval van ziekte is het evenwicht tussen bloedstollingsfactoren verstoord. Dit kan vervolgens lijden tot overmatige bloedingen, bijvoorbeeld in het geval van stollingsdeficiëntie zoals hemofilie, of tot veneuze trombose (VT) in het geval van een hypercoagulante staat.

In 2003 is er een tweede isovorm van TF ontdekt. Als gevolg van een frameshift door alternatieve splicing mist dit eiwit, asTF, het transmembraan domein, en heeft het een unieke C-terminale staart. Daardoor is asTF, in tegenstelling tot membraangebonden TF, een oplosbaar eiwit. Het is aangetoond dat asTF angiogenese stimuleert door te binden aan integrine $\alpha v\beta 3$, hetgeen migratie van endotheelcellen te bevordert. Daarnaast bindt het aan $\alpha 6\beta 1$ integrine om capillairen te vormen. In ziekteprocessen zoals kanker zorgt asTF voor versnelde tumorgroei door te binden aan $\beta 1$ integrines. In borstkanker vertoont asTF signaal transductie een synergisme met de oestrogeen receptor (ER) signaleringsroute om verdere groei van borstkanker te versnellen. Interessant is dat in alvleeskliertumoren, asTF de stollingsactiviteit van cellen en daarvan afgesnoerde blaasjes (microvesicles; MVs) verhoogt,

maar of asTF daadwerkelijk stollingseigenschappen heeft staat nog ter discussie. Ondanks dat het N-terminale gedeelte van asTF identiek is aan normaal gespliced TF (full-length TF; flTF) en het een bindingsplaats heeft voor FVII, mist asTF een bindingsplaats voor FIX en FX. Desondanks is expressie van asTF gevonden in pathologische bloedstolsels. Daarom is het eerste gedeelte van dit proefschrift gewijd aan het bepalen of asTF daadwerkelijk stollingseigenschappen heeft, en of er een samenspel is tussen asTF en flTF in de initiatie van de stolling op endotheelcellen.

Vaak wordt er in kankerpatiënten een verhoogde pro-coagulante staat waargenomen en deze patiënten hebben een verhoogd risico op kanker-geassocieerde trombose (cancer-associated thrombosis; CAT). CAT is de tweede meest voorkomende doodsoorzaak bij kankerpatiënten, na de gevolgen van kanker zelf. Helaas is de diagnose van CAT in deze patiënten ook geassocieerd met een slechtere prognose en verhoogde sterftekans, iets dat simpelweg niet verklaard kan worden door de gevolgen van VT zelf. Deze verkleinde kans op overleven zou beïnvloed kunnen worden door verhoogde activatie van cellulaire en circulerende stollingsfactoren, factoren die ook een rol spelen bij trombose. Activatie van deze stollingsfactoren leidt vervolgens tot activatie van bepaalde transmembraan eiwitten, zogenaamde Protease-activated Receptors (PARs), die mogelijk een rol spelen bij kankerprogressie. Normaliter worden stollingsfactoren gemaakt in de lever, maar stollingseiwitten geproduceerd door tumorcellen zijn ook in staat tumorgroei, angiogenese en metastase te beïnvloeden. Het tweede gedeelte van dit proefschrift behandelt daarom de effecten van stollingsfactoren op tumorprogressie. Er gaat vooral aandacht uit naar de rol van TF-afhankelijke signaaltransductie in borstkanker metastase. Dit is relevant aangezien TF vaak gezien wordt als de onmiskenbare link tussen kanker en stolling. In het laatste gedeelte van dit proefschrift worden genetische en niet-genetische factoren besproken die uiteindelijk kunnen leiden tot CAT. We beschrijven een nieuw muismodel om de effecten van tumor ontwikkeling op trombus formatie te bestuderen. Ten slotte, laten wij in een 'unbiased approach' zien welke (nieuwe) moleculaire mechanismen en biologische signaleringsroutes betrokken zijn bij CAT in een 'proof-of-principle' studie.

1. Alternatief gespliced Tissue Factor heeft geen functie in hemostase

Vijftien jaar na de ontdekking van asTF is de rol van dit eiwit in de bloedstolling nog steeds onduidelijk. Reeds uitgevoerde studies laten zien dat, ondanks het missen van een functionele bindingsplaats voor FX, asTF toch minimale stollingscapaciteit bezit in de aanwezigheid van fosfatidylserine. Histochemische studies wijzen uit dat asTF aanwezig is aan

de rand van een groeiende trombus in muismodellen. Dit suggereert dat asTF een functie heeft in bloedstolling. Helaas bevatten de studies die tot nu toe waren uitgevoerd een aantal belangrijke nadelen die het interpreteren van de stollingsfunctie van asTF bemoeilijken; i) stimuleren van endotheelcellen met cytokines een behandeling die doorgaans leidt tot TF synthese- leidde niet tot detecteerbare asTF niveaus in endotheel cellen of in het supernatant en/of ii) experimenten aan de stollingsfunctie van asTF werden uitgevoerd in de afwezigheid van flTF, terwijl asTF expressie in de afwezigheid van flTF nooit is waargenomen. Daarom moeten de conclusies die getrokken worden uit deze studies met terughoudendheid worden geïnterpreteerd. Om het samenspel tussen asTF en flTF te bestuderen in endotheelcellen, werden deze eiwitten tegelijkertijd tot expressie gebracht waarna verschillen in stollingsactivatie van flTF werd onderzocht in **hoofdstuk 2**. De resultaten lieten zien dat relatief lage asTF concentraties geen effect hebben op flTF-afhankelijke stolling, niet op cellen noch op MVs. Co-lokalisatie experimenten lieten zien dat asTF en flTF zelfs in gescheiden cellulaire compartimenten voorkomen.

Gebaseerd op de resultaten in **hoofdstuk 2** kan er geconcludeerd worden dat asTF een beperkte rol heeft in stollingsactivatie, met minimale stollingsactiviteit en geen regulerende rol richting flTF. Desondanks is het mogelijk dat asTF een rol speelt in hemostase. Tot nu toe is asTF gedetecteerd in bloedstolsels en op bloedplaatjes (waar het de stollingspotentiaal zou kunnen verhogen). Binding van asTF aan β1 integrines stimuleert interacties tussen endotheelcellen en monocyten; met name deze monocyten zijn nauw betrokken bij de initiatie en propagatie een stolsel. Eerder werk heeft laten zien dat behandeling van microvasculaire endotheelcellen (MVECs) met asTF leidt tot verhoogde expressie en activiteit van cel adhesie moleculen zoals E-selectin, VCAM-1 en ICAM-1 om verdere monocyt binding te bevorderen. Dit ondersteunt het concept dat asTF monocyt functie in trombus vorming kan beïnvloeden. Trombus vorming zou verder nog bevorderd kunnen worden, door een 'klittenband' functie van asTF op cellen en MVs. Collier en collega's hebben eerder laten zien dat endotheelcellen reeds afgesnoerde TF^+ MVs kunnen internaliseren en deze pool aan TF terug naar het celmembraan recyclen, waardoor de stollingsactiviteit van deze cellen omhoog gaat. asTF kan mogelijk de fusie van TF^+ MVs met endotheelcellen bevorderen door integrines te activeren.

Als laatste lijkt asTF betrokken bij β1 integrine-afhankelijke verhoogde afsnoering van MV's in alvleeskliertumorcellen, zonder de expressie niveaus van flTF te veranderen. In ons eigen onderzoek zagen wij geen asTF-afhankelijke verhoging van TF^+ MVs. Wellicht speelt het celtype een belangrijke rol in dit proces. Daarnaast verhoogt β1 integrine expressie op

MVs de TF-afhankelijke stollingsactivatie, omdat β1 integrine het macromoleculaire substraat bindingsplaats op TF optimaal positioneert. Ditzelfde model zou ook kunnen gelden in het geval van asTF. Het zou daarom interessant zijn om de effecten van asTF op VT ***in vivo*** te testen - in het aanwezigheid van een asTF-blokkerend antilichaam - teneinde de bijdrage van asTF aan trombus vorming te bestuderen. Als laatste is het aan te bevelen om asTF plasmaspiegels van VT-patiënten en controle individuen te bepalen zodat associaties tussen asTF niveaus en het risico op VT bepaald kan worden.

Onze studie toonde ook aan dat extreem hoge asTF concentraties leidden tot een verlaging van flTF expressie in non-raft membraan domeinen. Deze reductie in flTF op het celoppervlak resulteerde in verlaagde TF stollingsactiviteit zoals gemeten met FXa generatie experimenten. Het moet vermeld worden dat buitensporige concentraties van asTF zorgden voor verhoogde stress in het endoplasmatisch reticulum, wat tot uiting kwam door verhoogde BiP niveaus. We vonden ook dat asTF eiwit in een proteasoom-afhankelijke manier wordt afgebroken. Op basis van deze experimenten concluderen we dat extreem hoge asTF concentraties resulteren in de herkenning van 'incorrect' gevouwen TF en als gevolg daarvan tot flTF afbraak.

2. Stollingsfactoren dragen bij aan tumorprogressie

De bijdrage van stollingsfactoren aan tumorprogressie is uitgebreid bestudeerd. Stollingsfactoren kunnen tumorgroei beïnvloeden via diffusie van deze factoren vanuit bloed naar het tumormilieu vanwege lekkende bloedvaten. Echter, recent is ook gerapporteerd dat tumorcellen zelf in staat zijn om stollingsfactoren te maken. De rollen van bloed- en tumor-specifieke stollingsfactoren op tumorprogressie worden uitvoerig besproken in **hoofdstuk 3**. Het wordt duidelijk dat stollingsfactoren merkbaar bijdragen aan twee specifieke kenmerken van kanker: angiogenese en metastase. Zoals eerder beschreven, wordt angiogenese geïnduceerd door asTF in een β1 integrine-afhankelijke manier. Echter, daarnaast wordt angiogenese ook bevorderd door flTF/FVIIa- en trombine- afhankelijke activatie van Protease-activated Receptors (PARs), wat leidt tot verhoogde expressie en uitscheiding van proangiogene moleculen zoals IL8, VEGF en angiopoietine-1. Na intravasatie is overleving van de tumorcel in de bloedbaan sterk afhankelijk van stollingsactivatie door flTF teneinde een fibrine/plaatjes-rijke schild om de tumorcel te vormen. Dit schild beschermt de tumorcel tegen het immuunsysteem en andere schadelijke invloeden. Recentelijk werd tevens gerapporteerd dat von Willebrand factor (vWF) metastasering bevordert. Activatie van endotheelcellen door tumorcellen leidt tot het uitscheiden van vWF-draden, en deze draden kunnen de bloedplaatjes activeren en binden. Dit leidt uiteindelijk tot een binding-

splaats voor de tumorcel. Op deze manier kan de tumorcel ontsnappen aan de bloedstroom, het weefsel binnendringen en een secundaire tumor vormen.

Tissue Factor signalering ondersteunt metastase

Om te kunnen metastaseren moeten tumorcellen een verandering ondergaan van een epitheliale naar een mesenchymale staat (epithelial-to-mesenchymal transition; EMT;). Het EMT proces is ook dynamisch gelinkt aan het ontstaan van kankerstamcellen (cancer stem cell; CSC;). Zoals al eerder gerapporteerd, beïnvloedt TF de migratie en CSC-eigenschappen van tumorcellen. Daarom hebben wij besloten om het mechanisme waarop TF deze processen beïnvloedt, te bestuderen. De resultaten van dit onderzoek staan beschreven in **hoofdstuk 4**. Wij laten zien dat Mab-10H10, een TF antilichaam dat specifiek TF signalering remt en niet de bloedstollings-functie, borstkanker metastasering remt via i) regulatie van EMT-geassocieerde (transcriptie) factoren met als gevolg een epitheliaal fenotype, ii) verminderde tumor-initiërende capaciteit door een vermindering van het aantal kankerstamcellen en iii) regulatie van integrine functie om zo de mesenchymale-staat verder te onderdrukken. Specifiek resulteerde behandeling van borstkankercellen met Mab-10H10 voor een dissociatie van het TF/α3β1 integrine complex en tot een associatie van TF met α6β1 en α6β4 integrines. Dit laatste leidde tot vorming van focal adhesions en activatie van focal adhesion kinases. Dit leidde uiteindelijk tot een epitheliale cel morfologie met verminderde tumorigene eigenschappen en metastasering.

In ons onderzoek hebben wij gebruik gemaakt van de borstkankercellijn MDA-MB-231-mfp. Er moet vermeld worden dat deze cellijn ook lage hoeveelheden van asTF tot expressie brengt. In het onderzoek beschreven in dit hoofdstuk hebben wij niet onderzocht welke rol asTF heeft in metastasering. Tevens hebben we in **hoofdstuk 4** geen onderzoek gedaan naar potentiële, synergistische eigenschappen van asTF in de context van flTF functie. asTF wordt frequent tot overexpressie gebracht in alvleesklier- en borstkankertumoren, waar het - op een β1 integrine-afhankelijke manier - angiogenese en proliferatie bevordert. Tevens stimuleert asTF metastasering in alvleesklierkankermodellen. In borstkanker komt asTF voornamelijk voor in ER^+ tumoren, en in deze tumoren vertoont asTF synergie met ER functie, hetgeen uiteindelijk leidt tot verhoogde celdeling. Van belang is dat in eerdere studies remming van β1 integrine met een antilichaam, niet leidde tot volledige remming van asTF-afhankelijke proliferatie. Dit suggereert dat asTF mogelijk ook andere integrines kan binden teneinde borstkankerprogressie te beïnvloeden. Wij hebben in **hoofdstuk 4** reeds aangetoond dat α6β4 integrine expressie omhoog gaat na Mab-10H10 behandeling, en we achten het mogelijk dat asTF α6β4 integrine kan binden en activeren om zo verder bij

te dragen aan de epitheliale morfologie van de kankercellen. Dit is consistent met eerdere studies die laten zien dat MVEC cellen in de aanwezigheid van asTF een verhoogde expressie vertoonden van cellulaire adhesie moleculen. Het belang van interacties tussen asTF en β4 integrine voor celadhesie, capillair formatie en migratie van endotheelcellen is eerder onderzocht en uitgesloten, maar de functie van β4 integrine en asTF in kankercellen is nog niet uitgezocht.

Het is onwaarschijnlijk dat asTF een dominante rol speelt in kanker-geassocieerde angiogenese in het borstkanker model dat gebruikt is dit proefschrift; wij hebben eerder aangetoond in dit model dat asTF de proliferatie van tumorcellen beïnvloed, en niet de angiogenese. Echter asTF lijkt wel degelijk een rol te hebben in angiogenese in een borstkanker model op basis van MCF-7 cellen – een cellijn die normaliter geen flTF tot expressie brengt - en in alvleesklierkanker modellen waarin werd aangetoond dat asTF angiogenese stimuleert op een α6β1integrine-afhankelijke manier. Een aantal factoren zouden deze tegenstrijdigheid kunnen verklaren: i) asTF expressie niveaus in MDA-MB-231-mfp zijn laag ten opzichte van de artificiële overexpressiemodellen in andere onderzoeken; ii) asTF zou betrokken kunnen zijn bij fysiologische angiogenese maar niet bij pathologische angiogenese in de aanwezigheid van flTF en iii) flTF vangt alle β1 integrine receptoren weg van het celmembraan, waardoor het binding van asTF aan integrines en daarmee intracellulaire signalering voorkomen wordt. Het zou interessant zijn om bovenstaande mogelijkheden uit te zoeken om zo een mogelijk samenspel tussen asTF en flTF in tumor-geassocieerde angiogenese aan het licht te brengen.

Tot nu toe was de focus van dit proefschrift voornamelijk gericht op veranderingen binnen de kankercel voorafgaand aan metastase. Een ander proces dat betrokken is aan metastase, maar niet onderzocht binnen de context van dit proefschrift, is de afsnoering van MVs en hun opname door distaal gelegen cellen. Volgens de 'seed-and-soil' hypothese van Stephen Paget, waarin hij metastaserende cellen vergelijkt met plantenzaadjes, kunnen tumorcellen alleen uitgroeien in een metastatische niche als het tumormilieu gunstig voor deze cellen is. In de afgelopen jaren is aangetoond dat MVs orgaan-specifieke metastase kunnen veroorzaken door de pre-metastatische niche te veranderen ten gunste van de metastaserende cel. Deze voorbereiding door MVs voorafgaand aan metastase is afhankelijk van integrine expressieprofielen op de MVs. Zo dragen αvβ5 integrine-positieve MVs voornamelijk bij aan lever-specifieke metastase, terwijl α6β1 en α6β4 integrine-positieve MVs met name bijdragen aan de 'homing' van circulerende tumorcellen naar de longen. Er is uitvoerig onderzoek gedaan naar TF$^+$ MVs met betrekking tot tumorprogressie. Da Rocha Rondon en collega's

hebben recentelijk met behulp van het CRISPR/Cas9 editing aangetoond dat TF knock-out in MDA-MB-231 cellen zorgt voor verminderde afsnoering van MVs. Bovendien is aangetoond dat fosforylatie van TF op de aminozuren Ser253 en Ser258 fungeert als een soort "aan-en-uit" knop voor het opnemen van TF in de MVs. Wij stellen daarom voor dat inhibitie van TF signalering met Mab-10H10 zou kunnen leiden tot een vergelijkbaar vermindering van MV afgifte, te meer omdat Mab-10H10 cytoplasmatische fosforylatie van TF door PAR2 voorkomt. Daarnaast zou het interessant zijn om te bestuderen of TF een rol speelt in de opname van integrines in MVs, en zo orgaan-specifieke metastasering zou kunnen beïnvloeden.

3. Het achterliggende moleculaire mechanisme van kanker-geassocieerde trombose

Kanker-geassocieerde trombose is een vaak voorkomende complicatie en draagt bij aan hoge morbiditeit en mortaliteit. Doordat de mechanismen die ten grondslag liggen aan CAT onvolledig begrepen worden, is het een uitdaging om de patiënten die een verhoogd risico hebben op trombose te selecteren voor profylactische antistollingsmedicatie. Vandaar dat er aandacht uitgaat naar CAT in het derde gedeelde van dit proefschrift. In **hoofdstuk 5** is een muismodel ontwikkeld om effecten van kanker op trombose te onderzoeken. De meest-gebruikte muismodellen voor VT zijn erg invasief; teneinde VT te induceren moeten de dieren een operatie ondergaan waarbij de aderen blootgelegd moeten worden. Een ander nadeel van de huidige diermodellen is dat ze de pathologische aard van trombus vorming niet goed nabootsen. Het veelgebruikte ijzerchloride model berust op de inductie van oxidatieve stress in de vaten. Ondanks dat dit een relatief simpel model is, bootst het niet de klinische en biologische factoren na die het ontstaan van een trombus bepalen. In het 'inferior vena cava (IVC) stenosis' model wordt de vena cava gedeeltelijk afgebonden, waarbij schade aan de vaten wordt voorkomen. In dit stenose model is het ontstaan van VT afhankelijk van immuuncellen zoals leukocyten en neutrofielen, waarbij neutrofielen pro-trombotische 'Neutrophil Extracellular Traps' kunnen uitstoten. Dit model is dan ook een betere representatie van Diep Veneuze Trombose (DVT) in mensen. Helaas is dit model onderhevig aan enorme variatie in de uitkomsten, en ontstaat het bloedstolsel tegen de richting van de bloedstroom in. Op basis van deze overwegingen hebben wij een nieuw niet-invasief spontaan VT model ontwikkeld waarin aanmaak van antitrombine in de lever wordt uitgeschakeld, door middel van intraveneuze siRNA injecties. Verlaagde expressie van antitrombine leidt tot een verstoord evenwicht in het stollingsmechanisme met VT en bloedingen in de hoofden van de muizen als gevolg. In **hoofdstuk 5** constateren wij dat muizen met borstkanker gedeeltelijk gered worden van deze hemostatische afwijkingen. Deze pro-trombotische be-

handeling had op korte termijn geen effecten op tumoreigenschappen, zoals tumorgroei en metastase. Daarnaast zorgde de aanwezigheid van een borsttumor voor verhoogde niveaus aan bloedplaatjes, fibrinogeen en een systemische ontstekingsmediatoren. Dit alles werd niet beïnvloed door verlaging van antitrombine. Nader onderzoek van de organen liet een verhoogde vorming van fibrine zien in de levers van muizen zonder een tumor, terwijl de muizen die kanker ontwikkelden hoge fibrine vorming lieten zien in de tumor. Een interessante bevinding was dat er minder vaak macrovasculaire trombose werd aangetroffen in de grote aderen van deze muizen, daar waar vorming van deze grote trombi juist een fenotype is dat geassocieerd is met verlaagde antitrombine niveaus in muizen.

In tegenstelling tot de hypothese, werd er minder vaak een trombose-geassocieerd fenotype geobserveerd in muizen met een tumor. Dit zou verklaard kunnen worden door verhoogde aanwezigheid van bloedplaatjes in het plasma van deze muizen. Wij stellen nu de hypothese dat het beschermende werking van de aanwezigheid van een tumor wordt veroorzaakt door een gebrek aan complete consumptie van plaatjes in muizen die borstkanker hebben. De verhoogde aantallen bloedplaatjes in deze muizen zou in mensen gelijk zijn aan trombocytose. Ter ondersteuning van deze bevindingen, trombocytose is geassocieerd met metastasering en inflammatoire borstkanker. Daarnaast suggereert het fenotype wat zich voordoet in muizen met tumoren als diffuse intravasale stolling (DIC; disseminated intravascular coagulation), wat is gedefinieerd door bijvoorbeeld lage antitrombine niveaus, fibrine producten, consumptie van bloedplaatjes, veneuze trombose en/of bloedingen. DIC komt voor in 5% van alle borstkanker gevallen en is de meest extreme vorm van hyperstolling. Aangezien deze extreme vorm werd waar genomen, zou het van groot belang zijn om het MDA-MB-231 borstkanker model te combineren met het klassieke IVC stenose model. In muis alvleesklierkanker modellen werden er grote bloedstolsels ontdekt in muizen met tumoren vergeleken met muizen zonder tumoren. Daarnaast was het ontstaan van een trombus afhankelijk van TF^+ MVs. Echter, wij vonden in ons borstkankermodel geen aanwijzingen voor een TF-afhankelijke hyper-coagulante fenotype, die het bestuderen van CAT in een TF^+ MVs onafhankelijke manier rechtvaardigt.

Een andere aanpak om betrokkenheid van belangrijke 'sleutelfactoren' en biologische signaleringsroutes te bestuderen in CAT is het bestuderen van tumor gen expressie en deze expressie profielen te koppelen aan VT. Het onderzoek beschreven in **hoofdstuk 6** is de allereerste studie die aantoont dat tumoren van patiënten met zowel darmkanker (CRC; colorectal cancer) en VT een ander gen expressie profiel vertoont dan in tumoren van patiënten die alleen darmkanker hebben. Een inflammatoire staat werd aangetoond in tu-

moren van patiënten met CAT, en verhoogde niveaus van fibrine afzettingen waren aanwezig in tumoren van patiënten met VT. Verhoogde fibrine producten kunnen dienen als een matrix voor tumor-geassocieerde bloedvatvorming, of kunnen uitzaaiing bevorderen, hetgeen allemaal kan bijdragen aan verminderde overleving van patiënten met CAT. Wanneer patiënten met VT werden onderverdeeld in de 2 groepen: VT vòòr (max 1 jaar), en VT rondom (max 3 maanden) kanker diagnose, werden verschillende expressie profielen gevonden. Dit zou kunnen komen door kanker-gerelateerde behandelingen, omdat signaleringsroutes die gerelateerd zijn aan metabolisme verhoogd waren in de 'VT rondom diagnose' groep.

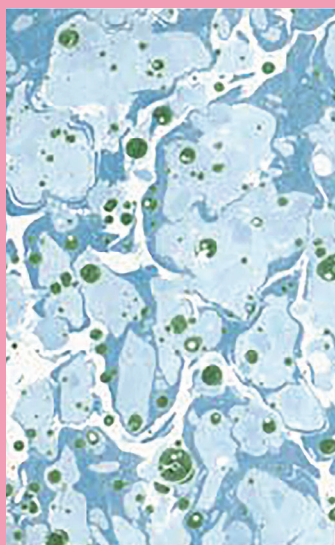
Om het pathofysiologische mechanisme van CAT in zijn geheel te kunnen begrijpen, ongeacht de kankerbehandeling, zijn de top 3 genen die differentieel tot expressie kwamen in CRC patiënten met VT voorafgaand aan kankerdiagnose erg interessant voor verder onderzoek, te meer omdat er verbanden gelegd kunnen worden met factoren die gerelateerd zijn aan de stolling. Reg4 is een voorbeeld van een gen dat hoger tot expressie kwam in de VT vòòr diagnosegroep; dit eiwit kan in afwezigheid van calcium binden aan heparine, een van nature in het lichaam voorkomend stolling remmend molecuul. Spink4 is een Kazal-type serine protease inhibitor en zou in staat kunnen zijn om stollingsfactoren te remmen. Ten slotte is co-expressie van Reg4 en spink4 strikt gereguleerd in inflammatoire darmaandoeningen, wat suggereert dat een (chronische) pro-inflammatoire staat verder kan bijdragen aan VT, wellicht via verhoogde expressie van TF. Eén op de vijf mensen met een inflammatoire darmaandoening wordt gediagnosticeerd met kanker. Tevens hebben zij een 2-3 maal verhoogde kans op het krijgen van VT. Daarnaast is verhoogde expressie van Reg4 geassocieerd met tumorprogressie, uitzaaiing en een verlaagde kans op overleving. Een wat directer verband met stollingsregulatie is er bij het eiwit ***SERPINA1***, dat codeert voor het eiwit a1-antitrypsine(A1AT). A1AT kan direct en indirect trombus formatie stimuleren door verschillende remmers van het stollingssysteem, zoals geactiveerd eiwit C te remmen. Complexvorming van A1AT met elastase zou systemisch neutrofiel activatie tot gevolg kunnen hebben en VT kunnen veroorzaken via Neutrophyl Extracellulair Traps. Bovendien is de aanwezigheid van A1AT in fibrine-rijke bloedstolsels aangetoond, wat een directe rol voor dit eiwit in VT suggereert. Met betrekking tot kanker is A1AT overexpressie gevonden in verscheidene tumoren zoals hersen-, darm- en maagkanker. In recente studies werden ook patiënten met verhoogd risico op VT geïncludeerd, alhoewel objectief vastgestelde VT in deze patiënten geen primair eindpunt was. Daardoor is het nog onduidelijk of er directe associaties zijn tussen A1AT plasmaniveaus en VT.

Helaas hebben de meeste studies die geprobeerd hebben biomarkers voor CAT te vinden zich met name gericht op stollingsfactoren, met een nadruk op TF in kanker-geassocieerde trombose. Een nog belangrijkere conclusie is dat wetenschappers en artsen, wellicht onterecht, geprobeerd hebben om klassieke risicofactoren voor VT te extrapoleren naar CAT, wat uiteindelijk heeft geresulteerd in weinig succes in het vinden van biomarkers die CAT voorspellen. Verschillende voorspel- en risicomodellen zijn ontwikkeld om te kunnen voorspellen welke kankerpatiënten een verhoogd risico hebben op VT, helaas, met een lage nauwkeurigheid. In **hoofdstuk 7** pleiten wij om te stoppen met het gebruiken van klassieke risicofactoren als voorspellers van CAT, met name, omdat studies met behulp van plasma proteomics, laat zien dat eiwitprofielen in plasma van patiënten met CAT, verschillen van de profielen in plasma van patiënten met alleen kanker of VT. Ten slotte hebben wij de verbanden tussen mutaties en verhoogd risico op CAT besproken. Omdat het een zeer 'biased' en tijdrovende benadering is om alle genen te onderzoeken met betrekking tot CAT, stellen wij daarom een objectieve aanpak voor om moleculaire profielen in patiënten met CAT te bepalen, zoals beschreven in **hoofdstuk 6**.

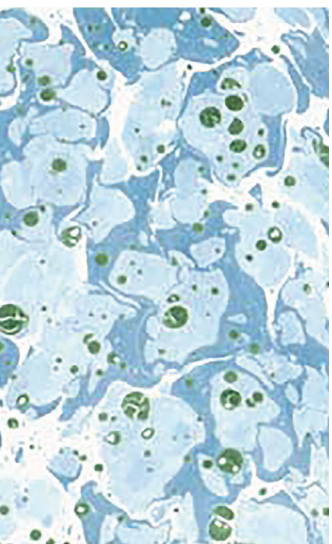
Conclusies en toekomstperspectieven

Dit proefschrift beschrijft i) de functie van asTF in hemostase, ii) de bijdrage van stollingsfactoren aan tumorprogressie, en iii) het verbreedt ons standpunt met betrekking tot kanker-geassocieerde trombose. Remming van TF signaaltransductie met het antilichaam Mab-10H10 zorgt voor verminderde EMT- en CSC-geassocieerde transcriptie programma's, tumor-initiërende capaciteit en metastase in triple negatieve borstkanker (TNBC; triple negative breast cancer) cellijn. Omdat dit een tumor variant is die zeer lastig is te behandelen en het een hoge kans op recidief heeft, zou het interessant zijn om TF signalering als therapeutisch aangrijpingspunt te nemen. Tweeledige behandeling van TNBC met conventionele chemotherapie en Mab-10H10 zou kunnen resulteren in een betere behandelingsstrategie, omdat dit zowel gericht is tegen de kankerstamcellen en de zeer proliferatieve dochtercellen.

Daarnaast laten wij een 'proof-of-principle' onderzoek zien waarin wordt beschreven hoe er 'unbiased' gezocht kan worden naar nieuwe biomarkers in CAT patiënten. Uitbreiding van deze studie door deze biomarkers te valideren in andere patiëntcohorten en andere tumor typen zal zorgen voor nieuwe inzichten in het mechanisme dat ten grondslag ligt aan kanker-geassocieerde trombose. Uiteindelijk zal dit leiden tot een beter voorspellingsmodel om de patiënten met een verhoogd risico op VT te selecteren en diegenen die wellicht profijt kunnen hebben van profylactische antistolling te behandelen.



Publications
Dankwoord
Curriculum Vitae



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CURRICULUM VITAE

Betül Ünlü was born on the 20th of January 1986 in Zaanstad, the Netherlands. In 2006 she finished secondary education at the Bertrand Russell College in Krommenie. Subsequently she started a Bachelor in Chemistry at the VU University Amsterdam. During her Bachelor-internship she has studied the toxicity of several nonsteroidal anti-inflammatory drugs, using yeast as a model, under the supervision of dr. Jolanda van Leeuwen and dr. Chris Vos. This project led to her first publication as a co-author in the journal ***Toxicology in vitro***.

Between 2010 and 2013 Betül pursued a Master in Biomolecular Sciences at the VU University Amsterdam, specifically the tracks Biological Chemistry & Molecular Cell Biology. Her first internship, in the group of prof. dr. Martine J. Smit, was on the elucidation of the CXCR4 and CXCR7 signaling axis in cancer, under the supervision of dr. Azra Mujić-Delić. In 2012 she started with an external internship in the laboratory of prof. dr. Henri H. Versteeg at the Leiden University Medical Center, on the role of Tissue Factor and ectopic coagulation factor VIIa in breast cancer, with dr. Chris Tieken as a supervisor.

After this second internship she did not leave the group, but became a group member of prof. dr. Henri H. Versteeg in the Tissue Factor-team and worked as a PhD-student in the department of Internal Medicine, division of Thrombosis and Hemostasis. During this PhD program, she has spent several months in the laboratory of prof. dr. Wolfram Ruf at the Scripps Research Institute (La Jolla, California, USA), in order to learn new techniques and work with specific mouse strains. In addition, Betül has received grants from the Dutch Cancer Society and 'Stichting de Drie Lichten' to support her research in the USA. Furthermore, work described in this thesis has been presented at several national and international congresses, where she also was awarded with travel grants from 'Stichting Stimulering Biochemie The Netherlands', a Young Investigator Award by the International Society on Thrombosis and Haemostasis, best abstract and best oral presentation award by the Dutch Society on Thrombosis and Hemostasis.

Since the 1st of July 2018, Betül works as a post doc in the research group of prof. dr. Jolanda van Leeuwen at the University of Lausanne in Switzerland. Here, she will map molecular mechanisms of genetic tumor suppressor interactions in cancer cells. This research will increase the understanding of the complex networks of genetic interactions underlying tumor progression.