Contact System Activation and Cancer: New Insights in the Pathophysiology of Cancer-Associated Thrombosis

E. Campello1  M.W. Henderson2  D.F. Noubouossie2  P. Simioni1  N.S. Key2

1 Thrombotic and Hemorrhagic Disease Unit, Department of Medicine, University of Padova, Padova, Italy
2 Division of Hematology/Oncology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States

Abstract

Cancer induces a systemic hypercoagulable state that elevates the baseline thrombotic risk of affected patients. This hypercoagulable state reflects a complex interplay between cancer cells and host cells and the coagulation system as part of the host response to cancer. Although the tissue factor (TF)/factor VIIa pathway is proposed to be the principal initiator of fibrin formation in cancer patients, clinical studies have not shown a consistent relationship between circulating TF levels (often measured as plasma microvesicle-associated TF) and the risk of thrombosis. A renewed interest in the role of the contact pathway in thrombosis has evolved over the past decade, raising the question of its role in the pathogenesis of thrombotic complications in cancer. Recent observations have documented the presence of activation of the contact system in gastrointestinal, lung, breast and prostate cancers. Although the assays used to measure contact activation differ, and despite the absence of standardization of methodologies, it is clear that both the intrinsic and extrinsic pathways may be activated in cancer. This review will focus on recent findings concerning the role of activation of the contact system in cancer-associated hypercoagulability and thrombosis. An improved understanding of the pathophysiology of these mechanisms may lead to personalized antithrombotic protocols with improved efficacy and safety compared with currently available therapies.

Keywords
► contact activation
► cancer
► hypercoagulability
► intrinsic pathway
► thrombosis

Introduction

Patients with cancer are at increased risk to develop venous thromboembolism (VTE), an association that is commonly known as Trousseau’s syndrome.1,2 The clinical manifestations of cancer-associated VTE include deep vein thrombosis and pulmonary embolism, as well as visceral or splanchic vein thrombosis.3 Indeed, cancer is one of the best-established risk factors for VTE.4,5 It has been estimated that ~20% of all first VTE events are associated with cancer.5 According to a recent U.K. cohort study that included 6,592 cancer-associated VTEs, the incidence rate of first VTE in patients with active cancer was 5.8 (95% confidence interval [CI], 5.7–6.0) per 100 person-years and the overall incidence rate for recurrence was 9.6 (95% CI, 8.8–10.4) per 100 person-years.6 Finally, cancer-associated thrombosis is linked with a worse prognosis, and thromboembolism is the second leading cause of death in cancer.5

Several risk factors for VTE usually coexist in cancer patients. These may include certain comorbidities, surgery,
immobility, tumour histology and stage, the presence of indwelling central venous catheters and chemotherapy and/or some molecular targeted therapies. These risk factors, which may be classified as patient-, tumour- or treatment-related, may additively exceed the threshold for clinically overt thrombosis (Fig. 1).

Tissue factor (TF) is the physiologic activator of coagulation in vivo. We and others have reported elevated levels of TF in the circulation of animal models and in patients with cancer. However, although circulating levels of TF correlate with mortality, it does not always correlate with markers of systemic hypercoagulability, or the occurrence of thrombosis. This suggests that other pathways modulate thrombogenesis in cancer. This review will focus on the hypercoagulable state in cancer with particular reference to recent findings concerning the potential contribution of the contact system (CS) of coagulation.

The Contact System

FXII-Dependent Contact Activation

The CS refers to a proteolytic pathway consisting of the zymogens factor XII (FXII) and prekallikrein (PK), and the non-enzymatic cofactor, high molecular weight kininogen (HK). Some definitions also include zymogen FXI. All component proteins are synthesized and secreted by the liver. Contact of plasma with negatively charged surfaces induces a conformational change in zymogen FXII resulting in a small amount of auto-activated FXII (FXIIa; α-FXIIa), which, in turn, cleaves PK to generate kallikrein (KAL). The conformational change in FXII, together with reciprocal activation of FXII by formed KAL and HK, leads to further formation of FXIIa. The activation of FXII and PK generates a potent activation feedback loop that overcomes inactivation of these enzymes by the principal CS inhibitor, the serpin C1 esterase inhibitor (C1INH).

The end result of FXIIa generation may be activated FXI (FXIa) and/or vasoactive and pro-inflammatory kinins, such as bradykinin (BK). FXIa initiates a series of Ca2+-dependent proteolytic events that lead to thrombin generation, and production of a fibrin clot. Following the initial generation of thrombin (by whatever mechanism), a powerful amplification mechanism accelerates thrombin formation in a FXI-dependent manner. Additionally, α-FXIIa can be further cleaved by KAL to generate β-FXIIa, which retains the ability to activate PK, but not FXI or FXII, and is able to dissociate from the surface (contact phase). C1INH targets both FXIIa and KAL, accounting for ~93% of plasma FXIIa or β-FXIIa inhibition. Furthermore, antithrombin, α2-macroglobulin and α2-antiplasmin also inhibit FXIIa to a lesser extent (Fig. 2).

Apart from glass surfaces, many other negatively charged surfaces or polyanionic molecules, including silica, kaolin, ellagic acid and sulphated polysaccharides, can accelerate contact activation. Certain glycosaminoglycans (GAGs), such as dermatan sulphate, chondroitin sulphate-E and heparin can also initiate contact activation in vitro or in vivo. However, over the past decade, intensive research efforts have focused on endogenous ‘natural activators’ of the CS. Several damage-associated molecular entities have been suggested to directly drive CS activation during vascular injury and infection including extracellular nucleic acids, misfolded aggregated proteins, mast cell heparin and pathogen-related molecules, such as endotoxin. Additionally, inorganic polyphosphate (polyP), a linear polymer of phosphates that is present in many infectious microorganisms and is also secreted by mast cells and platelets, has received the most attention. PolyP accelerates blood clotting and slows fibrinolysis, in a manner that is highly dependent on polymer length. Very long-chain polyP (found in many bacteria) is an especially potent trigger of the contact pathway, but aggregated shorter chain polyP in the form of Ca2+-dependent nanoparticles may also activate FXII.

Recently, it was demonstrated that membrane-associated platelet polyphosphate condensed into insoluble spherical nanoparticles on the surface of activated platelets potently activates factor XII. Finally, extravascular matrix proteins such as laminin and collagen are capable of assembling and activating the CS to drive coagulation under flow conditions.

The FXII-Independent Activation Pathway

An alternate activation mechanism of the CS, independent of FXII, but involving Zn2+ and HK-dependent PK activation on endothelial cells, has been described. It has been observed that
Measuring Activation of the Contact System in Plasma

Investigation of CS activity can be broadly defined as methods that address the potential for exogenous activation, and those that focus on analysis of in vivo activation. Tools such as clotting assays or substrate cleavage can be used to assess a patient’s capacity for CS activation using known initiators, as well as discovery of novel putative physiological activators. Evidence for in vivo activation is predominantly assayed via enzyme-linked immunosorbent assay (ELISA), though Western blot and mass spectrometry have been specialized use. The most common criticisms applied to these assays are the short half-life of the complexes and the potential for ex vivo activation during collection. Minimization of pre-analytical activation through use of benzamidines or specific CS inhibitors with blood collection is critical and should be considered in study design, since FXII may be rapidly activated ex vivo by contact with blood drawing equipment.

To our knowledge, there are currently no clinically standardized assays to quantify in vivo activation of the CS, presenting an unmet need in cancer-associated thrombosis research.

Clotting Assays

The activated partial thromboplastin time assay (aPTT) uses an anionic surface to activate FXII following the addition of phospholipid and calcium. Shortened aPTT times (likely driven primarily by elevated FVIII levels) have been linked to an elevated risk of thrombosis, although not specifically in cohorts with cancer. One-stage clotting assays based on aPTT using deficient plasma may be used to detect FXI and XII activity. A reduction in plasma FXII activity has been regarded as a marker of consumption and, thus, indirect evidence of activation of the CS. However, given the global nature of the aPTT-based tests, more specific assays are required to probe the role of the contact pathway in disease states, including cancer-associated thrombosis.

Substrate Cleavage/Chromogenic Assays

Factors XIIa, XIa and KAL are serine proteases and their enzymatic function can be assessed by cleavage of synthetic peptides to generate a chromogenic or fluorogenic signal. The most commonly used substrate is S-2302 (H-D-Pro-Phe-Arg-pNA), which is sensitive to cleavage by FXIIa and KAL (with a nearly identical Km and Kcat), and to a much lesser extent, FXIa. This substrate mimics the last three amino acids in the BK sequence of kininogen, and may therefore be used to address the role of FXIIa and KAL in liberation of BK from HK. These substrates may be used to assay the activation of potential initiators of the CS in plasma, as demonstrated in a recent report describing prostatic tumour cell-derived exosomes (prostasomes). However, detection of in vivo active enzymes using substrates is not practical nor quantifiable. Furthermore, the cross-reactivity of currently available substrates is problematic; although several more specific substrates for CS enzymes have been developed, the use of specific enzyme inhibitors to minimize the possibility of cross-reactivity should be considered. For example, corn
trypsin inhibitor (CTI) or soybean trypsin inhibitor (STI) may be added to sodium citrate before blood collection to inhibit FXIIa or KAL, respectively. \(^{45,46}\) Similarly, ethylenediaminetetraacetic acid (EDTA) will chelate zinc, \(^{47}\) a required cation in the contact pathway, \(^{48}\) and thus collection of samples in citrate is preferred.

**ELISA**

One of the earliest reports identifying the role of the CS in cancer used capture ELISA to quantify CS proteins in a cohort of gastrointestinal cancer patients. \(^{49}\) Depending on the targeted epitopes, these assays may not be able to distinguish between zymogen, enzyme or inhibited protein. However, combining this approach with evidence of reduced zymogen activity indicates prior CS activation. ELISAs able to distinguish activation states offer a greater benefit when analysing clinical samples. Originally developed as radioimmunoassays, \(^{50}\) these assays have been modified to ELISA formats that detect CS proteins bound to their physiologic inhibitors including C1-inhibitor esterase (C1INH), \(\alpha_1\)-antitrypsin, antithrombin, \(\alpha_2\)-antiplasmin and \(\alpha_2\)-macroglobulin (\(\rightarrow\) Fig. 2). ELISAs for enzyme-inhibitor complexes have been used in various disease states, although not in the setting of cancer-associated thrombosis, to our knowledge (\(\rightarrow\) Fig. 2). \(^{51}\) Certain reports concluded that circulating complexes do not correlate with disease activity, because of their short half-life. \(^{52}\) However, the half-lives of these complexes are estimated in the range of 30 to 50 minutes, \(^{53}\) which can still detect chronic CS activation. By analogy, thrombin-antithrombin (TAT) complexes have a half-life of \(\approx 15\) minutes in plasma, yet are an accepted biomarker of ongoing coagulation activation in vivo. \(^{54}\) Indeed, investigations in patients with endotoxaemia, myocardial infarction and amyloidosis have revealed measurable changes in contact activation using CS enzyme-inhibitor complexes. \(^{39,51,55,56}\) More recently, heavy chain-only nanobodies specific for FXIIa have been used to distinguish not only between the activation states of FXII, but also between the \(\alpha\) and \(\beta\) isoforms. \(^{57}\) Finally, HK can be cleaved by either FXIIa or KAL to release HK fragments. An ELISA has been developed to detect cleaved HK in plasma as a marker of CS activation (via KAL) due to its longer half-life (\(\approx 9\) hours), \(^{58}\) but has yet to be applied to cancer-associated thrombosis research.

**Western Blot**

An alternative approach for detecting activation of the CS activation in plasma is Western blotting. Here, detection of enzyme-inhibitor complexes or cleavage of HK (an indirect measurement of BK release) provide a semi-quantitative measurement of CS activation. \(^{59}\) Western blotting can offer advantages such as the speciation of FXIIa (\(\alpha\) or \(\beta\)) when analysed under reducing conditions, or complexes with inhibitors under non-reducing conditions, without using specific monoclonal antibodies or the aforementioned nanobodies. \(^{60}\) Detection of heavy chain-only HK by Western blotting has shown sensitivity to concentrations as low as 5 ng/mL, \(^{61}\) with discernible differences in the plasma of rodent models of BK-mediated diseases. \(^{62}\) However, due to the low throughput of Western blotting, clinical samples are not typically analysed using this approach.

**Mass Spectrometry**

The major inflammatory effector of the CS is BK, a 9 amino acid peptide generated from the cleavage of HK by KAL. Detection of BK versus FXIa (or FXIa-inhibitor complex) provides a potentially valuable measurement of the inflammatory versus procoagulant endpoints of CS activation. Unfortunately, BK has a circulating half-life \(< 20\) seconds \(^{63}\) due to its rapid metabolism by angiotensin-converting enzyme I (ACE) in the lungs, which greatly limits its detection when it is generated in vivo. However, mass spectrometry may be used to quantify the major stable metabolite, BK 1–5. \(^{64}\) Given the role of BK in tumour progression, \(^{65,66}\) and evidence for thrombotic protection in mice deficient in the BK2 receptor, \(^{64}\) this underutilized approach should be considered in the evaluation of clinical samples.

**Clinical Evidence of Contact System Activation in Cancer**

As already mentioned, early evidence of CS activation in cancer was presented in 1990. \(^{49}\) CS activation was evaluated in 69 patients with gastrointestinal cancer (12 with gastric, 15 with pancreatic and 42 with colon cancer), 33 of who had liver metastases, and in 118 healthy controls recruited from blood donors (\(\rightarrow\) Table 1). Antigen levels of FXII, PK, HK and C1INH were measured by immunohemochromic assays; activity levels of PK and C1INH were measured by chromogenic assays. Values of FXII, PK, HK and C1INH were expressed as a percentage of human standard plasma pool values. FXII, PK and HK antigens were decreased in patients with gastrointestinal cancer (\(84 \pm 28\%\), \(74 \pm 19\%\) and \(86 \pm 14\%\), respectively) compared with the control group (\(94 \pm 27\%\), \(88 \pm 18\%\) and \(98 \pm 14\%\), respectively), but only PK and HK values were statistically different (\(p < 0.05\) for both). PK activity was significantly decreased compared with controls (\(74 \pm 21\%\) vs. \(101 \pm 17\%, p < 0.05\)). C1INH antigen and activity were significantly increased in cancer compared with controls (\(p < 0.05\) for both comparisons). Interestingly, in the subgroup of patients with metastatic colon cancer, FXII, PK and HK levels were significantly decreased (\(78 \pm 18\%\), \(75 \pm 13\%\) and \(77 \pm 9\%\), respectively) compared with controls (all \(p < 0.05\)). C1INH was significantly increased both in patients with and without metastases compared with controls in both the immunological and functional assays (\(p < 0.05\)). The authors concluded that patients with intestinal cancer manifest reduced contact factor levels with markedly elevated inhibitor levels. Battistelli et al measured plasma activities of fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI and FXII in 73 patients with non-metastatic colorectal cancer (48 colon and 25 rectum) and in 67 matched controls. \(^{43}\) They showed that the mean plasma activity of fibrinogen (\(400 \pm 113\) mg/dL), FVIII (\(145 \pm 50\%\)), FIX (\(127 \pm 29\%\)) and FV (\(131 \pm 65\%\)) were significantly higher in colorectal cancer patients than in control subjects (\(287 \pm 74\) mg/dL, \(92 \pm 35\%\), \(109 \pm 22\%\) and \(108 \pm 35\%\), respectively), while FVII (\(102 \pm 25\%\) vs. \(118 \pm 34\%, p = 0.004\)) and FXII (\(96 \pm 26\%\) vs.
113 ± 3%, \( p = 0.003 \) levels were significantly decreased. Interestingly, FVII was highly correlated with FXII (\( p < 0.01 \)) only in cancer patients. The authors concluded that decreased FVII and FXII activity may be indices of intravascular coagulation activation in colorectal cancer. More recently, Pan et al.\(^6\) measured CS activation in 10 lung cancer patient plasmas compared with normal pooled plasma. PK, HK and C1INH were quantified by Western blot. The authors concluded that CS activation was present, as evidenced by cleaved C1INH and C1INH/protease complexes in all 10 patients, with absent HK in 9 patients, decreased amounts of PK in 6 and KAL/protease complex in 5. Moreover, the authors confirmed the same CS activation pattern in 11 colon, 9 breast, 3 pancreatic and 1 renal cancer patients. In particular, all of the pancreatic cancer patient samples showed an absence of detectable HK, whereas only one of the colon cancer plasmas had undetectable HK, and this occurred in a patient with stage IV disease (\( \text{Table 1} \)).

### Mechanisms of Contact System Activation in Cancer

Although several observations documenting activation of the CS in different cancers have been published, the responsible mechanism(s) is poorly understood. As previously mentioned, the variety of assays that have been used makes it difficult to compare results. \( \text{Table 2} \) summarizes the reported mechanisms of CS activation in cancer.

### Microvesicles

Microvesicles (MVs) are submicron, lipid bilayer membrane particles, shed by various cells upon activation or apoptosis. Our group and others have described elevated levels of circulating MVs, including exosomes (MVs of 50–100 nm in diameter) derived from tumour and host blood cells types in cancer patients.\(^68\)–\(^71\) Cancer chemotherapy can potentially induce MV release from tumour, blood or endothelial cells.\(^72\) Red cell and platelet transfusions administered to cancer patients are additional sources of MVs. MVs and exosomes may play a major role in tumourigenesis, tumour progression, metastasis and cancer-associated thrombosis.\(^68\)–\(^71,73,74\) As already mentioned, the prothrombotic role of MVs has been mainly ascribed to TF-bearing MVs.\(^69,70,73,75,76\) However, in a cohort of women with breast cancer, circulating annexin-V positive MVs were elevated and correlated with the stage of the tumour. When analysed according to cellular origin, platelet-derived MVs represented the vast majority (> 80%); their level correlated with plasma levels of prothrombin fragment 1.2, suggesting a role in systemic hypercoagulability.\(^3\) In this study, TF-MV levels were very low and not different from that of women with benign breast

### Table 1: Studies of contact system activation in patients with cancer

<table>
<thead>
<tr>
<th>Patients</th>
<th>Patients/controls</th>
<th>Cancer subtype</th>
<th>Analytes</th>
<th>Main results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>69 gastrointestinal cancer (33 with liver metastasis)</td>
<td>69/118</td>
<td>12 gastric, 15 pancreatic, 42 colon</td>
<td>FXII, PK, HK, C1INH immunological and functional assays</td>
<td>1. FXII, PK and HK activity decreased 2. C1INH increased 3. CS activation more pronounced in patients with liver metastasis</td>
<td>49</td>
</tr>
<tr>
<td>73 colorectal cancer without metastasis</td>
<td>73/67</td>
<td>48 colon, 25 rectum</td>
<td>Fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII activity</td>
<td>1. FVII and FXII activity decreased 2. FVII highly correlated with FXII</td>
<td>43</td>
</tr>
<tr>
<td>34 cancer (11 advanced disease)</td>
<td>34/NPP</td>
<td>10 lung, 11 colon, 9 breast, 3 pancreatic, 1 renal</td>
<td>PK, KAL/protease complexes, HK, C1INH and C1INH/protease complexes by Western analysis</td>
<td>1. Cleaved C1INH and C1INH/protease complexes, absence of HK, decreased amounts of PK, and KAL/protease complexes (primarily in lung cancer patients) 2. Thrombin generation correlated with KAL/protease complexes</td>
<td>67</td>
</tr>
<tr>
<td>20 prostate cancer</td>
<td>20/20</td>
<td>20 prostate</td>
<td>FXIIIa activity, thrombin generation</td>
<td>Humanized antibody 3F7 inhibited FXIIIa activity and reduced thrombin generation in normal plasma samples spiked with prostates from pancreatic cancer patients</td>
<td>44</td>
</tr>
</tbody>
</table>

Abbreviations: C1INH, C1esterase inhibitor; FXII, factor XII; HK, high molecular weight kininogen; KAL, kallikrein; NPP, normal pool plasma; PK, prekallikrein; TF, tissue factor.
tumours, although the correlation with prothrombin fragment was seen only in patients with metastatic cancer. Red blood cell and platelet-derived MVs have previously been shown to activate coagulation through the contact pathway in the context of blood product storage and human endotox-aemia, although here again, the precise molecular trigger for this event remains to be determined.

In addition to blood cell-derived MVs, certain tumour-derived exosomes have also been shown to interact with the CS. Exosomes spontaneously released or induced by treatment with doxorubicin triggered thrombin generation even in the presence of inhibitory TF antibodies.

In a mouse model of breast cancer, tumour-derived exosomes cooperated with neutrophils primed by tumour-derived G-CSF to generate NETs and enhance thrombosis (further discussed below). Additionally, exosomes secreted by prostate cancer cells (prostasomes) trigger thrombin generation in vitro in a dose-dependent manner, and induce lethal pulmonary embolism in mice. Moreover, the addition of a recombinant FXIIa inhibitor significantly reduced peak and total thrombin generated by prostasomes, and the combined application of FXIIa and TF inhibitors completely blunted thrombin generation. Polyphosphates were found on the surface of prostasomes. Treatment of prostasomes with specific inhibitors of polyphosphates or with polyphosphate degrading enzymes abrogated prostasome-induced FXIIa generation in vitro and protected mice from prostasome-induced lethal pulmonary embolism. However, since similar effects were also observed following inhibition of TF, both the intrinsic and extrinsic pathways seemed to contribute to thrombosis in this model. Further data are required in a diverse range of cancers to understand the role that circulating exosomes/MVs may play in CS activation.

### Cell-Free DNA, Histones and Neutrophil Extracellular Traps

Elevated circulating levels of cell-free DNA (cfDNA) and histones, the major molecular components in nucleosomes/chromatin, are found in cancer patients. They are associated with adverse outcomes and are positively correlated with markers of in vivo coagulation activation such as plasma TAT and D-dimer levels. The mechanisms of extracellular release and the cellular origin of cfDNA and

### Table 2 Major proposed mechanisms of contact activation in cancer

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Mechanism suggested</th>
<th>Ref</th>
</tr>
</thead>
</table>
| Gastrointestinal with or without liver metastasis; no VTE | 1. Proteolytic enzymes such as plasmin, collagenase and cathepsin  
2. Kallikrein produced by cancer cells  
3. Imbalance of CS linked with metastatic process | 49   |
| Colorectal without metastasis; no VTE   | Intravascular coagulation activation mediated by both extrinsic and intrinsic pathways                                          | 43   |
| Miscellaneous                           | 1. High levels of MVs  
2. Prothrombotic role mainly ascribed to TF-bearing MVs  
3. Red blood cell and platelet-derived MVs have been shown to activate CS                                                   | 69,70,75,76 |
| Melanoma cell line                      | Exosomes spontaneously released or induced by treatment with doxorubicin triggered thrombin generation even in the presence of inhibitory TF antibodies | 80   |
| Prostate cancer                         | 1. Prostasomes secreted by prostate cancer cells activated intrinsic pathway via PolyP  
2. Blocking FXIIa reduced the prothrombotic potential of prostasomes                                                      | 44   |
| Acute myeloid leukaemia                 | Increased levels of cell-free plasma DNA indicative of contribution of the contact pathway to systemic coagulation activation in the total patient cohort and in patients with lower TF procoagulant activity | 85   |
| Breast cancer                           | Cell-free DNA released from epirubicin-treated whole blood significantly elevated thrombin generation in a dose-dependent manner via activation of the contact pathway | 95   |
| Miscellaneous                           | 1. Increased plasma H3Cit in active cancer with stroke compared with stroke without cancer  
2. H3Cit positively correlated with plasma TAT                                                                               | 102  |
| Miscellaneous                           | 1. High GAGs (glucosamine and galactosamine) levels expressed by tumoural tissue (mainly lung) or by endothelial cells may activate CS  
2. Carcinoma mucins may induce CS activation                                                                                | 67   |

Abbreviations: CS, contact system; F, factor; GAGs, glycosaminoglycans; H3Cit, citrullinated histone H3; MVs, microvesicles; PolyP, polyphosphate; TAT, thrombin-antithrombin complexes; TF, tissue factor; VTE, venous thromboembolism.
histones are unclear, and probably vary according to tumour type, patient co-morbidities and chemotherapy. In cancer patients, cfDNA may be either tumour-derived or released from apoptotic or necrotic non-tumoural tissues, such as neutrophils. Cancer chemotherapy is associated with increased plasma levels of cfDNA. Multiple groups, including our own, have reported FXII-dependent procoagulant activity of purified DNA in vitro. Specifically, Swystun et al showed that cfDNA purified from epirubicin-treated whole blood ex vivo significantly elevated thrombin generation in a dose-dependent manner by a mechanism involving activation of the contact pathway. Purified histones promote platelet activation and aggregation, and trigger thrombin generation in a platelet-dependent manner. They also induce phosphatidylserine expression on red blood cells and impair thrombomodulin-dependent protein C activation, leading to enhanced thrombin generation in plasma ex vivo. Noteworthily, it is unclear if DNA and histones circulate in their free forms. It is also unknown if DNA or histones bound to other blood components affect their respective procoagulant activities observed in vitro.

Cancer cells secrete various types of cytokines that modify neutrophil biology, leading to changes in neutrophil counts and state of activation, including the release of neutrophil extracellular traps (NETs). Animal models have suggested that NETosis plays a major role in cancer-associated thrombosis. In a mouse model of mammary carcinoma, tumour-bearing mice developed a leukaemoid reaction and spontaneous delayed onset thrombosis within the lungs. Interestingly, the percentage of circulating hypercitrullinated neutrophils increased at day 21 post-tumour injection and decreased at the time that thrombosis occurred. Furthermore, the disappearance of hypercitrullinated neutrophils from the circulation coincided with the appearance of hypercitrullinated histone H3 (H3Cit) in the plasma of these mice. Hypercitrullination of histones, which is believed to be mediated by neutrophil PAD4 enzyme, has been proposed to be a specific marker of NETosis. Hence, the observations in this model were thought to indicate a major role of tumour-induced NETs in cancer-associated thrombosis. In another study, orthotopic injection of the same cancer cell line into mice led to rapid development of metastases. Tumour-bearing mice exhibited a reduced time to jugular vein occlusion during venous thrombosis induced by Rose Bengal/laser photocoagential injury compared with control mice, as well as reduction in time to arterial occlusion triggered in the carotid artery by ferric chloride injury. Pre-treatment of mice with DNAse 1 abolished the differences between tumour-bearing and control mice during both venous and arterial thrombosis challenges, further suggesting a role of NETs in these models of cancer-associated thrombosis. In humans, Thålin et al reported a significantly higher level of plasma H3Cit in patients with active cancer and stroke than in patients with stroke without active cancer. In this study, plasma level of H3Cit positively correlated with plasma TAT, supporting a possible link between NETosis and cancer-associated thrombosis. However, the mechanisms by which NETs promote thrombosis are not fully understood and are likely multifactorial. NETs released intravascularly adhere to the vessel wall, where they resist flow and trap suspended and soluble components of the blood, including those of the clotting system. In this way, NETs facilitate interactions between coagulation components, enhance thrombin generation and increase thrombus size. These prothrombotic properties can be abrogated by preventing NET formation or by dismantling the NET scaffold, using DNase 1, for example. However, whether the intact NET macromolecular structure directly activates coagulation is controversial. In cancer, MVs, activated leukocytes and/or cancer cells themselves can provide circulating TF, which are trapped on NETs. Neutrophil elastase, an enzyme abundant in neutrophil cytoplasmic granules and in extruded NETs, has been shown to enhance thrombosis by inhibiting TF pathway inhibitor. Deficiency in FXII does not confer protection from thrombus formation in NET-dependent animal models of thrombosis. Several studies have reported CS activation using thrombin generation in platelet-poor plasma containing NETs generated ex vivo. However, in the presence of intact NETs, we did not observe any thrombin generation in platelet-free plasma, or in a purified CS reconstituted in buffer.

Activated Platelets
There is an extensive literature on the multiple roles of activated platelets in cancer-associated thrombosis. Activation of the CS by activated platelets has been observed since the early 1980s. Activated platelets can expose membrane-bound divalent ion-complexed polyphosphate nanoparticles, which resist circulating polyphosphatases and trigger FXII activation. Released platelet polyphosphates have also been reported to activate the CS in vitro and in vivo. A recent study by Riedl et al demonstrated platelet activation mediated by expression of podoplanin by brain tumours, likely through CLEC-2 signalling. However, whether tumour-podoplanin-CLEC-2-mediated platelet axis promotes thrombosis through CS activation has not been evaluated.

Glycosaminoglycans
At a molecular level, certain GAG species have been shown to activate FXII and the KAL-kinin pathway, with adverse clinical outcomes; the most notable example was when heparin batches contaminated by hypersulfated chondroitin sulphate moieties were inadvertently administered to patients. Whether tumour cells can similarly produce atypical GAGs that activate the CS is unclear. Pan et al measured levels of glucosamine- and galactosamine-containing glycans as a putative mechanism for CS activation in cancer patients. Galactosamine levels were increased in lung cancer but not in breast or pancreatic cancer patients compared with controls; on the other hand, glucosamine levels were increased in both lung and breast cancer patients compared with controls, although this was not observed in pancreatic cancer (~Table 2).
Catheter-Related Thrombosis

The use of venous catheters to facilitate chemotherapy, transfusions, parenteral nutrition and blood sampling is common in cancer patients. Up to 66 and 50% of patients with cancer and indwelling catheters develop insertion site thrombosis or pulmonary embolism, respectively.\textsuperscript{113,114} Reported risk factors for catheter-related thrombosis (CRT) in cancer patients include left-sided or superior vena cava insertion, chest radiotherapy, metastasis and elevated homocysteine levels.\textsuperscript{115} The mechanisms by which catheters promote thrombosis are incompletely understood. Vessel injury and stasis caused by catheter insertion, cancer-induced thrombocytopenia, and in certain cancer types can be of major concern in patients with chemotherapy-treatments are associated with bleeding side effects, which results in reducing the thrombogenicity of materials used to make medical devices in vitro, though available data in vivo are limited.\textsuperscript{116} Interestingly, it seems that anticoagulant agents that target serine proteases of the common pathway (FXa and thrombin) have limited capacity to prevent medical device-induced thrombosis in vivo and ex vivo.\textsuperscript{118,123} It has been postulated that medical device-driven CS activation generates FXa and thrombin in concentrations that overcome the inhibition by therapeutic doses of FXa and thrombin inhibitors.\textsuperscript{116} Using agents that inhibit CS activation might provide more efficient anticoagulation. However, further studies are required to evaluate the use of CS inhibitors for the prevention of medical device-induced thrombosis in clinical settings.

Although more evidence from basic, translational and clinical research is required, the potential contribution of CS activation in non-CRT thrombosis in cancer opens the door to novel therapeutic possibilities. Inhibition of the CS may protect against thrombosis without increasing the risk of bleeding, as previously shown by genetic or pharmacologic inhibition of FXIa in animals.\textsuperscript{124} Several classes of CS inhibitors are under development as thromboprotective and/or anti-inflammatory agents.\textsuperscript{125–147} (Table 3). Most of these agents have shown ability to inhibit CS in vitro or in experimental models of thrombosis in animals without cancer. Conceptually, targeting FXII or FXIa would be reasonable in settings where CS activation is the dominant mechanism of coagulation activation. As mentioned above, circulating TF is present in many cancers. TF is very efficient at initiating coagulation, but is also efficiently inhibited by TFPI.\textsuperscript{148} Thrombus formation then relies on the contribution of the intrinsic pathway for subsequent thrombin generation. This can be achieved by FXIa formation that will then activate FXI, or by the feedback activation of FXI by thrombin. Consequently, FXI or FXIa are good targets to interrupt the additive or synergistic effects of activation of both the extrinsic and intrinsic pathways. Moreover, such an approach seems to represent a reasonable compromise between preventing thrombosis with a lower risk of bleeding compared with commonly used heparins. For instance, reducing plasma FXI to 20% of normal levels using an antisense oligonucleotide (ASO ISIS-416858) was more effective than low molecular weight heparin in preventing VTE following knee replacement surgery, without any increase in intraoperative or postoperative bleeding.\textsuperscript{126,143,144} To date, no clinical trial has evaluated therapies targeting FXI/XIa to prevent thrombosis in the context of cancer.

Systemic administration of DNAse confers protection against experimental models of cancer-associated thrombosis.\textsuperscript{81} Thus, DNAse I represents a potential candidate for the prevention or treatment of thrombosis, as it does not directly affect haemostasis. However, convincing evidence supporting the contribution of NETs to human thrombosis, and clinical experience with systemic administration of DNAse I in humans are lacking, although recombinant DNAs I aerosol is used in humans with cystic fibrosis.\textsuperscript{147}

Therapeutic Implications of Contact System Activation in Cancer

Current practice recommends the use of heparins for the treatment and prophylaxis of VTE, as well as the treatment of symptomatic CRT in patients with cancer.\textsuperscript{121} These treatments are associated with bleeding side effects, which can be of major concern in patients with chemotherapy-induced thrombocytopenia, and in certain cancer types associated with a high risk of bleeding. The use of anticoagulation for routine prophylaxis of CRT is not recommended, as it largely fails to prevent CRT occurrence.\textsuperscript{115} Additionally, manufacturing catheters using biomaterials that are less thrombogenic would help to further reduce the incidence of CRT. Indeed, coating catheters with polyethylene glycol and CTI reduced protein adhesion, the prevention or treatment of thrombosis, as it does not directly affect haemostasis. However, convincing evidence supporting the contribution of NETs to human thrombosis, and clinical experience with systemic administration of DNAse I in humans are lacking, although recombinant DNAs I aerosol is used in humans with cystic fibrosis.\textsuperscript{147}
<table>
<thead>
<tr>
<th>Inhibitor class</th>
<th>Mechanism of action</th>
<th>Evidence</th>
<th>Disadvantages</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor XII/XIIa inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn trypsin inhibitor (CTI)</td>
<td>Kunitz-type reversible inhibitor of the FXIIa active site; purified from corn seeds</td>
<td>1. CTI-coated catheters inhibited FXIIa activity 2. CTI-coated catheters showed longer time to occlusion in a catheter thrombosis model in rabbits</td>
<td>1. Does not fully block contact activation at concentrations typically used (30–100 μg/mL) 2. High concentrations may inhibit also FXIa 3. Expensive</td>
<td>119,127</td>
</tr>
<tr>
<td>Recombinant FXIIa inhibitor rHA-infestin-4</td>
<td>Highly specific FXIIa inhibitor formed by the IV domain of Kazal-type serine protease inhibitor from the midgut of insect Triatoma infestans plus recombinant human albumin</td>
<td>1. Intravenous infusion abolished occlusive arterial thrombus formation in mice and rats 2. Prevented lethal PE, ischaemic stroke, thrombus formation on ruptured atherosclerotic plaques</td>
<td>1. Potentially immunogenic 2. Concentration-dependent inhibition of plasmin and FXa</td>
<td>128 129</td>
</tr>
<tr>
<td>Synthetic peptide H-D-Pro-Phe_Arg-chloromethyl ketone (PCK)</td>
<td>Irreversibly inhibits amidolytic FXIIa activity and KAL-mediated FXII activation</td>
<td>Prevented cerebral infarction in a mouse model</td>
<td>Unknown</td>
<td>130</td>
</tr>
<tr>
<td>Monoclonal antibodies targeting FXII/FXIIa 9A2 and 15H8</td>
<td>Monoclonal antibodies against the human FXII heavy chain that interfere with conversion to FXIIa</td>
<td>15H8 reduced fibrin deposition and limited platelet-rich thrombus growth in a collagen-coated thrombogenic graft in baboons</td>
<td>Unknown</td>
<td>131</td>
</tr>
<tr>
<td>Monoclonal antibody targeting FXIIa 3F7</td>
<td>Recombinant fully humanized antibody that binds FXIIa enzymatic pocket with high affinity</td>
<td>1. Abolished thrombus formation under flow in experimental mouse and rabbit models 2. Protected mice from cancer-induced lethal PE 3. Minimal immunogenic potential 4. Long half-life</td>
<td>Unknown</td>
<td>44,132,133</td>
</tr>
<tr>
<td>Antisense oligonucleotide</td>
<td>Reduces FXII hepatic synthesis</td>
<td>1. Reduced arterial and venous thrombosis in mice 2. Attenuated catheter-related thrombosis in rabbits</td>
<td>Slow onset: 3–4 wk of treatment required to lower FXII levels into the therapeutic range</td>
<td>120,126,134</td>
</tr>
<tr>
<td><strong>Factor XI/XIa inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease nexin-2 Kunitz-domain (PN2KPI) and Desmolaris</td>
<td>Kunitz-type FXIa active site inhibitors</td>
<td>Inhibited arterial thrombosis in mice</td>
<td>Desmolaris also inhibits FXa</td>
<td>135,136</td>
</tr>
<tr>
<td>4-carboxy-2-azetidinone BMS-262084</td>
<td>Irreversible inhibitor of FXIa active site</td>
<td>Inhibited thrombus formation in rabbit arterial and venous thrombosis model</td>
<td>Unknown</td>
<td>137</td>
</tr>
<tr>
<td>Tetrahydroquinoline (THQ) derivatives and Phenylimidazoles</td>
<td>Reversible inhibitor of FXIa active site</td>
<td>Unknown</td>
<td>Unknown</td>
<td>138,139</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Inhibitor class</th>
<th>Mechanism of action</th>
<th>Evidence</th>
<th>Disadvantages</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal antibody O1A6 (aXIMAb)</td>
<td>Inhibits FXI activation by binding FXI/FXIa Apple3 domain</td>
<td>Limited acute arterial thrombus growth and blood vessel occlusion in a baboon AV shunt model</td>
<td>Unknown</td>
<td>140</td>
</tr>
<tr>
<td>Monoclonal antibody 14E11</td>
<td>Inhibits FXI activation by FXIIa by binding to FXI Apple2 domain</td>
<td>Reduced thrombus formation in baboon vascular graft model and disseminated intravascular coagulation in mouse peritonitis model</td>
<td>Unknown</td>
<td>141,142</td>
</tr>
<tr>
<td>Antisense oligonucleotide IONIS-416858</td>
<td>Complementary to human and rhesus macaque FXI mRNA - reduces FXI hepatic synthesis</td>
<td>Slow onset: 3–4 wk of treatment required to lower FXI levels into the therapeutic range</td>
<td>Slow onset: 3–4 wk of treatment required to lower FXI levels into the therapeutic range</td>
<td>143,144</td>
</tr>
<tr>
<td><strong>Other inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polysphosphate inhibitors</td>
<td>Multifunctional cationic groups in the core of a dendritic polymer; positive charges responsible for binding and inhibition of polyP</td>
<td>1. Thromboprotective in mouse models of arterial thrombosis 2. Reduced bleeding compared with heparin 3. Nontoxic</td>
<td>1. Thromboprotective in mouse models of arterial thrombosis 2. Reduced bleeding compared with heparin 3. Nontoxic</td>
<td>145,146</td>
</tr>
<tr>
<td>Polycationic compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>Recombinant human DNase I</td>
<td>Treatment with DNase I abolished thrombus formation in a murine breast cancer model</td>
<td>Treatment with DNase I abolished thrombus formation in a murine breast cancer model</td>
<td>82,147</td>
</tr>
</tbody>
</table>

*Abbreviations: AV, arteriovenous; PE, pulmonary embolism; VTE, venous thromboembolism.*
Conclusion

Increasing interest in the contact pathway of coagulation has focused on a possible role in the pathogenesis of thrombosis. Preliminary observations have noted the presence of activation of the CS in gastrointestinal, lung, breast and prostate cancers. However, assays used to measure CS activation differed among studies. A standardized approach that would best quantify in vivo CS activation in clinical samples has yet to be developed. Several candidates that are known to activate the CS in vitro are found in the circulation of patients with cancer. However, further research is needed to establish what biological surfaces or molecular component(s) promote CS activation in human cancer. It is possible that the surface varies with the type of tumour. For instance, polyphosphate-bearing prostasomes seem to be a candidate in prostate cancer. More studies are required to understand if the type, stage, grade and treatment of cancer modulate CS activation. Although NETosis appears to play a role in cancer-associated thrombosis in animals, it remains unclear if this effect is due to direct CS activation. As TF is up-regulated in many cancers, it seems reasonable to propose that concomitant activation of both the intrinsic and extrinsic pathways acts synergistically to produce a highly prothrombotic state in cancer (► Fig. 3). It is tempting to speculate that while TF is the primary initiator, CS activation contributes to the amplification of thrombin generation. Future research should focus primarily on the standardization of methods to measure CS in clinical samples. This advance would further our understanding of the mechanisms by which CS activation contributes to non-CRT in humans. For CRT where CS activation is thought to play a major role, novel

Fig. 3 Molecular activators of coagulation in cancer. Coagulation activation in cancer-associated thrombosis may be explained by contributions from both the tissue factor ("extrinsic") and FXII/FXI-dependent ("intrinsic") pathways. Tissue factor–bearing microvesicles may be released into the circulation by various tumour types and promote thrombin generation and ultimately thrombosis. FXII may be activated in vivo by a variety of negatively charged molecules. These could include phosphatidylserine (e.g. on microvesicles), glycosaminoglycans, polyphosphate, collagen, nucleic acids and misfolded proteins. Activation of the contact system in cancer would promote the generation of thrombin, thereby exacerbating thrombotic risk.
therapeutic options targeting contact pathways should be evaluated in well-designed clinical trials.

Conflict of Interest
None declared.

Acknowledgements
Support from the ISTH Training Fellowship Program (to EC) is gratefully acknowledged. NSK was supported by NIH grant R01-HL095096.

References
16 de Maat S, Tersteege C, Herczenik E, Maas C. Tracking down contact activation - from coagulation in vitro to inflammation in vivo. Int J Lab Hematol 2014;36(03):374–381
17 Renné T, Schmaier AH, Nickel KD, Blomback M, Maas C. In vivo roles of factor XII. Blood 2012;120(22):4296–4303
32 de Maat S, de Groot PG, Maas C. Contact system activation on endothelial cells. Semin Thromb Hemost 2014;40(08):887–894
40 Ramström S. Clotting time analysis of citrated blood samples is strongly affected by the tube used for blood sampling. Blood Coagul Fibrinolysis 2005;16(06):447–452


Factors XI and XII as targets for new anticoagulants.


134 Weitz JI, Fredenburgh JC. Factors XI and XII as targets for new anticoagulants. Front Med (Lausanne) 2017;4:19