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NEUTROPHIL EXTRACELLULAR TRAPS IN CANCER AND CANCER-ASSOCIATED THROMBOSIS

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Stockholm 2022

Neutrophil extracellular traps in cancer and cancer-associated thrombosis
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Aulan, Danderyds sjukhus, 3rd June 2022, at 9.00.

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POPULAR SCIENCE SUMMARY OF THE THESIS

After almost two centuries of research into the mechanisms behind the development of thrombosis in cancer patients, many aspects of this complication are still largely unknown. Neutrophils, a part of our innate immune defense, can extrude their DNA coated with prothrombotic enzymes when strongly stimulated. This process, called neutrophil extracellular trap (NET) formation, has been shown to cause blood clots in animal cancer models.

Patients with unexplained venous thromboembolism (VTE) have a high risk (5-10%) of occult cancer. Large trials where these patients undergo extensive imaging have not shown a significant benefit of screening all these patients. To address this, a risk score termed the RIETE score was developed. We evaluated this risk score in all patients diagnosed with VTE at Danderyd Hospital during the year 2014 (Study I). Although the score performed well when excluding women, the score did not identify VTE patients at a high risk of being diagnosed with cancer in the following two years, indicating that new approaches are warranted.

Identifying and quantifying NETs has been proven difficult. In order to investigate the role of NETs in cancer and cancer-associated thrombosis, we developed and validated a novel method of quantifying a NET-specific complex, nucleosomal citrullinated histone H3 (H3Cit-DNA) in human plasma (Study II).

We proceeded to quantify H3Cit-DNA, along with several other biomarkers reflecting coagulation and neutrophil activation, to investigate which markers were associated with poor prognosis in a cohort of 106 palliative cancer patients (Study III). H3Cit-DNA and markers of neutrophil activation were associated with poor prognosis.

Finally, we prospectively recruited 500 patients presenting with VTE and analyzed H3Cit-DNA and several other biomarkers in plasma (Study IV). After adjustments of known risk factors, only H3Cit-DNA was associated with a cancer diagnosis during follow-up, corroborating that NETs have a role in the development of cancer-associated thrombosis, and that NET markers such as H3Cit-DNA could be potentially useful in cancer diagnostics.

To summarize, there are currently no reliable risk scores for identifying VTE patients with a high risk of occult cancer. We discovered that H3Cit-DNA is elevated in advanced cancer and in patients presenting with VTE and an underlying malignancy by developing an assay that quantifies the NET marker H3Cit-DNA in human plasma, adding to the mounting evidence of the significance of NETs in cancer and cancer-associated thrombosis.

ABSTRACT

Cancer is associated with a hypercoagulable state, and venous thromboembolism (VTE) may be the first sign of occult cancer. Cancer screening of all patients presenting with VTE would, however, overload the healthcare system and burden patients with unnecessary investigations. Current data suggest that neutrophil extracellular traps (NETs), prothrombotic nuclear content released by neutrophils upon strong stimulation, are central in cancer biology. This thesis aimed at a clinical investigation of the role of coagulation in advanced cancer and the role of NETs in cancer-associated thrombosis.

In Study I, we evaluated the recently developed RIETE risk score to identify patients presenting with VTE and a simultaneous high risk of occult cancer. The risk score failed to identify VTE patients with a high risk of occult cancer, illustrating the need for the development of risk score models in this population.

In Study II, we developed an enzyme-linked immunosorbent assay for the quantification of nucleosomal citrullinated histone H3 (H3Cit-DNA), a protein-DNA complex generated during NET formation. The assay was rigorously validated revealing high accuracy. All assay components are furthermore commercially available, enabling rapid dissemination and implementation of the assay within the field of NETs research.

Study III was an exploratory study investigating several biomarkers reflecting neutrophil activation, NET formation, coagulation, and fibrinolysis and their association with mortality in 106 terminal cancer patients. Markers of neutrophil activation and NETs were associated with mortality in univariate and multivariate Cox regression. Several prior studies have revealed that markers of coagulation and fibrinolysis are associated with prognosis in cancer patients. However, no studies have investigated terminal cancer patients, and to our surprise, we did not find an association between poor prognosis and markers of coagulation and fibrinolysis.

Study IV was a prospective cohort study of 500 patients presenting with acute VTE. Venous blood was sampled at the time of VTE, and markers of NETs and neutrophil activation were analyzed. H3Cit-DNA and cell-free DNA were associated with cancer diagnosis during a one-year follow-up in univariate analyses, but only H3Cit-DNA remained significant after adjustments in multivariate analyses, which could indicate a role of NETs in the development of cancer-associated thrombosis.

In summary, there are as of date no accurate risk scores identifying VTE patients with underlying cancer. Through the development of an assay quantifying the NET marker H3Cit-DNA in human plasma, we found that H3Cit-DNA is elevated in advanced cancer and in patients presenting with VTE and an underlying cancer, contributing to the growing evidence of the role of NETs in cancer and cancer-associated thrombosis. Further research will determine the diagnostic potential of NETs.

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I. Thålin C, **Rosell A**, Lundström S, Wallén H. Neutrofilernas märkliga fångstnät - Immunförsvarets Dr Jekyll och Mr Hyde [The neutrophil's multifaceted traps - the Dr Jekyll and Mr Hyde of the immune system]. Lakartidningen. 2019 May 21;116:FI3S.

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IV. Ng H, Havervall S, **Rosell A**, Aguilera K, Parv K, von Meijenfeldt FA, Lisman T, Mackman N, Thålin C, Phillipson M. Circulating Markers of Neutrophil Extracellular Traps Are of Prognostic Value in Patients With COVID-19. Arterioscler Thromb Vasc Biol. 2021 Feb;41(2):988-994. doi: 10.1161/ATVBAHA.120.315267. Epub 2020 Dec 3. Erratum in: Arterioscler Thromb Vasc Biol. 2021 Jun;41(6):e384.

V. Havervall S, **Rosell A**, Phillipson M, Mangsbo SM, Nilsson P, Hober S, Thålin C. Symptoms and Functional Impairment Assessed 8 Months After Mild COVID-19 Among Health Care Workers. JAMA. 2021 May 18;325(19):2015-2016. doi: 10.1001/jama.2021.5612.

VI. Mackman N, Hisada Y, Grover SP, **Rosell A**, Havervall S, von Meijenfeldt F, Aguilera K, Lisman T, Thålin C. Response by Mackman et al to Letter Regarding Article, "Patients With COVID-19 Have Elevated Levels of Circulating Extracellular Vesicle Tissue Factor Activity That Is Associated With Severity and Mortality-Brief Report". Arterioscler Thromb Vasc Biol. 2021 Jun;41(6):e381-e382. doi: 10.1161/ATVBAHA.121.316203. Epub 2021 May 26.

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CONTENTS

LIST OF ABBREVIATIONS

CAT	cancer-associated thrombosis
CV	coeffient of variation
DNase	deoxyribonuclease
DOAC	direct oral anticoagulant
DVT	deep vein thrombosis
eDNA	extracellular DNA
ELISA	enzyme-linked immunosorbent assay
EV	extracellular vesicle
G-CSF	granulocyte-colony stimulating factor
H3Cit	citrullinated histone H3
H3Cit-DNA	nucleosomal citrullinated histone H3
IL-1β	interleukin-1β
IL-1R	interleukin-1 receptor
IQR	interquartile range
LLOQ	lower limit of quantification
LMWH	low-molecular weight heparin
LPS	lipopolysacharide
NE	neutrophil elastase
NETs	neutrophil extracellular traps
MPN	myeloproliferative neoplasms
MPO	myeloperoxidase
PAD4	peptidylarginine deiminase 4
PE	pulmonary embolism
SD	standard deviation
sP-selectin	soluble P-selectin
TAT	thrombin-antithrombin complexes
TF	tissue factor
ULOQ	upper limit of quantification
VTE	venous thromboembolism

LITERATURE REVIEW

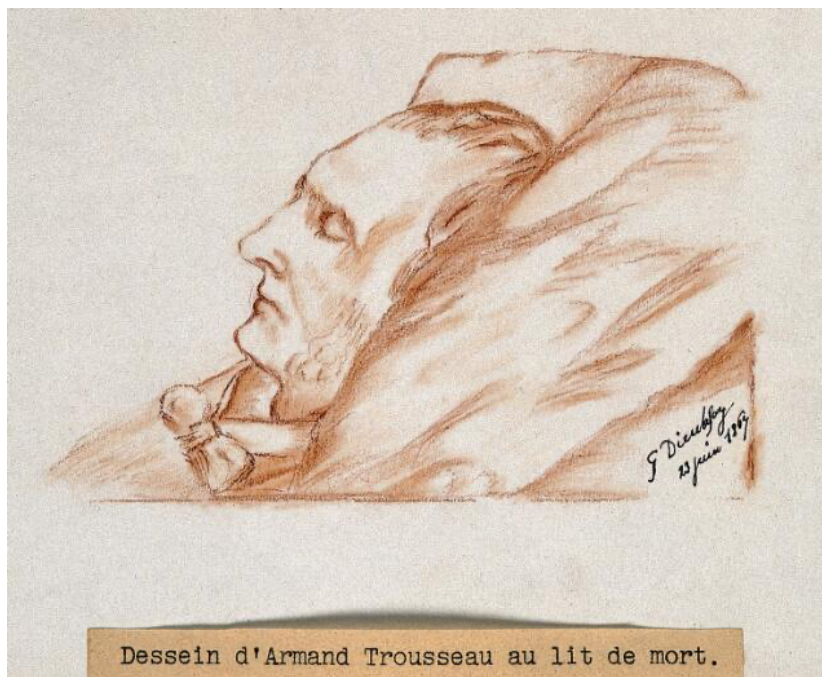## 1.1 CANCER-ASSOCIATED THROMBOSIS

The link between malignant disease and thrombosis was described already in 1823 by Jean-Baptise Bouillaud (2). In 1865, Armand Trousseau further elaborated on the hypercoagulable state associated with cancer and suggested that a change in blood composition was the cause (3).

"You recollect that we have studied painful white oedema, not only in recently delivered women, but also, and more frequently, in persons of both sexes affected with pulmonary phthisis or internal cancer. Today, I propose to speak to you of this affection which always has, as its primary cause, a special alteration of the blood…"

Armand Trousseau, 1865.

Adding to the list of famous scientists succumbing to their disease of study, Trousseau detected migratory thrombophlebitis months prior to receiving the diagnosis of gastric cancer that would be his end (Figure 1).

Dessein d'Armand Trousseau au lit de mort.

Figure 1. Trousseau on his deathbed drawn with chalk by one of his students, Georges Dieulafoy (4).

More than a decade and a half later, the mechanisms behind cancer-associated thrombosis (CAT) are still not fully elucidated.

CAT encompasses venous thromboembolism (VTE) [which includes deep vein thrombosis (DVT) and pulmonary embolism (PE)], arterial thrombosis, and disseminated intravascular coagulation. VTE is the most common type of CAT, with cancer patients having a four to nine-fold increased risk of VTE compared to the general population (5-7). An estimated 20-

30% of all first VTEs are associated with cancer (8). Following acute unprovoked VTE, 5-10% of patients are diagnosed with cancer within a year (9, 10).

Clinical factors, such as treatment (including chemotherapy (11), radiotherapy (12), surgery (13), and hormonal treatment (14)), immobilization and venous stasis due to local tumor compression (15), contribute to thrombotic risk in cancer patients.

In addition to clinical factors, cancer-specific prothrombotic pathways and host immune responses can tip the scale towards the development of thrombosis.

Through expression of procoagulant proteins, such as tissue factor (TF), cancer cells can activate the host coagulation system. Local clotting at the site of the tumor can be due to TF expression. Shedding of extracellular vehicles (EVs) from cancer cells that expose TF or other procoagulant proteins can promote thrombosis (16). By release of inflammatory mediators (17), proangiogenic factors (18), and platelet aggregation agonists (19), the procoagulant potential of host cells can be altered.

The rate of VTE greatly varies between cancer types (20), indicating that different mechanisms and pathways are present in different cancer types. The highest risk of VTE is seen in pancreatic, stomach, primary brain, and lung cancer (21).

Direct activation of platelets through the expression of transmembrane glycoprotein podoplanin is associated with thrombosis in glioma (22), and podoplanin expression in glioma was positively correlated to D-dimer levels and negatively correlated to platelet count (23). Generally, thrombocytosis is associated with an increased risk of VTE in cancer patients (24), although in brain tumors an opposite association has been observed (25), possibly linked to podoplanin-mediated platelet activation and consumption.

Leukocytosis is associated with CAT (26) and is frequent in lung and colorectal cancer (27). Cancer cells can release and express inflammatory mediators and myeloid growth which induces leukocytosis.

Cancer-associated VTE is a significant predictor of poor prognosis. This association is seen irrespective of cancer type and stage (20, 28, 29). In a study from 2006 incorporating a range of different types of cancer, VTE was associated with a median overall relative risk of 3.7 for mortality (range of hazard ratios for cancer types stratified by stage, 1.3-14.4). More recent data from patients treated with immune checkpoint inhibitors revealed a transient hazard ratio for death of 3.1 in patients with VTE (95% CI 2.1-4.6) (28).

A meta-analysis combining patient-level data from 14 randomized controlled trials investigating prophylactic low-molecular weight heparins (LMWHs) vs placebo observed no effect on mortality with an adjusted relative risk of 0.99 (95% CI 0.93-1.06) (30). This could reflect a prothrombotic phenotype that is associated with more aggressive disease, and that treatment with LMWHs may reduce the risk of VTE but not alter that phenotype.

Direct oral anticoagulants (DOACs) have revolutionized the treatment of VTE. Since the CLOT trial that demonstrated the superiority of LMWHs over warfarin in treating VTE patients with cancer (31), LMWHs have been the anticoagulant of choice in cancer patients. In recent years, however, DOACs have demonstrated non-inferiority vs LMWHs (32-34) in preventing recurrent VTE in patients with active cancer. This is reflected in guidelines that now often recommend both LMWHs and DOACs for cancer patients with acute VTE (35, 36).

1.2 VENOUS THROMBOEMBOLISM AS THE FIRST SIGN OF OCCULT CANCER

"I have long been struck with the frequency with which cancerous patients are affected with painful oedema in the superior or inferior extremities, whether one or other was the seat of cancer. This frequent concurrence of phlegmasia alba dolens with an appreciable cancerous tumor, led me to the inquiry whether a relationship of cause and effect did not exist between the two, and whether the phlegmasia was not the consequence of the cancerous cachexia."

Armand Trousseau, 1865.

Current guidelines recommend a limited screening approach for all patients presenting with an unprovoked VTE (37-39). This limited screening approach consists of thorough medical history and physical examination, laboratory investigations (complete blood count, calcium, urinalysis, and liver function tests), and chest X-ray. Age- and gender-specific cancer screening (colon, breast, cervix, and prostate) should also be performed according to national recommendations.

A VTE is classified as provoked if it is associated with either a transient (recent surgery, trauma, hospital stay, estrogen use, pregnancy) or a permanent (active cancer, inflammatory disease) risk factor. The scientific and standardization subcommittees on control of anticoagulation and predictive and diagnostic variables in thrombotic disease of the International Society on Thrombosis and Haemostasis (ISTH) have released a classification document to further improve comparability (40), as these provoking factors have not always been consistent across studies.

Individual prospective randomized trials have failed to show the benefit of extensive screening with computed tomography (CT) of the abdomen/pelvis or positron emission tomography (PET)/CT compared to a limited screening approach (41-44). A recent meta-analysis showed that more cancers are detected at initial screening in the extended screening group compared to limited screening (10). As follow-up in most studies was limited to one or two years, it is unclear if this translates into improved long-term patient outcomes, but no individual studies report a survival benefit of extensive screening compared to limited screening (41-45).

To improve the identification of VTE patients with a high risk of cancer, the *Registro Informatizado Enfermedad TromboEmbolica* (RIETE) score was developed in 2017 (46).

To date, extensive screening of all patients with unprovoked VTE has not proven to be beneficial compared to limited screening to detect occult cancer in VTE patients as the number needed to screen is high (91 in the SOME trial (41)). The addition of blood biomarkers to clinical characteristics could potentially yield a stronger risk score, similarly to recently developed risk scores to estimate the risk of VTE in cancer patients (47) and the risk of bleeding in patients receiving anticoagulation for atrial fibrillation (48).

1.3   PROGNOSIS IN ADVANCED CANCER

Physicians consistently overestimate survival for cancer patients (49, 50). Both patient and clinician decision-making are impacted by life expectancy, which coupled with inflated survival estimates could lead to unnecessary interventions in patients with poor prognosis. Objective tools for estimating survival in patients with advanced cancer are therefore needed. Current prediction models such as Palliative Performance Scale (51), Palliative Prognostic Score (52), and the Palliative Prognostic Index (53), incorporate subjective assessments which makes them prone to a similar bias as clinicians' survival estimates. There is therefore a need for objective prognostic tools to aid clinicians and patients in palliative care (54).

1.4   NEUTROPHIL EXTRACELLULAR TRAPS

In a pivotal paper, a role for NETs in the development of CAT was proposed (55). Strongly stimulated neutrophils will extrude their chromatin in a process referred to as neutrophil extracellular trap (NET) formation (56). Through NADPH-oxidase, high levels of intracellular reactive oxygen species are generated, activating the enzyme peptidyl-arginine deiminase 4 (PAD4) which citrullinates arginine residues on histones (57). This citrullination causes a reduction in histone positive charge, and chromatin decondensation (57). At the same time, neutrophil elastase (NE) and myeloperoxidase (MPO) enter the nucleus, causing further chromatin decondensation (58). After altering nuclear shape, the nuclear membrane breaks, and chromatin reaches the cytoplasm. Once outside the nucleus, more proteases attach, the plasma membrane ruptures, and chromatin is subsequently released extracellularly as a NET (59) in a process termed lytic NET formation.

To date, three main mechanisms of NET formation have been described: lytic NET formation, which results in the death of the neutrophil, and viable NET formation, which is further divided into nuclear or mitochondrial viable NET formation. Viable NET formation was described in 2012, where the neutrophil survived anucleated (60) and was able to continue engulfing pathogens. Apart from releasing nuclear DNA, mitochondrial DNA can also be released through viable NET formation (61).

The strict requirement of PAD4 in NET formation is under debate. Several in vivo studies using PAD4$^{-/-}$ mice observed impaired NET formation in influenza A (62), methicillin-resistant *S. aureus* (63), *P. aeruginosa* (64), LPS (62), ionomycin (96), deep vein thrombosis models (65), cancer conditioned media and G-CSF (66). Another study, however, observed NET formation in PAD4$^{-/-}$ mice when stimulated with LPS, PMA, and *Klebsiella pneumonaie* (67). However, studies highlight a difference between mouse and human neutrophils,

indicating that PAD4 may be more important in mice than in humans (68). In both mouse and human neutrophils, phorbol myristate acetate stimulation induces the release of uncitrullinated NETs (69). These discrepancies are likely partly due to methodological differences but could also indicate that there are PAD4-independent pathways of NET formation.

1.5 NETS IN CANCER

Neutrophils have long before the discovery of NETs been implicated in promoting metastasis (70). Moreover, in a cecal ligation and puncture model of infection, NETs trap circulating tumor cells in the vasculature and increase liver metastasis (71). Injection of LPS resulted in a similar effect. Surgical stress using an ischemia/reperfusion model resulted in an increased NET formation and metastasis, an effect that was abolished when mice were treated with DNase I or PAD4-inhibitors (72). However, surgery or infection is not required for NET induction in cancer. In a mice model of breast cancer, spontaneous NET formation triggered metastasis formation, an effect that was inhibited by treatment with DNase-coated nanoparticles (73).

NETs markers are elevated in cancer patients as compared to healthy controls (74, 75) and hospitalized non-cancer patients (76). High levels of the circulating NET marker MPO-DNA were increased in esophageal cancer with involvement of lymph nodes compared to those without (77). In hepatocellular carcinoma, increased levels of MPO-DNA and intratumoral NETs were associated with poor prognosis and metastasis (78). In breast cancer, NE-DNA complexes were increased in metastatic disease compared to localized disease (79). NET induction in cancer is thought to be mediated by granulocyte-colony stimulating factor (G-CSF) (55), interleukin-8 (80), soluble P-selectin (81), inflammasomes (82), interleukin-1β (IL-1β) (82), and tumor-educated platelets (83) (Figure 2).
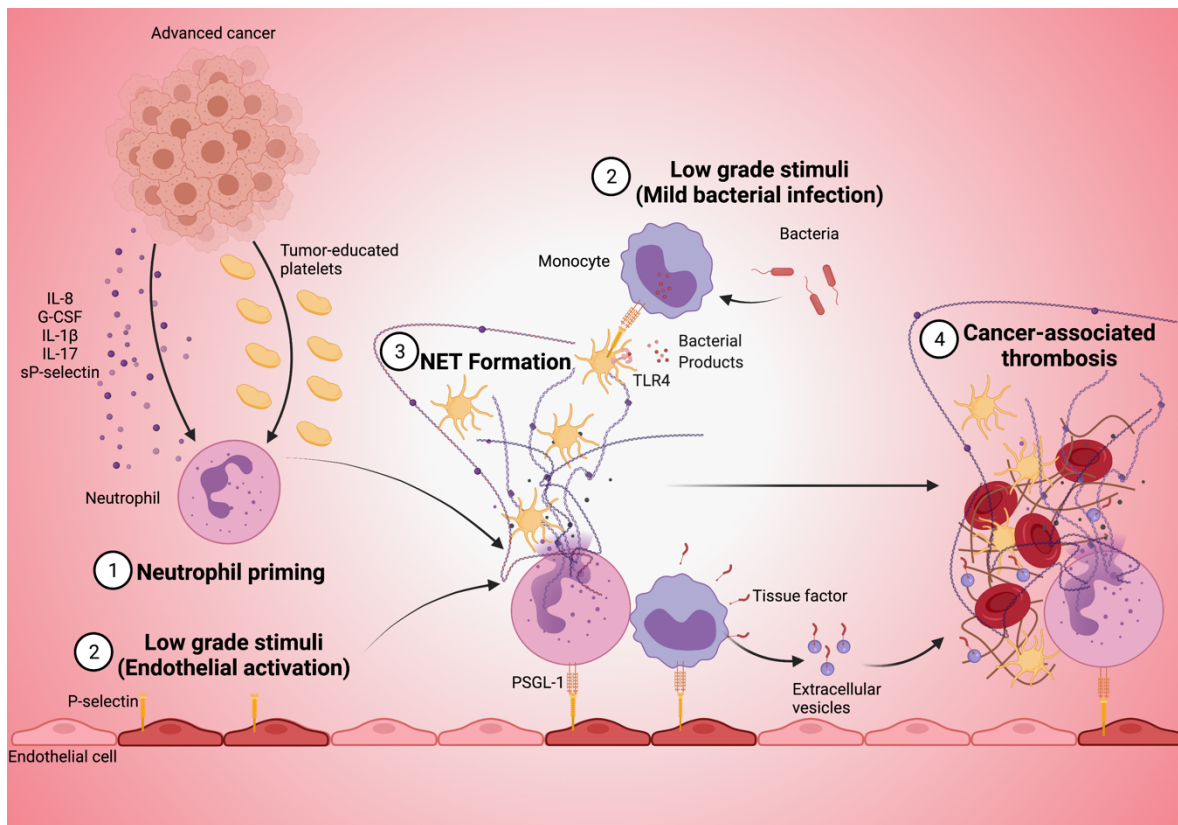
Figure 2. Cancer-associated thrombosis and neutrophil extracellular traps

1. The tumor primes neutrophils toward NET formation through the secretion of soluble mediators (G-CSF, IL-1β, IL-8, sP-selectin) and tumor-educated platelets. 2. Low-grade stimuli trigger neutrophils to adhere to the endothelium. 3. The threshold for NET formation is reached and neutrophils generate NETs. 4. NETs trap platelets, red blood cells, and extracellular vesicles with tissue factor activity and occlude vessels, promoting CAT.

IL-8; interleukin-8, G-CSF; granulocyte-colony stimulating factor, IL-1β; interleukin-1β, sP-selectin, soluble P-selectin, PSGL-1; P-selectin glycoprotein ligand-1, TLR4; Toll-Like Receptor 4, NET; Neutrophil extracellular trap.

1.6   DETECTION AND QUANTIFICATION OF NET FORMATION

Accurate detection and quantification of NET formation in blood and tissue is a difficult and not yet standardized endeavor. Conventional microscopy with immunodetection of extracellular DNA (eDNA) and histones is one of the most widely used methods for NET detection. On the other hand, eDNA and granular proteins are also generated from sources other than NETs (84-86). As a result, staining for the co-localization of NET components (eDNA, NE, MPO, citrullinated histones) is advised. (87, 88). Flow cytometry detecting NET components on cells and EVs are also used to quantify NET formation (55, 89). However, these methods are not standardized and are hampered by operator subjectivity during the data-analysis stage (90). Commercial and in-house enzyme-linked immunosorbent assays (ELISAs) can be used to quantify NET components in blood, allowing for higher throughput and easier pre-and post-analytical handling than flow cytometry samples. The presence of NE, MPO, nucleosomes and eDNA in neutrophils does, however, not necessarily indicate NET formation in vivo, as it could represent neutrophil activation without NET formation.

Similar to immunostaining, the co-localization of cfDNA and neutrophil granules, such as MPO-DNA, is thereby recommended also in ELISA approaches. According to a recent study, however, the widely used MPO-DNA ELISA assay is prone to errors and provides questionable specificity for NET detection (91). The assay failed to detect in vitro generated NETs spiked in plasma, and several samples displayed high signals when an isotype control antibody were used instead of the capture antibody, indicating interference in plasma (91). The previously developed ELISA for citrullinated histone H3 (H3Cit) (92) may be more specific but has issues such as lot-to-lot variability and low antibody specificity.

1.7 PROTHROMBOTIC EFFECTS OF NETS

In 2013, the term immunothrombosis was coined (93) describing the interplay between innate immunity and thrombosis to hinder the dissemination of pathogens. Promoting thrombosis is an important immunoprotective effect of NETs (94), but NETs are also implicated in endothelial and organ damage in murine models of sepsis (95, 96). Fuchs et al showed that NETs act as a scaffold for platelets ultimately leading to the platelet aggregation (97), and NETs also capture procoagulant EVs, EVs with TF activity, and von Willebrand factor (97-100). Furthermore, NETs are coated with NE and cathepsin G, which inactivates tissue factor pathway inhibitor (94). In baboon (101) and mouse (98, 102) experimental models of DVT, NETs are abundant in thrombi.

$PAD4^{-/-}$ mice, as well as wild-type mice treated with deoxyribonuclease (DNase) 1, displayed reduced thrombin activity and vessel occlusion in sepsis compared to control mice (96), and in mice DVT models these approaches also resulted in reduced thrombosis (65, 98, 102).

These results indicate a prothrombotic effect of eDNA in mice. Furthermore, in a mouse model with compromised endogenous DNase activity, NETs occluded vessels independently of the coagulation cascade (103). Taken together, evidence suggests that NETs promote vascular occlusion in the absence of cancer. Although there is a study reporting that intact NETs enhanced thrombin generation in plasma (104), another study reported conflicting results (105). Factor XII is activated by binding to negatively-charged surfaces such as eDNA (106). As the net charge of eDNA is reduced when in complex with positively charged histones, it is unlikely that intact NETs activate Factor XII.

1.8 CLINICAL STUDIES EXAMINING NETS AND CAT

Co-culturing neutrophils derived from healthy persons with human pancreatic cancer cells (AsPC-1) induces NET formation. The same results were achieved when healthy neutrophils were co-cultured with either platelets or AsPC-1 cell culture medium, suggesting that cancer cells secrete factors that directly or indirectly, through platelets, trigger NET formation. The addition of NETs released from cancer patients increased fibrin generation in control plasma in vitro, and increased levels of thrombin-antithrombin (TAT) complexes, effects which were reduced by DNase 1-treatment (74). In patients with different cancers, NETs were detected in coronary, cerebral, and pulmonary microthrombi (107). In addition, circulating levels of H3Cit correlated with levels of TAT and sP-selectin, suggesting a connection between NETs

and activation of coagulation in cancer patients (107). Another study observed abundant histone-DNA complexes in thrombi from cancer patients (108). Furthermore, as compared to healthy controls, plasma levels of circulating nucleosomes and eDNA were higher in cancer patients but did not correlate with plasma levels of TAT. No more NET-specific analyses were performed for this study, limiting the conclusions of the plasma analyses. In myeloproliferative neoplasms (MPN), higher levels of MPO-DNA were observed in patients with a history of thrombosis compared to those without (109).

In the Vienna Cancer and Thrombosis Study, circulating H3Cit was higher in a cancer population that developed VTE (110). Although not powered for subgroup analysis, the study indicated that NETs could be especially important in promoting thrombosis in pancreatic and lung cancer, as these were the only cancer types where H3Cit was predictive of VTE in subgroup analyses.

1.9   ANIMAL STUDIES INVESTIGATING THE ROLE OF NETS IN CAT

The first implication for the involvement of NETs in the development of CAT was demonstrated by Demers et al (55) using murine models of lung cancer, breast cancer (4T1), and chronic myelogenous leukemia. They proposed that cancer produces a systemic milieu in which neutrophils are more prone to NET release, needing just minor stimuli to trigger NET formation. Low-dose lipopolysaccharide (LPS) administration induced NET formation in tumor-bearing mice, as measured by higher levels of H3Cit in plasma and pulmonary microthrombosis, as opposed to in control mice, establishing a lower threshold for NET formation in cancer. A similar phenotype with neutrophilia and a drop in platelet count could be replicated in control mice given repeated injections of G-CSF followed by LPS. In 4T1 tumor-bearing mice, daily treatment with a neutralizing anti-G-CSF antibody diminished the phenotype. Mice with RIP1-Tag2 insulinoma and MMTV-PyMT tumors display an accumulation of NETs in vasculature leading to vascular occlusions and impaired function in organs distant from the tumor (111). This phenotype could be reversed by treatment with DNase-1 or with the PAD4 inhibitor GSK484 (111, 112). In plasma and thrombi from nude mice bearing human pancreatic cancers, neutrophilia and higher levels of H3Cit and eDNA have been observed (113). DNase-1 administration or neutrophil depletion decreased venous thrombus size in tumor-bearing but not control mice. NETs were found in arterial and venous thrombi from mice with 4T1 tumors (114). Furthermore, DNase-1 also prevented arterial thrombosis in both tumor-bearing and control animals, as well as lowered the extent of venous thrombosis in tumor-bearing mice. Accordingly, DNase 1-treatment decreased thrombus size in mice with 4T1 tumors (115). However, longer treatment with DNase-1 was associated with lower survival, which was partly mitigated by the concurrent use of broad-spectrum antibiotics. Interleukin-1 receptor (IL-1R) blockade has been investigated in mice with 4T1 tumors (82). Mice with 4T1 tumors had high IL-1β and G-CSF expression and neutrophilia when compared to WT mice. IL-1R blockade, DNase 1, and PAD4 inhibitor GSK484 all reduced NET formation and thrombus weight in mice with 4T1 tumors.

In animal models of MPN, NETs have been linked to CAT (116). Jak2^{WT} transplanted with Jak2^{V617F} bone marrow or Jak2^{V617F} mice developed spontaneous pulmonary thrombosis and displayed elevated NET formation compared to control mice. This phenotype was absent when mice were transplanted with PAD4-deficient Jak2^{V617F} bone marrow. Treatment with DNase-1 or the JAK inhibitor Ruxolitinib decreased thrombus size in Jak2^{V617F} in the inferior vena cava stenosis model of DVT. Taken together, these results suggest a role of NETs in the development of thrombosis in MPN.

2 RESEARCH AIMS

Animal models have established a role of NETs in CAT. The overall aim of this thesis was to explore the possible role of neutrophil activation and NETs in CAT and prognosis in advanced cancer patients.

Specific aims:

- To assess the performance of recently proposed risk scores in identifying VTE patients at high risk of occult cancer (Study I)

- To determine the prevalence of occult cancer and clinical variables associated with occult cancer in a cohort of patients seeking acute care due to VTE. (Study I+IV)

- To develop a robust and highly specific assay to detect and quantify H3Cit-DNA in human plasma (Study II).

- To determine the levels of H3Cit-DNA in a terminal cancer cohort using the novel H3Cit-DNA assay and determine whether high levels are associated with poor prognosis (Study III).

- To investigate the relationship between circulating markers of NETs and markers of coagulation in terminal cancer patients. (Study III)

- To determine if markers of NETs are associated with cancer-associated VTE. (Study IV).

- To determine the value of circulating markers of NETs in identifying VTE patients at high risk of occult cancer (Study IV).

3 MATERIALS AND METHODS

3.1 PATIENTS AND STUDY DESIGN

3.1.1 Study I

Patients diagnosed with DVT and/or PE between January 1 and December 31, 2014, at the departments of medicine and cardiology at Danderyd hospital in Stockholm, Sweden, were identified and included in a retrospective cohort study. Patients under the age of 18 or patients not residing in Stockholm County were excluded. Patients were identified using ICD10-codes I80-82 and I26 to search hospital registries; all medical records were evaluated to verify the diagnosis of VTE. Only the first VTE diagnosis was considered if a patient had multiple VTE diagnoses throughout the study period.

Baseline variables and routine laboratory data were extracted from medical records.

Provoking factors were adopted from the ISTH's Scientific and Standardization Committee (40). Patients with a provoked VTE had either a major transient risk factor during the three months preceding the diagnosis of VTE (i.e. cesarean section, surgery with general anesthesia for more than 30 minutes, or confined to bed in hospital for at least three days with an acute illness), or a minor transient risk factor during the two months preceding the diagnosis of VTE (i.e. estrogen therapy, pregnancy, puerperium, surgery with general anesthesia for less than 30 minutes, admission to hospital for less than 3 days with an acute illness, leg injury resulting in reduced mobility for at least 3 days, and confined to bed for at least 3 days out of hospital [i.e. bedridden]) or a long-term risk factor (active cancer or a non-malignant illness linked to a more than twofold increase in the likelihood of recurrent VTE after quitting anticoagulant medication [e.g. inflammatory disease]).

Cancer was considered active if any of the following conditions were met: (1) no potentially curative anti-cancer therapy was administered; (2) evidence of recurrent or progressive disease; or (3) ongoing anti-cancer therapy (40). In addition to the ISTH-proposed provoking factors, we included long-distance travel of more than 6 hours in the week preceding diagnosis, thoracic outlet syndrome (TOS), and upper extremity DVT with unilateral catheter as provoking factors.

Patients were monitored for 24 months after index VTE. Individual medical records were used to acquire cancer diagnoses. The great majority of primary care providers and hospitals in Stockholm County are covered by the medical records. As a result, we expect to have likely covered all cancer cases. Patients with cancer at baseline (within ten days after VTE) would not have benefited from a screening program and were thus omitted from the outcome analyses. There was no structured screening procedure in place; instead, individuals were screened for cancer based on the clinical judgment of the attending physician. The RIETE score was calculated retrospectively, and it had no impact on clinical practice.

The RIETE score was applied to all included patients without known active cancer. Seven items are included in the score; male sex (+ 1p), age > 70 years (+ 2p), chronic lung disease (+ 1p), anemia (+ 2p, defined as hemoglobin < 130 g/L in men and < 120 g/L in women), elevated platelet count (+ 1p, defined as platelet count $\geq 350*10^9$/L), postoperative status (− 2p) and prior VTE (− 1p). Patients receiving three or more points are classified as having a high risk of occult cancer whereas 2 points or less puts patients into a category with a low risk of occult cancer.

3.1.2 Study II

The aim of the study was to overcome the prior issues of the H3Cit ELISA, mainly high variability between standard curves and batch-to-batch variability of polyclonal antibodies.

3.1.2.1 *In vitro enzymatically modified histones versus semi-synthetic designer nucleosomes*

Standard curves of histone H3 citrullinated in vitro using PAD4 were created as previously described (92). Using two distinct lots of PAD4, two lots of in vitro citrullinated H3 were produced and stored at -80°C. Three separate lots of human semi-synthetic H3R2,8,17Cit designer nucleosomes (dNucs; EpiCypher #16-1362) were aliquoted at -80°C for comparison. ELISA was used to assess inter-lot variability and presented as F(DFn, DFd). The stability of semi-synthetic H3R2,8,17Cit histones and nucleosomes was tested by spiking into undiluted human plasma.

3.1.2.2 *H3Cit-DNA Protocol and step-by-step standardized validation*

In summary, microplates were coated with a monoclonal anti-histone H3 (citrulline R8) antibody (Abcam, Cat# 232,939) and detected with a monoclonal anti-DNA antibody (Cell Death ELISAPLUS, Roche). Semi-synthetic recombinant nucleosomes with citrulline at the 2, 8, and 17th arginine residues of histone H3's N-terminus (EpiCypher, Cat#16–1362) were used as standard curve.

Due to the lack of a verified reference method, trueness and uncertainty could not be determined. The working range of the assay was defined by the lower and upper limits of quantification (LLOQ and ULOQ, respectively). Precision was calculated by running one plasma sample in six repetitions on the same plate (intra-assay) and four plasma samples in triplicate on four distinct days (inter-assay), with acceptable values for the coefficient of variation (CV) of 10% and 15%, respectively. Two undiluted plasma samples were spiked with H3R2,8,17Cit dNucs at concentrations of 2000 ng/mL to assess dilution linearity. Serial dilutions of spiked plasma samples (in standard diluent) were conducted until the predicted concentration was less than the LLOQ, and the samples were tested in duplicate on the same plate. The results are reported as the percent recovery for the estimated concentration at each dilution within the working range. A recovery rate of 80 percent to 120 percent was accepted. Serial dilutions of two plasma samples exhibiting high endogenous quantities of H3Cit-DNA complexes were used to assess parallelism (in standard diluent). In the same run, neat

samples and serial dilutions were examined in duplicate and the dilution factor was adjusted for. The CV was computed for each sample based on the results of the neat sample and the dilutions, with an accepted CV of <20%. Four aliquots of plasma samples with a concentration of H3Cit-DNA complexes within the working range were collected to test recovery. One aliquot was left undiluted, while the other three were diluted at a ratio of 1:2, 1:4, and 1:8. (in standard diluent). In the same run, ten microliters of H3R2,8,17Cit dNuc were added to the samples at an anticipated concentration of 400 ng/mL and evaluated in triplicate. The results are provided as %recovery, with an acceptable recovery range of 80% to 120%. The assay's selectivity was determined by comparing H3R2,8,17Cit dNucs to unmodified recombinant nucleosomes in the same run.

3.1.3 Study III

Between October 2016 and May 2018, 106 cancer patients were recruited prospectively at the palliative care unit at Stockholms Sjukhem in Stockholm, Sweden. Inclusion criteria were active cancer, intact cognition, and the ability to understand spoken and written Swedish. When research personnel was available, inclusion was carried out twice a week. There were no exclusion criteria in place. Data on demographics, comorbidity, and ongoing medical treatment were gathered from hospital records. As controls, 31 healthy and age-matched individuals without prior cancer diagnosis were included.

3.1.4 Study IV

In this prospective study, patients diagnosed with DVT and/or PE at Danderyd hospital in Stockholm, Sweden, between May 16, 2017, and March 27, 2020, who lived in Stockholm County were eligible for inclusion within 4 days of VTE.

Patients were included during office hours on weekdays when research personnel was available. Electronic medical records and interviews were used to obtain information on demographics, comorbidity, and ongoing medical therapy. All procedures were carried out in line with the Helsinki Declaration. Every patient provided written informed consent. The study was approved by the regional ethical review board in Stockholm (Dnr 2017/260-31/4, 2017/1834-32, and 2018/115-32). Provoking factors were adopted from ISTH, as described above with the addition of varicose vein surgery as a provoking factor.

All ambiguous cases, whether an inflammatory disease was to be considered provoking, whether a cancer was to be considered active, and all leg injuries that were not fractures were presented to two senior consultants specialized in coagulation disorders who were blinded to outcome and exposure variables.

Patients were followed for a year after the index VTE. Individual medical records were used to obtain cancer diagnoses (excluding non-melanoma skin cancer, essential thrombocythemia, polycythemia vera, and primary myelofibrosis). Almost all primary care providers and hospitals in Stockholm County are covered by the medical records. As a result, we expect to have covered all cancer cases. Patients were screened based on the clinical

judgment of the attending physician; no structured screening method was used. Furthermore, an exploratory analysis was done that excluded all patients who died or were diagnosed with cancer within 10 days after VTE, since these individuals would not likely benefit from any screening program.

3.2   LABORATORY DATA

3.2.1   Blood sampling

For Studies II, III, and IV, a venous blood sample was taken at study enrollment. Platelet-poor plasma was generated from citrated whole blood by immediately centrifuging it for 20 minutes at 2000 x g at room temperature, then storing it at -80 °C until further analysis.

3.2.2   Laboratory analyses

H3Cit-DNA was quantified using the in-house capture ELISA developed in Study II (117). EV TF activity was quantified using a previously described in-house assay (118). Neutrophil elastase (NE) was measured using the PMN Elastase Human ELISA Kit (Abcam), cell-free DNA (cfDNA) using Quant-iT PicoGreen (Invitrogen), soluble P-selectin (sP-selectin) using the human sP-selectin/CD62P ELISA Kit (R&D Systems), IL-8 using the V-Plex Human IL-8 Kit (Meso Scale Diagnostics), G-CSF using the Quantikine Human G-CSF Immunoassay (R&D Systems), TAT using the Enzygnost TAT micro (Siemens), D-dimer using the Asserachrom D-DI (Diagnostica Stago) and PAI-1 using the Human PAI-1 Activity ELISA Kit (Molecular Innovations), all according to the manufacturers' instructions.

3.3   STATISTICAL ANALYSES

In general, continuous data were reported as medians, whereas categorical variables were provided as frequencies (percentages) (interquartile range [IQR]). To compare proportions, the Fisher exact test was utilized, and the student t test or the Mann Whitney U test was used to compare continuous variables. To test for normality of distribution, the D'Agostino and Pearson or Shapiro-Wilk normality tests were used, and statistical methods were chosen to fit non-normal distributions when necessary. Correlations were investigated using Spearman's rank correlation coefficient. The area under the curve was estimated for receiver operating characteristic curves. The circulating markers were used as continuous variables in Cox regression analysis, and they were dichotomized at the 95th percentile. To enable comparison, continuous biomarker variables were translated on a common scale with a mean of zero and a standard deviation (SD) of one (Z-standardization). The hazard ratio (HR) of a new cancer diagnosis was calculated using univariate and multivariate cause-specific Cox proportional hazards models. Kaplan-Meier curves were created, and a time-to-failure analysis was carried out. The log-rank test was used to compare curves. Statistical analyses were performed using STATA 16.1 (StataCorp, Houston, TX. USA) or GraphPad Prism 7/8 (GraphPad Software, Inc, La Jolla, CA, USA). A two-sided p-value < 0.05 was considered statistically significant in all studies.

For Study II, the extra sum-of-squares F test was used to compare curves.

For Study IV, a total of 28 events were required to achieve 80 % power to detect a difference in H3Cit levels between patients diagnosed with cancer during follow-up and those remaining cancer-free using a previously reported assay (29). An interim analysis was done since more than 28 events were observed after 500 enrolled participants. The effect of storage duration on circulating markers was investigated using linear regression.

3.4 ETHICAL CONSIDERATIONS

In Study I, no informed consent was obtained as it was a retrospective study of electronic medical records. Such a study always poses the risk of a research subject experiencing this as an invasion of privacy. As all the data is from the same center during a well-defined period, there is a risk that a research subject is aware that they are a part of the study material when reading the publication. As the patient material is large, there is no risk of identification of a research subject. Taken together, the breach of integrity is deemed acceptable. The study was approved by the regional ethical review board in Stockholm (Dnr 2017/2159-31/1).

For Study II, the study participants are the same as in Study III.

In Study III, the study provides no benefit to study participants. The venous blood draw can be technically demanding in terminal cancer patients. The only benefit is the potential for improved care in the future. Furthermore, the fact that researchers are also caregivers puts the patients in a position of dependency that may affect their decision to enroll. Patients had to be judged cognitively intact to be eligible for inclusion, however, this is a subjective assessment and mild confusion is frequent toward the end of life.

Provided that accurate information is provided, by agreeing to study inclusion, the patient judges that the altruistic benefits of study inclusion outweigh the pain of a venous blood draw. The study was approved by the regional ethical review board in Stockholm (Dnr 2015/1533–31/1, 2016/359–32, 2016/1102–32, 2016/2051–32/1, 2017/1837–32 and 2018/1845–32/1).

In Study IV, the patients are in an emergency department setting, and similarly to Study III, there is a risk of dependency as researchers are also providing care. The study provides no individual benefit for study participants but could possibly lead to improved care in the future. The study was approved by the regional ethical review board in Stockholm (Dnr 2017/260-31/4, 2017/1834-32, and 2018/115-32).

4 RESULTS

4.1 STUDY I

In Study, I, a total of 588 people was found to have a confirmed VTE. A total of 73 patients with active cancer, 9 patients who died within 10 days of VTE, 13 patients not residing in Stockholm County, and five patients who were lost to follow-up were excluded from further analysis. During the 24 months following the VTE, 47 patients (9.6%) had a new cancer diagnosis, with 94 percent (44/47 patients) receiving a diagnosis within the first 12 months (Figure 3a). Lung (17%), prostate (17%), ovarian (11%), and hematological malignancies (11%) were the most prevalent cancer sites among those diagnosed. Four of the cases (8.5%) were recurrences of a previously diagnosed but not considered active malignancy.

Patients who were diagnosed with cancer at the start of the study (≤10 days after VTE, n=16,) were not considered candidates for occult cancer screening. As a result, a total of 472 patients were included in subsequent outcome analyses. The median age of the patients in this group was 68 (IQR 53-78), and 261 (55%) of them were men (Table 1). 261 (55%) of the patients had an unprovoked VTE, 234 (50%) had DVT, 197 (42%) had PE, and 41 (8.7%) had simultaneous DVT and PE. During the 11-day – 24-month follow-up period following VTE, 31 of the 472 patients (6.6%) were diagnosed with cancer.

Table 1. Baseline characteristics of study participants

	No active or new cancer diagnosis (n=441)	Cancer diagnosis 11 days - 24 months after VTE (n=31)
Male sex, No. (%)	247 (56)	14 (45)
Age, median (IQR), y	68	71
	(51-78)	(61-78)
BMI, median (IQR)	26.0	25.4
	(23.5-28.8)	(21.9-28.3)
Prior cancer, No. (%)	42 (10)	11 (36)
Initial VTE presentation, No. (%)		
DVT	222 (50)	12 (39)
DVT+PE	38 (9)	3 (10)
PE	181 (41)	16 (52)
Risk factors for VTE[a], No. (%)		
No provoking factor (unprovoked)	239 (54)	22 (71)
Recent surgery	51 (12)	1 (3)
Hospital stay	58 (20)	4 (13)
Bedridden/immobilized	53 (17)	4 (13)
Long distance travel	23 (5)	0
Estrogen use	27 (6)	2 (7)
Leg injury	40 (9)	1 (3)
Inflammatory disease	30 (7)	1 (3)
Prior VTE, No. (%)	99 (22)	8 (26)
Prior unprovoked VTE, No. (%)	58 (13)	4 (13)
COPD, No. (%)	30 (7)	7 (23)

Smoking, No. (%)	41 (9)	5 (16)
Prior smoking, No. (%)	181 (41)	18 (58)
Diabetes mellitus, No. (%)	36 (8)	4 (13)
Prior stroke/TIA, No. (%)	50 (11)	3 (10)
Prior MI, No. (%)	27 (6)	2 (6)
Heart failure, No. (%)	33 (8)	1 (3)
Platelet count, median (IQR), 10^9/L	215 (174-263)	225 (163-250)
Hemoglobin, median (IQR), g/L	139 (127-149)	134 (127-148)
WBC count, median (IQR), 10^9/L	8.4 (7.1-10.3)	8.2 (6-9.8)

IQR, Interquartile range; BMI, Body mass index; VTE, Venous thromboembolism; DVT, Deep vein thrombosis; PE, Pulmonary embolism; COPD, Chronic obstructive pulmonary disease; TIA, transitory ischemic attack; MI, myocardial infarction; WBC, White blood cell; H3Cit-DNA, Nucleosomal Citrullinated Histone H3; cfDNA, cell-free DNA; NE, Neutrophil elastase. [a] The provoking factors pregnancy, cesarean section, DVT with unilateral catheter, varicose vein surgery, and thoracic outlet syndrome were present in less than 10 patients each and are not presented above.

As five patients did not have a platelet count or hemoglobin levels, the RIETE score's performance was examined in 467 patients. According to the RIETE score, 27% (126/467) of these individuals were classed as having a high risk for an occult malignancy (i.e. $\geq$3 points). The cumulative cancer incidence in the high-risk group was 10.4% during follow-up, compared to 5.8% in the low-risk group, P = 0.079. (Figure 3a). The score's sensitivity was 0.39 (95% CI 0.24–0.56), specificity was 0.74 (95% CI 0.70–0.78), negative predictive value was 0.94 (95% CI 0.91–0.96), and positive predictive value was 0.095 (95% CI 0.055–0.16). The RIETE score had a C-statistic of 0.56 (95% CI 0.45–0.67). The performance of the RIETE score in men and women is seen in Figure 3b, c.
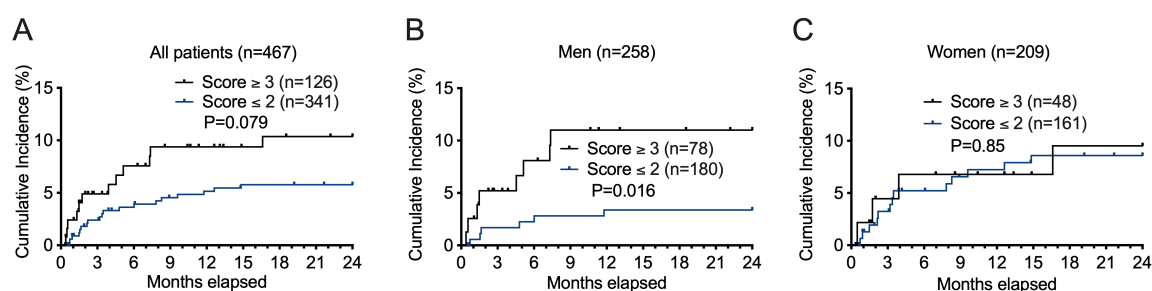

Figure 3. Cumulative incidence of new cancer diagnosis according to RIETE score.

A) Patients with a RIETE score $\geq$ 3 points versus $\leq$ 2 points. B) Male patients with a RIETE score $\geq$ 3 points versus $\leq$ 2 points. C) Female patients with a RIETE score $\geq$ 3 points versus $\leq$ 2 points.

4.2 STUDY II

As revealed by standard curves generated using two distinct lots of PAD4, in vitro PAD4-mediated citrullination can lead to considerable inter-lot variability of ELISA signal (Figure 4a). Spiking semi-synthetic citrullinated histone H3 into 100% human plasma, yielded almost no detectable recovery (Figure 4b). Nucleosomes carrying H3R2,8,17Cit, on the other hand, were restored at predicted levels (Figure 4b). The three different H3R2,8,17Cit dNucs lots

were highly similar (Figure 4c, d), implying that recombinant nucleosomes are appropriate for plasma-based immunosorbent assays.
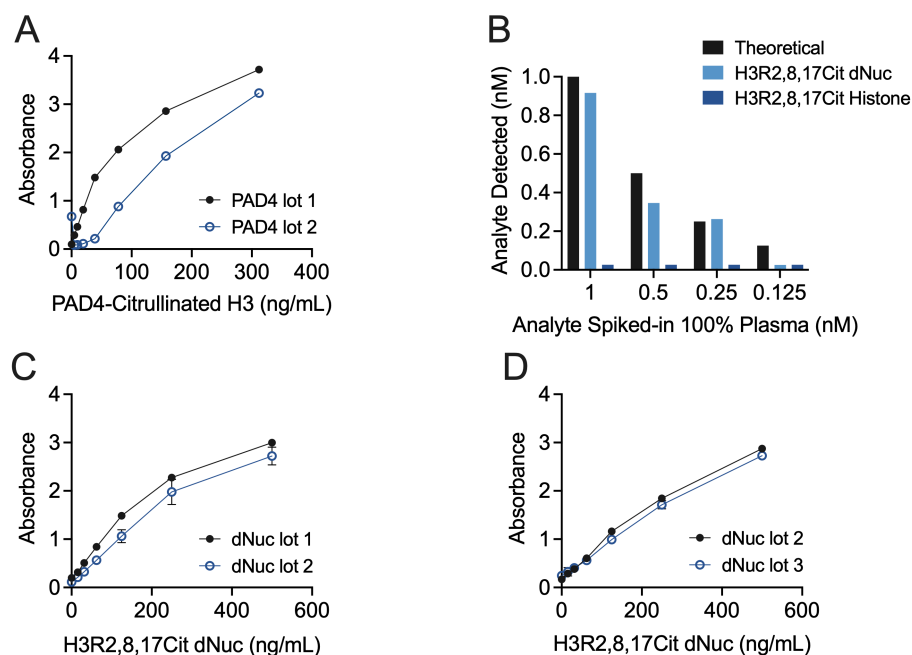
Figure 4. A) Two distinct lots of PAD4 were used to generate in vitro citrullinated histone H3 calibration standard curves, with standard curves of each preparation assayed by ELISA. The calibration curves produced by the lots are statistically different; $F_{(DFn, DFd)}$ 133.3 (4,6), $P < .0001$. B) After direct dilution into human plasma, recombinant H3R2,8,17Cit dNucs but not recombinant H3R2,8,17Cit histones are recovered at predicted amounts. C) ELISA standard curves for H3R2,8,17Cit dNucs lots 1 vs 2 show good inter-lot consistency. The curves do not vary statistically; $F_{(DFn, DFd)} = 2.186$ (4,34), $P = .0915$. D) ELISA standard curves for lots 2 vs 3 of H3R2,8,17Cit dNucs reveal no significant difference in the curves; $F_{(DFn, DFd)}$ 2.004 (4,10), $P = .1698$.

We created an ELISA that quantifies nucleosomal H3Cit complexes by combining a monoclonal capture antibody, a monoclonal double-stranded DNA detection antibody, and an H3R2,8,17Cit dNuc calibration standard (dubbed "H3Cit-DNA ELISA"). A systematic validation was carried out, with the working range, accuracy, linearity, parallelism, recovery, and selectivity all being evaluated (Figure 5a-f). The working range (LLOQ to ULOQ) was 20.5 ng/mL to 383.4 ng/mL. The results reveal that the accuracy is high, with intra- and inter-assay CVs of 3.3 and 8.9%, respectively (Figure 5a and b). Samples spiked beyond the ULOQ can be diluted into the working range (Figure 5c), and samples with high H3Cit-DNA endogenously can also be diluted into the working range (Figure 5d). Spiked concentrations of dNucs into plasma diluted into assay buffer could be recovered (Figure 5e). The detection of citrullinated nucleosomes but not unmodified nucleosomes demonstrated the assay's excellent selectivity (Figure 5f).
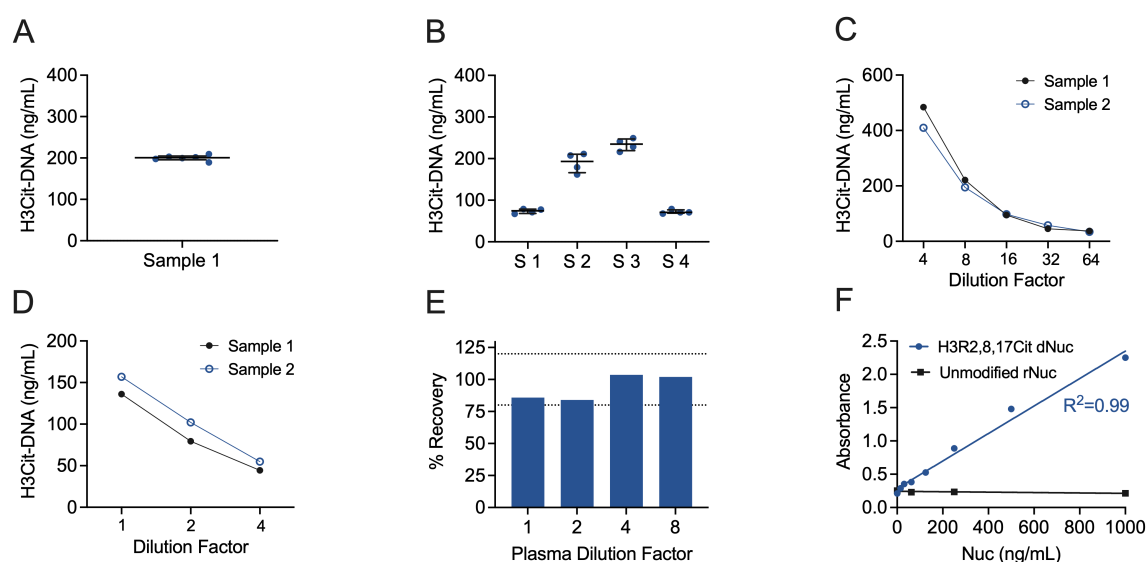
Figure 5. A) Intra-assay variability. The intra-assay CV for the same plasma sample in six repetitions on the same plate was 3.3%. B) Inter-assay variability. Four plasma samples (S1–S4) examined in duplicate on four different plates revealed an inter-assay CV of 7.4, 12.5, 6.2, and 6.5 %, respectively. The inter-assay CV as mean (SD) was 8.9% (2.9%). C) Dilution linearity. Two samples were spiked to 2000 ng/mL with H3R2,8,17Cit dNucs and serially diluted in assay buffer. The mean (SD) recovery for dilutions within the LLOQ and ULOQ working ranges was 88 % (18%). D) Parallelism. Two samples containing significant quantities of endogenous H3Cit-DNA complex were serially diluted in assay buffer. The mean (SD) CV was 16.9% (3%). E) Recovery. Different dilutions of plasma were prepared, and known concentrations of H3R2,8,17Cit dNucs were spiked to a theoretical concentration of 400 ng/mL. Mean (SD) recovery was 93.3% (10.4%). F) Selectivity. The ELISA detected H3R2,8,17Cit dNucs but not unmodified nucleosomes.

4.3   STUDY III

Among study participants with advanced cancer, the median age was 73 years (IQR 66–81), and 38 patients (36 %) were men. Breast (18%), prostate (16%), and lung (13%) were the most common tumor locations, with 89 percent of patients having known metastatic solid malignancy. The median survival time was 31 days, and 8 (7.5%) of the patients had not yet deceased at the end of the 180-day observation period.

We measured markers of neutrophil activation (NE), NET formation (H3Cit-DNA), and proposed NET inducers (sP-selectin, IL-8, and G-CSF) in patients with terminal cancer. Patients with terminal cancer had higher levels of NE, H3Cit-DNA, sP-selectin, IL-8, and G-CSF compared to healthy controls (Figure 6a–e). H3Cit-DNA levels were found to be elevated in all the tumor types studied. sP-selectin and IL-8 correlated moderately with NE and H3Cit-DNA, but not with G-CSF (Table 2). The neutrophil activation marker NE had a substantial correlation with H3Cit-DNA levels (Table 2). In univariate and multivariate adjusted Cox regression, NE and H3Cit-DNA were associated with poor prognosis (Figure 7).
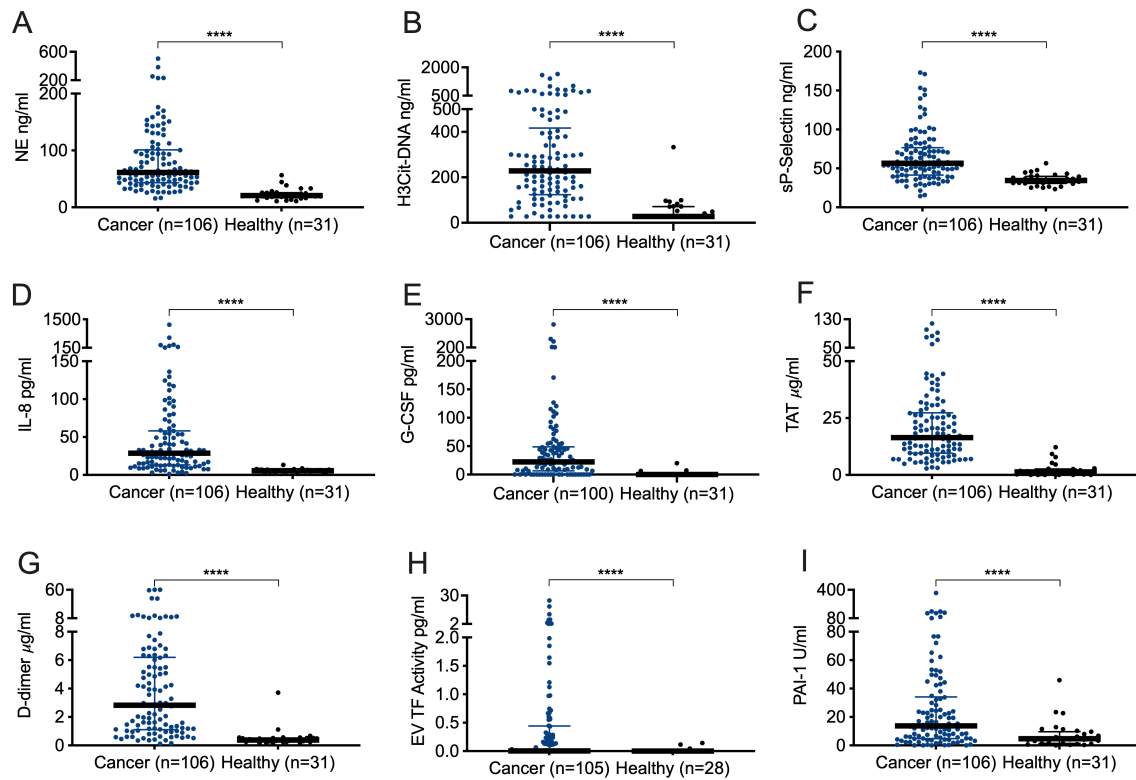
Figure 6. In patients with terminal cancer, plasma levels of markers of coagulation, fibrinolysis, neutrophil activation, and NETs, are higher than in healthy individuals. Lines represent the median with IQR. The Mann-Whitney U test was used to compare groups.
**** P < 0.0001.

Table 2. Correlations between measured circulating markers.

	sP-selectin	IL-8	H3Cit-DNA	NE	TAT	D-dimer	PAI-1	G-CSF	EV TF Activity
sP-selectin	1	0.320*	0.390*	0.487*	0.409*	0.304*	0.206*	-0.122	0.465*
IL-8	0.320*	1	0.228*	0.387*	0.065	0.273*	0.321*	0.187	0.373*
H3Cit-DNA	0.390*	0.228*	1	0.377*	0.172	0.235*	0.219*	-0.066	0.144
NE	0.487*	0.387*	0.377*	1	0.354*	0.243*	0.282*	0.037	0.388*
TAT	0.409*	0.065	0.172	0.354*	1	0.450*	0.106	0.028	0.287*
D-dimer	0.304*	0.273*	0.235*	0.243*	0.450*	1	-0.001	0.005	0.207*
PAI-1	0.206*	0.321*	0.219*	0.282*	0.106	-0.001	1	0.285*	0.265*
G-CSF	-0.122	0.187	-0.066	0.037	0.028	0.005	0.285*	1	0.033
EV TF Activity	0.465*	0.373*	0.144	0.388*	0.287*	0.207*	0.265*	0.033	1

sP-selectin soluble P-selectin, *IL-8* interleukin-8, *H3Cit* citrullinated histone H3, *NE* Neutrophil elastase, *TAT* Thrombin-antithrombin complex, *EV TF activity* extracellular vesicle tissue factor activity, *PAI-1* plasminogen activator inhibitor-1 activity, *G-CSF* granulocyte colony-stimulating factor. * *P* < 0.05.
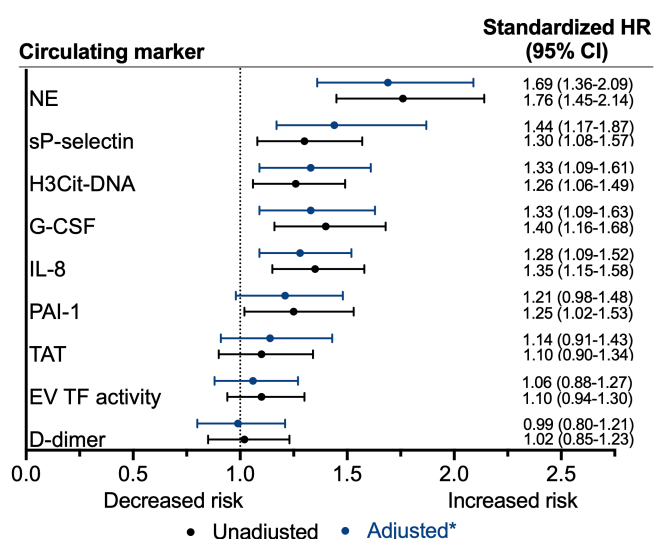
Figure 7. A forest plot of circulating markers as mortality risk predictors. *Adjusted for age, sex, metastatic disease, and medical treatment (oral anticoagulants, low molecular weight heparins [LMWHs], and corticosteroids)

HR, hazard ratio; sP-selectin, soluble P-selectin; IL-8, interleukin-8; H3Cit, citrullinated histone H3; NE, Neutrophil elastase; TAT, Thrombin-antithrombin complex; EV TF activity, extracellular vesicle tissue factor activity; PAI-1, plasminogen activator inhibitor-1 activity; G-CSF granulocyte colony-stimulating factor.

We measured procoagulant and anti-fibrinolytic circulation markers, as well as coagulation and fibrinolysis markers, in patients with terminal cancer to explore coagulation and fibrinolysis. Patients with terminal cancer had higher levels of EV TF activity, PAI-1, TAT, and D-dimer compared to healthy individuals (Figure 6f–i). In multivariate Cox regression, none of the coagulation and fibrinolysis markers were linked with poor prognosis, contrary to our expectation (Figure 7). We investigated correlations between NE and H3Cit-DNA with TAT, D-dimer, EV TF activity, and PAI-1 to see if there was a link between neutrophil activation, NETs, and coagulation/fibrinolysis (Table 2). D-dimer and PAI-1 were weak but significantly correlated with NE and H3Cit-DNA. EV TF activity and TAT correlated to NE, but not H3Cit-DNA.

4.4 STUDY IV

In study IV, a total of 500 patients were enrolled. 13 patients were excluded after a secondary review of medical records failed to confirm VTE, 2 patients did not meet inclusion criteria (one not included within 4 days of VTE and one not residing in Stockholm County), 3 patients were lost to follow-up, and 22 patients were excluded because citrated blood withdrawal was not possible at baseline. Patients with known active cancer at the time of VTE (n=45) were excluded. The remaining 415 patients in the primary outcome analysis were 263 men (63 percent) with a median age of 68 (IQR 53-77). At the time of inclusion, 203 (49%) of the patients had DVT, 51 (12%) of the patients had both DVT and PE, and 161 (39%) of the patients had PE. Anticoagulant medication had been started in 261 patients (63%) at the time of venous blood collection, and 218 (53%) of all VTEs were unprovoked.

Table 3. Baseline characteristics of study patients

	No active or new cancer diagnosis (n=386)	Cancer during follow-up (n=29)	Cancer diagnosis up to 10 days after VTE (n=14)	Cancer diagnosis 11- 365 days after VTE (n=15)
Male sex, No. (%)	143 (63)	20 (69)	10 (71)	11 (67)
Age, median (IQR), y	67 (51-77)	74 (68-80)	74 (70-76)	73 (63-85)
BMI, median (IQR)	26.6 (24.1-29.3)	25.9 (22.6-29.1)	26.2 (22.6 – 29.1)	25.6 (20.6-30.5)
Prior cancer, No. (%)	48 (12)	8 (28)	5 (36)	3 (20)
Initial VTE presentation, No. (%)				
DVT	190 (49)	13 (45)	3 (21)	10 (67)
DVT+PE	48 (12)	3 (10)	3 (21)	0 (0)
PE	148 (38)	13 (45)	8 (57)	5 (33)
Risk factors for VTE[a], No. (%)				
No provoking factor (unprovoked)	195 (51)	23 (79)	11 (79)	12 (80)
Recent surgery	54 (14)	3 (10)	2 (14)	1 (7)
Hospital stay	70 (18)	3 (10)	2 (14)	1 (7)
Bedridden/immobilized	46 (12)	4 (14)	2 (14)	2 (13)
Long distance travel	29 (8)	0 (0)	0 (0)	0 (0)
Estrogen use	22 (6)	0 (0)	0 (0)	0 (0)
Leg injury	67 (17)	0 (0)	0 (0)	0 (0)
Inflammatory disease	17 (4)	0 (0)	0 (0)	0 (0)
Prior VTE, No. (%)	84 (22)	3 (10)	0 (0)	3 (20)
Prior unprovoked VTE, No. (%)	47 (12)	1 (3)	0 (0)	1 (7)
COPD, No. (%)	18 (5)	5 (17)	2 (14)	3 (20)
Smoking, No. (%)	28 (7)	5 (17)	2 (14)	3 (20)
Prior smoking, No. (%)	151 (39)	17 (59)	10 (71)	7 (47)
Diabetes mellitus, No. (%)	38 (10)	3 (10)	2 (14)	1 (7)
Prior stroke/TIA, No. (%)	34 (9)	2 (7)	0 (0)	2 (13)
Prior MI, No. (%)	19 (5)	2 (7)	1 (7)	1 (7)
Heart failure, No. (%)	19 (5)	2 (7)	1 (7)	1 (7)
Platelet count[b], median (IQR), 10^9/L	227 (185-1272)	260 (179-322)	307 (205-383)	210 (176-282)
Hemoglobin[c], median (IQR), g/L	138 (126-148)	133 (117-150)	134 (98-150)	129 (118-150)
WBC count[d], median (IQR), 10^9/L	8.2 (6.5-10.2)	9.0 (7.1-10.9)	10.7 (8.8-12.9)	7.9 (7-9.6)
H3Cit-DNA, median (IQR), ng/mL	154 (106-261)	195 (87-285)	203 (102-513)	149 (82-252)
cfDNA, median (IQR), ng/mL	445 (391-510)	423 (364-590)	436 (368 – 653)	391 (351 – 547)
NE, median (IQR), ng/mL	31.6 (21.0-48.6)	41.4 (27.4-70.4)	58.6 (35.1 – 92.3)	35.6 (24.4 – 46.7)

IQR, Interquartile range; BMI, Body mass index; VTE, Venous thromboembolism; DVT, Deep vein thrombosis; PE, Pulmonary embolism; COPD, Chronic obstructive pulmonary disease; TIA, transitory ischemic attack; MI, myocardial infarction; WBC, White blood cell; H3Cit-DNA, Nucleosomal Citrullinated Histone H3; cfDNA, cell-free DNA; NE, Neutrophil elastase.

[a] The provoking factors pregnancy, cesarean section, DVT with unilateral catheter, varicose vein surgery, and thoracic outlet syndrome were present in less than 10 patients each and are not presented above.

[b] Platelet count was unknown in three patients

[c] Hemoglobin levels were unknown in two patients

[d] WBC count was unknown in three patients

A total of 29/415 patients (7.0%) were diagnosed with cancer during the one-year follow-up.

In the first 10 days after the VTE incident, half of the cancer cases (14/29) were diagnosed.

Table 3 shows the baseline characteristics of patients who had no active or new cancer diagnosis during follow-up and cancer during follow-up.

Colorectal cancer (17%), lung cancer (14%), pancreatic cancer (10%), breast cancer (10%), prostate cancer (10%), and upper gastrointestinal cancer (10%) were the most prevalent cancers discovered during follow-up. There were no malignancies found in patients under the age of 50.

When comparing patients who were diagnosed with cancer during follow-up to those who were not, average levels of H3Cit-DNA and cfDNA, but not NE, were significantly higher in patients with VTE who were diagnosed with occult cancer during follow-up (mean 326 [SD 414] ng/ml vs 217 [SD 197] ng/ml, p=0.0098, 516 [SD 235] ng/ml vs 462 [SD 113] ng/ml, p=0.028, and 53.8 [SD 6.9) ng/ml vs 43.3 (SD 2.35) ng/ml, p=0.23, respectively). There was a significant association between very high levels of H3Cit-DNA (>671 ng/ml) and cfDNA (>653 ng/ml) with cancer diagnosis during follow-up when dichotomized at the 95th percentile (Figure 8a, b). Notably, most malignancies were detected promptly after VTE in patients with very high levels of H3Cit-DNA and cfDNA, as shown by the Kaplan-Meier curve. During follow-up, there was a tendency toward a relationship between extremely high levels of NE (>106 ng/ml) and cancer diagnosis, albeit this result was not statistically significant (Figure 8c).
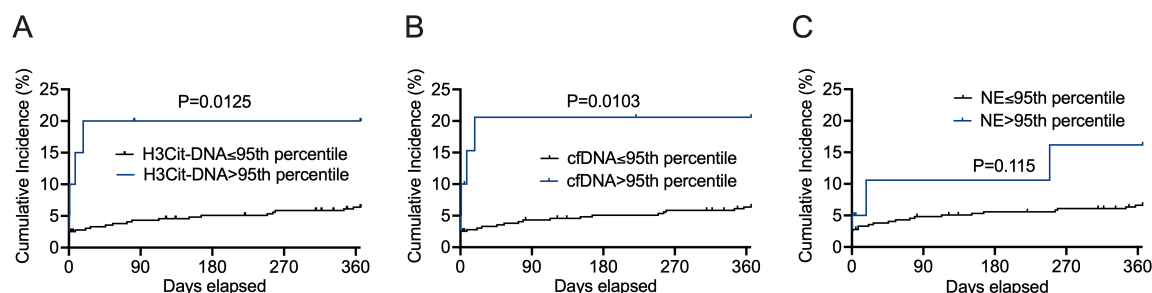
Figure 8. New cancer diagnosis during follow-up after acute VTE according to H3Cit-DNA, cfDNA, and NE levels.

VTE, Venous thromboembolism; H3Cit-DNA, Nucleosomal Citrullinated Histone H3; cfDNA, cell-free DNA; NE, Neutrophil elastase.

Continuous levels of H3Cit-DNA and cfDNA, but not NE, were linked with new cancer diagnosis in univariate Cox regression models (standardized HR of 1.38 [95% CI 1.09-1.76], 1.34 [95% CI 1.06-1.68] and 1.15 [95% CI 0.92-1.44], respectively (Table 5). Similarly, H3Cit-DNA and cfDNA levels above the 95th percentile were linked to new cancer diagnoses, but not NE (standardized HR of 3.50 [95% CI 1.22-9.02], 3.62 [95% CI 1.26-10.4], and 2.52 [95% CI 0.76-8.32], respectively). Five separate models with adjustments for age, gender, start of anticoagulant therapy, provoked VTE, previous malignancy, and COPD were built (Table 5). Consistently across all models, only circulating H3Cit-DNA levels were linked to the result.

Table 5. Time to cancer diagnosis and association with H3CitDNA, cfDNA, and NE (in univariable and multivariable cause-specific Cox regression models).

Analysis	Variable	HR	95% CI	*P*-value
Univariable	H3Cit-DNA (per SD increase)	1.38	1.09–1.76	0.007
	H3Cit-DNA (>95th percentile)	3.50	1.22–9.02	0.020
	cfDNA (per SD increase)	1.34	1.06–1.68	0.013
	cfDNA (>95th percentile)	3.62	1.26–10.4	0.017
	NE (per SD increase)	1.15	0.92–1.44	0.213
	NE (>95th percentile)	2.52	0.76–8.32	0.130
	Age (per 10-year increase)	1.51	1.14–2.00	0.004
	Female sex	0.77	0.35–1.69	0.514
	COPD	3.84	1.46–10.1	0.006
	Prior cancer	2.57	1.14–5.81	0.023
	Unprovoked VTE	3.53	1.44–8.67	0.006
Multivariable model 1 (adjusted for age and sex)	H3Cit-DNA (per SD increase)	1.28	1.01–1.62	0.044
	H3Cit-DNA (>95th percentile)	2.74	0.94–7.97	0.064
	cfDNA (per SD increase)	1.27	0.99–1.62	0.058
	cfDNA (>95th percentile)	2.64	0.90–7.7	0.076
Multivariable model 2 (adjusted for age and start of anticoagulant treatment)	H3Cit-DNA (per SD increase)	1.29	1.01–1.66	0.042
	H3Cit-DNA (>95th percentile)	2.95	1.02–8.55	0.046
	cfDNA (per SD increase)	1.25	0.98–1.61	0.071
	cfDNA (>95th percentile)	2.72	0.91–8.17	0.075
Multivariable model 3 (adjusted for age and provoked VTE)	H3Cit-DNA (per SD increase)	1.29	1.01–1.64	0.041
	H3Cit-DNA (>95th percentile)	3.09	1.07–8.92	0.037
	cfDNA (per SD increase)	1.34	1.04–1.73	0.024
	cfDNA (>95th percentile)	3.33	1.15–9.70	0.027
Multivariable model 4 (adjusted for age and prior cancer)	H3Cit-DNA (per SD increase)	1.30	1.02–1.66	0.033
	H3Cit-DNA (>95th percentile)	3.06	1.06–8.81	0.038
	cfDNA (per SD increase)	1.27	1.00–1.61	0.048
	cfDNA (>95th percentile)	2.60	0.89–7.60	0.080
Multivariable model 5 (adjusted for age and COPD)	H3Cit-DNA (per SD increase)	1.29	1.00–1.65	0.047
	H3Cit-DNA (>95th percentile)	2.90	1.00–8.39	0.049
	cfDNA (per SD increase)	1.25	0.97–1.60	0.080
	cfDNA (>95th percentile)	2.49	0.85–7.30	0.096

SD, standard deviation; H3Cit-DNA, Nucleosomal Citrullinated Histone H3; cfDNA, cell-free DNA; NE, Neutrophil elastase, COPD, Chronic obstructive pulmonary disease; VTE, Venous thromboembolism.

5 DISCUSSION

The work in this thesis highlights the importance of establishing further risk stratification tools in the identification of VTE patients with an increased risk of occult cancer. We provide further evidence of the role of NETs in cancer progression and cancer-associated thrombosis. Through the implementation of our thoroughly validated in-house generated H3Cit-DNA ELISA, we further demonstrate that circulating NET markers are elevated in VTE patients with occult cancer.

5.1 CURRENT RISK STRATIFICATION

In Study I, the incidence of cancer during follow-up after VTE was high after both provoked and unprovoked VTE. The RIETE score failed to identify individuals at high risk for occult cancer, owing mostly to poor performance in women. These findings corroborate the need for novel risk scores to identify VTE patients at risk of occult cancer.

Most guidelines recommend only limited cancer screening restricted to patients with unprovoked VTE (38). We observed a high rate of cancer both in provoked and unprovoked VTE, similarly to a recent study (46). Should the current practice of restricting occult cancer screening to patients with only unprovoked VTE be continued? If a patient presents with other risk factors for cancer such as high age, previous smoking, or prior cancer diagnosis and provoked VTE, I would consider a limited screening approach.

In line with prior data (119, 120), most (94%, 44/47) cancers were diagnosed within a year after VTE. The RIETE score was established to identify individuals at high risk of receiving a new cancer diagnosis within the next 24 months, and many prior studies used a 24-month follow-up. As a result, we planned to follow study patients for 24 months, although a one-year follow-up seems sufficient in studies going forward. A risk with a longer follow-up period is that it also increases the probability of incorporating cancer diagnoses that are unrelated to the index VTE.

Many recent studies on occult cancer in VTE patients exclude individuals who have had cancer at any point (46, 121) or during the last 5 years (44). A lower threshold for occult cancer screening in patients with prior cancer presenting with acute VTE should be considered.

In contrast to previous validations (122-124), the RIETE score was unable to identify a subset of individuals at high risk of occult cancer. In the male subgroup, the score did detect such a subset, but overall performance was low. The RIETE score's limited discriminative power was mostly owing to poor performance in women. Furthermore, in clinical practice components of the RIETE score such as age, anemia, thrombocytosis, chronic lung disease, and provocation by recent surgery are often considered when clinicians assess the risk of malignancy after VTE.

The study's key strength is the low risk of selection bias of the registry approach, as opposed to prospective trials which tend to include younger patients and have a risk of inclusion bias. The study included 96% of all participants in the database who were eligible for cancer screening. However, the retrospective aspect of our study, as well as the relatively small sample size, restrict our findings.

Taken together, our findings demonstrate that individuals with both unprovoked and provoked VTE had a high risk of new cancer diagnoses. Although the RIETE score was effective in males, the performance in women and in general is not promising enough to warrant implementation into clinical practice. As a result, additional risk score models are required.

5.2   CIRCULATING MARKERS OF NETS IN CANCER AND CAT

NETs are investigated and implicated in various disease states. However, a lack of reliable techniques has impeded the interpretation of sometimes contradicting data, highlighting the necessity for standardized tests. Our validated in-house ELISA for quantification of H3Cit (92) using in vitro enzymatically modified histones suffers from a high degree of variability, as well batch-to-batch variability of polyclonal antibodies used.

In study II we highlight the limitations of utilizing histones enzymatically citrullinated in vitro (or free histones in general) as calibration standard curves in human plasma and show the clear benefit of recombinant designer nucleosomes. Lastly, we present a reliable test that uses recombinant nucleosomes as calibration standard, as well as carefully chosen, specific monoclonal antibodies. The ability to detect H3Cit-DNA levels properly and reliably in human plasma samples is demonstrated by rigorous methodological validation of the assay performance parameters.

Importantly, while the current work focused on validating the assay for citrated plasma, H3Cit has been suggested as a sepsis biomarker (125), and elevations were detected in serum samples. Some analytes have been discovered to have different stability in plasma vs serum samples, particularly when centrifugation is delayed (126). Future studies should systematically validate the H3Cit-DNA assay for serum samples, similar to what we did here for citrated plasma.

H3Cit is extensively utilized as a NET marker. However, a significant issue that must be addressed is the absence of standardized methodologies for detecting and quantifying this post-translational modification. Furthermore, citrullinated histones can potentially originate from other sources than neutrophils – some tumors express PAD4 (127, 128) This study highlights the significance of developing more strict validations of presently utilized methodologies, as well as a unique approach to ensuring more robust and repeatable data that can be compared across disease contexts and laboratories. We hope that this assay quantifying H3Cit-DNA will be an important tool in elucidating the role of NETs in disease.

In study III, we further establish an association between neutrophil activation and NETs and prognosis in individuals with advanced cancer. Using our novel and carefully validated assay from Study II for detecting circulation H3Cit-DNA (117), our findings were consistent with our earlier study (76). Despite the well-known link between malignancy and thrombosis, we could not identify an association between coagulation and fibrinolysis markers and poor prognosis in patients with terminal cancer.

The levels of plasma H3Cit-DNA and the neutrophil activation marker NE were highly correlated. Furthermore, sP-selectin, which has been shown to induce NET formation through interaction with P-selectin glycoprotein ligand-1 (95), correlated to H3Cit-DNA. However, this could also be due to NET formation triggering platelet activation (97). IL-8 also correlated strongly to H3Cit-DNA, in line with its ability to induce NET formation (56). However, an origin of H3Cit-DNA complexes from tumor cells cannot be ruled out. PAD4 tumoral expression is of course still a possible source of H3Cit-DNA (128) which also has been shown to lead to the secretion of NET-like constructs containing H3Cit (127).

In our patient cohort, none of the markers of coagulation or fibrinolysis were linked to a poor prognosis. This is in contrast to the findings of numerous earlier studies (129-136). Important to note is that the patients in this study were palliative cancer patients with a very short median survival (31 days), compared to 264 days or longer in previous trials. A procoagulant phenotype in the earlier stages of cancer predicts a dismal prognosis. As these patients have terminal cancer and an extremely poor prognosis most patients have that procoagulant phenotype, which might explain why coagulation and fibrinolysis markers have little predictive value in this population.

As this was an exploratory study, the limited sample size makes comparing tumor subtypes and relationships between circulating markers with venous or arterial thromboembolism difficult. Other limitations include the possibility of bias and confounding variables (e.g. treatment). We used Cox regression with the circulating markers as continuous variables to reduce the bias that occurs with dichotomizing continuous variables.

Conclusively, our findings reveal that neutrophil activation and NET markers, but not coagulation and fibrinolysis markers, are substantially linked to an unfavorable prognosis in individuals with advanced cancer. The absence of relationships between NET marker H3Cit-DNA and markers of coagulation and fibrinolysis implicates that neutrophils and NETs could contribute to an unfavorable prognosis via mechanisms unrelated to coagulation. Further and larger studies should be conducted to evaluate neutrophil activation and NET markers in the search for objective prognostic models and new treatment targets in cancer.

After identifying the need for new biomarkers to identify VTE patients with a high risk of occult cancer in Study I, we finally performed a prospective observational cohort study. In Study IV, we document an association between high H3Cit-DNA levels and cancer identified during a one-year follow-up in patients with acute VTE. After controlling for established risk

variables such as age, sex, prior cancer, provoked VTE, and COPD, this association remained significant only for H3Cit-DNA.

The moderate correlations between H3Cit-DNA, cfDNA, and NE are consistent with earlier published research not investigating acute VTE (75, 76, 107, 137). However, it is crucial to emphasize that only H3Cit-DNA should be regarded as a NET-specific marker, as higher cfDNA and NE may not always indicate NET formation. cfDNA might come from other sources, such as apoptotic or necrotic cells, and NE is only a reflection of neutrophil activity (138, 139). Neutrophil activation and NET formation are inflammatory processes, and several studies have shown a strong correlation between inflammatory markers such as TNF-α and interleukins with NET markers (75, 76). Inflammatory markers are often increased during VTE (140). Because inflammation triggers neutrophil activation and NET formation, these markers are expected to be elevated in acute VTE. This elevation may be masking an elevation caused by occult cancer. Considering the foregoing, we expected that extremely high levels of H3Cit-DNA, cfDNA, and NE would be related to cancer diagnosis and dichotomized the variables at the 95th percentile.

In recent clinical trials investigating image-based cancer screening, the rate of cancer diagnosis was lower than predicted in power calculations (41, 44). A recent review and meta-analysis emphasized the decreasing incidence of occult cancer over time following VTE (141). We discovered that almost half of the cancer cases were diagnosed nearly immediately after the VTE event (in this study defined as cancer diagnosed up to and including ten days after VTE), which is consistent with previous research (10, 41, 142, 143). In keeping with recent clinical studies, we observed a low incidence (3.8%) of occult cancer after this interval, corroborating with the previously indicated trend of a decreasing incidence of occult cancer following VTE.

There are various limitations to this study. H3CitDNA, cfDNA, and NE were only measured at the time of VTE diagnosis. The occurrence of VTE is expected to impact the levels of these biomarkers. It is feasible that measures taken at a later timepoint following VTE will render different results, most likely pointing to a greater link between the biomarkers and cancer found during follow-up.

The similar baseline characteristics of included patients compared to the Study I evaluating all patients diagnosed with VTE at the same site throughout 2014 (143), indicate low risk of selection bias.

While high H3Cit-DNA levels were linked to new cancer diagnoses after VTE, H3Cit-DNA alone does not appear to be very useful as a risk predictor in identifying VTE patients with a high risk of hidden cancer. Nonetheless, our research suggests that H3Cit-DNA has the potential to be used as a cancer diagnostic marker in combination with other biomarkers and clinical variables and should be considered for multi-analyte cancer screening assays.

6 CONCLUSIONS

- The RIETE score failed to identify VTE patients with a high risk of occult cancer, highlighting the need for new risk models.
- Plasma levels of H3Cit-DNA can be quantified in human plasma using a novel and rigorously validated ELISA implementing solely commercially available reagents.
- This highly specific assay can be used to study the role of NETs in various disease settings.
- Markers of neutrophil activation and NETs are elevated in terminal cancer patients and associated with poor prognosis.
- There was no association between markers of NETs and markers of coagulation and fibrinolysis in terminal cancer patients, indicating that NETs contribute to a poor prognosis independently of coagulation.
- High levels of H3Cit-DNA are associated with occult cancer in patients with VTE, corroborating a role of NETs in the pathogenesis of cancer-associated thrombosis.

7 FUTURE PERSPECTIVE

There is a need for new approaches to identify patients presenting with VTE with a high risk of occult cancer - and in general to detect cancer at an earlier stage - in order to improve clinical outcomes. Accumulating evidence, including the results in this thesis, strongly indicates a role of NETs in cancer progression and CAT.

Further studies should investigate the potential of NETs as cancer diagnostic markers in other populations with a high risk of underlying cancer, such as patients presenting with non-specific symptoms. Combining several biomarkers and clinical factors into a multi-analyte risk score is likely to yield a higher sensitivity for cancer detection compared to a single analyte, and NET markers should be considered for inclusion in such a test. Ultimately, targeting NET formation could also potentially be a route to reducing the burden of CAT.

8   ACKNOWLEDGEMENTS

David, Viktor, Isak, Felix, Jesper, and Julius – thank you for making med school so much fun!

The Bästis-gang - **Vincent, Harald, Fredrik, Jakob, Johan, Mattias, and Anton** – thank you for keeping my mind off work!

Charlotta, Gustaf, Fanny, and Maja, my family, thank you for always supporting and believing in me. **Fanny and Ruben**, my new family - thank you Ruben for sleeping so well that I managed to write this thesis, and thank you, Fanny, for your endless support and helping me prioritize. I love you.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Trots nästan två århundraden av forskning kring mekanismerna bakom cancer-associerad trombos, är orsaken fortfarande inte helt säkerställd. Neutrofiler, vita blodkroppar delaktiga i vårt medfödda immunförsvar, kan utsöndra sitt DNA när de stimuleras starkt. I djurmodeller har *neutrophil extracellular traps* (NETs) visat sig orsaka blodproppar hos cancerdjurmodeller.

Patienter med oförklarlig venös tromboembolism (VTE) har en hög (5–10%) risk för dold cancersjukdom. Randomiserade kliniska prövningar har inte visat någon vinst med utförlig screening jämfört med begränsad screening för dessa patienter. Det har nyligen utvecklats en riskmodell för att välja ut vilka patienter som bör genomgå utförlig screening. I Studie I har vi utvärderat den hos alla patienter som diagnosticerats med VTE på Danderyds sjukhus under 2014. Riskmodellen lyckades inte välja ut patienter med hög risk för dold cancer, vilket indikerar att nya metoder behövs.

Att mäta förekomsten av bildningen av NETs i blod är svårt och ännu inte standardiserat. I Studie 2 utvecklade vi en ny metod för att mäta ett protein som är specifikt för NETs-bildning, citrullinerat histon H3-DNA (H3Cit-DNA).

I Studie III utvärderade vi denna metod på patienter med avancerad cancersjukdom, och det visade sig att de patienterna med höga nivåer av H3Cit-DNA hade en dålig prognos.

I Studie IV inkluderas 500 patienter med VTE på Danderyds sjukhus. I samband med inklusion lämnades blodprov, och vi mätte H3Cit-DNA. Patienter med höga nivåer av H3Cit-DNA hade en högre risk att diagnosticeras med cancer under en uppföljning på ett år. Detta bekräftar de data från djurmodeller att NETs verkar ha en roll vid utvecklingen av cancer-associerad trombos, samt indikerar att H3Cit-DNA skulle kunna ha potential som diagnostisk cancermarkör.

Sammantaget talar dessa resultat för att bildningen av NETs bidrar till bildningen av cancerassocierad trombos, men bidrar också till en dålig prognos hos cancerpatienter. Framtida studier bör fokusera både på att utforska vidare potentialen av markörer för NETs som diagnostiska cancermarkörer, men också om blockering av bildningen av NETs kan vara en behandling mot cancer eller dess komplikationer.

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