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TROPONIN ELEVATION IN ACUTE STROKE

- CLINICAL CHARACTERISTICS AND THE LINK TO CANCER-ASSOCIATED NEUTROPHIL EXTRACELLULAR TRAPS

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- clinical characteristics and the link to cancer-associated neutrophil extracellular traps

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In loving memory of my grandmother Marianne Fries

ABSTRACT

Elevated plasma levels of troponin, a marker of myocardial injury, are a frequent observation in stroke patients. Despite several reports on an adverse short-term prognosis, however, the significance of troponin elevation in stroke is still controversial, and the myocardial injury often lacks a clear etiology. The aim of this thesis was to determine patient characteristics, including long-term prognosis, in these patients, as well as to explore possible underlying pathomechanisms.

In a retrospective cohort analysis of 247 stroke patients (**Study I**), troponin elevation was significantly associated with age, comorbidity burden, and stroke severity. Stroke patients with troponin elevation also had a higher prevalence of electrocardiographic changes suggestive of myocardial ischemia on admission. A 5-year follow-up period revealed an almost 2-fold increased risk of mortality, with an adjusted hazard ratio of 1.90 (95% CI 1.34-2.70).

In an explorative case-control study (**Study II**), we furthermore suggest that cancer may be a contributing factor to the poor prognosis in these patients, showing a significant prevalence of underlying cancer among ischemic stroke patients with high troponin elevations. Plasma analyses were strongly supportive of a hypercoagulable state in these patients, and histopathological investigations revealed widespread arterial microthrombi in several organs including the heart. Neutrophil activation, with the release of highly pro-coagulant extracellular chromatin, referred to as *neutrophil extracellular traps* (NETs), has recently been proposed to play a central role in cancer-associated venous thromboembolism. We therefore proceeded to investigate the role of NETs in the cancer-associated hypercoagulable state seen in the ischemic stroke patients with high levels of plasma troponin as well as an underlying malignancy. As with markers of coagulation, plasma markers of NETs were significantly elevated in these patients, and there were significant positive correlations between the two. Histopathological investigations further supported the role of NETs in the thrombotic state by immunodetection of NET markers in arterial microthrombi. To assess a circulating NET burden in these patients, a novel ELISA-based assay to quantify the NET-specific marker H3Cit in plasma was developed, and subsequently standardized and methodologically validated (**Study III**) revealing a high specificity, precision and stability of the assay.

These results support cardiologic work-up and more aggressive prevention measures in stroke patients with troponin elevation. They furthermore suggest that an underlying cancer should be considered in ischemic stroke patients with unexplainably high plasma levels of troponin. Finally, we link this hypercoagulable state to NETs, and therefore encourage further studies to explore whether markers of NETs could serve as novel diagnostic and prognostic tools in the setting of cancer-associated arterial thrombosis. To this end, we suggest a novel ELISA-based assay to quantify the NET-specific marker H3Cit in plasma.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original papers, which will be referred to as **Paper I-IV**:

I. **Thålin C**, Rudberg AS, Johansson F, Jonsson F, Laska AC, Nygren AT, von Arbin M, Wallén H, Aspberg S. Elevated Troponin Levels in Acute Stroke Patients Predict Long-term Mortality.
J Stroke Cerebrovasc Dis 2015;24:2390-6.

II. **Thålin C**, Blomgren B, Mobarrez F, Lundstrom A, Laska AC, von Arbin M, von Heijne A, Rooth E, Wallén H, Aspberg S. Trousseau's Syndrome, a Previously Unrecognized Condition in Acute Ischemic Stroke Associated With Myocardial Injury.
J Ivestig Med High Impact Case Rep 2014 Jun 24;2(2):2324709614539283. doi: 10.1177/2324709614539283

III. **Thålin C**, Demers M, Blomgren B, Wong SL, von Arbin M, von Heijne A, Laska AC, Wallén H, Wagner DD, Aspberg S. NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation.
Thromb Res 2016;139:56-64

IV. **Thålin C**, Daleskog M, Paues Göransson S, Schatzberg D, Lasselin J, Laska AC, Kallner A, Helleday T, Wallén H, Demers M. Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma.
Submitted.

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute Coronary Syndrome
AF	Atrial Fibrillation
AMI	Acute Myocardial Infarction
CAD	Coronary Artery Disease
CD142	Tissue Factor specific, Cluster of Differentiation
cfDNA	Cell free DNA
CHF	Chronic Heart Failure
CI	Confidence Interval
CK18	Cytokeratin 18
CT	Computed Tomography
CV	Coefficient of Variation
ECG	Electrocardiography
ELISA	Enzyme-Linked Immunosorbent Assay
G-CSF	Granulocyte Colony-Stimulating Factor
H3Cit	Citrullinated Histone H3
HR	Hazard Ratio
hsTnI	High Sensitive Troponin I
hsTnT	High Sensitive Troponin T
ICB	Intracerebral Bleeding
MPO	Myeloperoxidase
MPs	Microparticles
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NIHSS	National Institute of Health Stroke Scale
O.D.	Optical Density
OR	Odds Ratio
p value	Probability Value
PAD4	Peptidylarginine Deiminase 4
PE	Pulmonary Embolism
ROS	Reactive Oxygen Species

RR	Relative Risk
SD	Standard Deviation
sP-selectin	Soluble P-selectin
TAT	Thrombin-Antithrombin Antigen
TF	Tissue Factor
TF+MPs	Tissue Factor Positive Microparticles
TFPI	Tissue Factor Pathway Inhibitor
TnI	Troponin I
TnT	Troponin T
TOAST	Trial of Org 10172 in Acute Stroke Treatment
VTE	Venous Thromboembolism
VWF	Von Willebrand Factor

1 INTRODUCTION

The cardiac biomarkers troponin I and T are specific indicators of myocardial injury. As such, they are essential in the diagnosis of myocardial infarction (1), but are also known to be elevated in a portion of patients with acute stroke (2). Most studies report an association between troponin elevation in acute ischemic stroke and poor short-term outcome. However, although troponin elevation reflects myocardial injury, it does not reveal the etiology, and multiple mechanisms have been proposed to play a role. Although the current AHA/ASA guidelines for the early management of patients with acute ischemic stroke recommend assessment of cardiac biomarkers in all ischemic stroke patients (3), this is routine in far from all stroke centers. Furthermore, there are no established guidelines on how to interpret troponin elevation in ischemic stroke, or how these patients should be treated and followed-up. Further knowledge of the different pathomechanisms could lead to new insights as to how these patients should be handled. The work in this thesis explores clinical characteristics and prognosis in ischemic stroke patients with troponin elevation, with a focus on the previously unrecognized contribution of cancer and neutrophil activation.

1.1 THE TROPONIN COMPLEX

The troponin protein complex is a regulatory protein controlling the calcium-mediated interactions between actin and myosin in striated muscle. It consists of three subunits; C, I and T. Unlike cardiac troponin C (cTnC), which is identical to the troponin C expressed in skeletal muscle, troponin T (TnT) and I (TnI) are specific to the heart and thus play a central role in the diagnosis of myocardial infarction (1). The half-life of TnT and TnI in the blood is 2 hours (4), they appear in blood 3-6 hours after myocardial injury, peak after 12-24 hours and return to baseline after 7-10 days (5). The majority of TnI and TnT are structurally bound in the 3-unit complex (troponin I, T and C), and the degradation of the myofibril in myocyte damage results in a slow release of TnI and TnT over many days (6). A smaller portion; 6-8% for TnT and 3-4% for TnI, are free cytoplasmic components, which account for the detection of TnI and TnT in plasma during early stages of myocardial damage (6). Although the troponin release does not indicate the underlying mechanism of injury, it is considered to indicate acute or chronic myocardial damage (4). Measuring troponin serially is one way to distinguish between acute and chronic myocardial injury. Acute myocardial injury is more likely to present with dynamic patterns of plasma troponins (7), whereas chronic conditions present with stable elevations of troponin.

In recent years, high sensitive troponin assays have been developed, both for troponin I (hsTnI), and troponin T (hsTnT). These high-sensitivity assays have a substantially higher sensitivity than the conventional assays, allowing measurement of TnI and TnT in ng/l rather than µg/l. However, although they have proven superior in diagnosing acute coronary syndrome (ACS) (8) by detecting even minor myocardial damage, they also pose increasing dilemmas in interpreting elevations in conditions other than ACS.

1.2 TROPONIN ELEVATION IN ACUTE ISCHEMIC STROKE

1.2.1 Prognostic significance

It has long since been known that acute stroke may be followed by electrocardiographic (ECG)-changes as well as elevation of different cardiac proteins in plasma (9, 10). One of the first studies on troponin elevation in ischemic stroke patients was published 2000 by James et al (11), reporting a three-fold increased risk of in-hospital mortality as well as a two-fold increased risk of discharge to institutional care if TnT > 0.1 µg/l. The prognostic value of troponin elevation in ischemic stroke patients has since then been confirmed by several studies, the three largest to date by Peddada et al (12) comprising 1,145 ischemic stroke patients (OR 4.28, 95% CI 2.40-7.63, of in-hospital mortality if hsTnI > 0.12 ng/ml), Scheitz et al (13) comprising 1,016 ischemic stroke patients (RR 2.3, 95% CI 1.1-4.7, of in-hospital mortality in patients with dynamic hsTnT elevations), and Lasek-Bal et al (14) comprising 1,068 first-ever ischemic stroke patients (RR 3.05, 95% CI 1.65-5.65, for 30 days mortality if hsTnI > 0.014 ng/ml). Other studies have, however, reported on the lack of association between troponin elevation and an increased mortality (15-18); but these studies were smaller, and the conflicting results may be due to different assays, cut-off levels and inclusion criteria. The pooled analysis of 15 studies by Kerr et al in 2009 (2), including 2,901 stroke patients, revealed an independent association between troponin elevation and mortality (OR 2.9; 95% CI 1.7–4.8), suggesting that elevated troponins in acute stroke is indeed associated with an increased risk of mortality. Although few studies report cause of death, the study by Di Angelantonio et al (19), comprising 330 ischemic stroke patients with a follow-up of six months, showed that 2/3 of the deceased patients in the group with high elevations of troponin (TnI > 0.4 ng/mL) died of cardiac deaths in contrast to 1/3 of the deceased patients in the group with normal levels of troponin (TnI<0.1 ng/mL). Likewise, the recent study by Stahrenberg et al (20) showed an association between troponin elevation in ischemic stroke patients and cardiovascular event and mortality. Most studies, however, explored the short-

term outcome (mortality or functional outcome), leaving limited knowledge of the long-term prognosis of these patients. We therefore sought to determine the long-term prognosis of stroke patients with troponin elevation in **Study I.**

1.2.2 Pathophysiology

The majority of stroke patients have substantial comorbidity, among them renal insufficiency and congestive heart failure (CHF). Renal insufficiency and CHF are recognized causes of stable and chronic troponin elevation (21, 22), and may thus be important confounders contributing to elevated troponin levels in acute stroke patients. Other suggested causes are precedent atrial fibrillation (AF) (23), concomitant ACS (24), and a neurologically induced myocardial injury due to sympathoadrenal activation (16). These previously proposed mechanisms are summarized in *Figure 1*, and discussed in more detail below.

AF is a common source of cardiac emboli to the brain, causing 20-40% of all ischemic stroke events (25, 26), and patients with AF have a 5-fold increased risk of developing an ischemic stroke (26). AF is also associated with troponin elevation as a result of a "demand ischemia" with myocardial stress due to increased and variable heart rates (27-29). Several recent studies have reported on a higher prevalence of new onset AF (diagnosed on admission or during in-hospital cardiac monitoring) in ischemic stroke patients with troponin elevation (23, 30-32), with a four to six times increased risk of the detection of new onset AF. The largest study by Scheitz et al, 2015 (30), comprising 1,228 ischemic stroke patients without known AF on admission, provided an optimal cut-off value of hsTnT > 17 ng/L to predict new onset AF. Likewise, troponin elevation in patients with known AF has been associated with an increased risk of developing stroke. A sub study of cardiac biomarkers in over 6,000 patients with AF in the clinical trial Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) (33) found an independent association between TnI levels and the risk of stroke, with an almost doubled risk in the highest TnI group (≥0.040 µg/L). In line with this, the Apixaban for Reduction in Stroke and other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) trial (34), found an almost 2-fold increased risk for stroke or systemic embolism in the highest hsTnI group (≥10.1 ng/L). These large clinical trials, however, studied patients with AF and at least one additional risk factor for stroke, as all patients were treated with anticoagulants.

Type 1 acute myocardial infarction (AMI), i.e. thrombus formation on acute ruptured atherosclerotic plaques or embolization, may be another cause of troponin elevation in

ischemic stroke patients, supported by the similar risk factors and the high prevalence of coronary atherosclerosis in patients with cerebral infarction (35). Indeed, several studies report significantly higher incidence of ST segment deviations suggestive of acute myocardial ischemia on admission electrocardiography (ECG) in ischemic stroke patients with troponin elevation (2, 19, 24, 36-39), and the above mentioned pooled analysis of 15 studies by Kerr et al (2) reported a 3-fold increase in the prevalence of ECG changes suggestive of myocardial ischemia in stroke patients with troponin elevation (OR 3.03; 95% CI 1.49– 6.17). The study by Raza et al (40), comprising 200 ischemic stroke patients, showed an association with the diagnosis of non-fatal AMI and TnI>0.4 ug/L (41.2 vs. 3.3 %, p<0.001). A few studies also show an association between troponin elevation and echocardiographic wall motion abnormalities in these patients (31, 38, 39, 41), although only Song et al (38) restrain the definition of echocardiographic abnormality to focal wall hypokinesia suggestive of AMI. Two recent studies investigated coronary vessel status in these patients, presenting contradicting results; Mochmann et al, 2016 (42), and Zeus et al, 2016 (43). The case-control study by Mochmann et al compared coronary angiographic findings in 29 patients with acute ischemic stroke and hsTnT elevation with age- and sex-matched patients presenting with non-ST-segment-elevation ACS (NSTE-ACS) with similar baseline hsTnT levels. Despite the angiographic evidence of a coronary culprit lesion in 25% of the acute ischemic stroke patients with troponin elevation, they were significantly less frequent than in patients with NSTE-ACS (80%), and half of the acute ischemic stroke patients with troponin elevation had no angiographic evidence of coronary artery disease (CAD). On the contrary, however, the cohort study by Zeus et al, including 84 consecutive ischemic stroke patients with troponin elevation and abnormal ECG and/or clinical symptoms of ischemia found that hsTnT $\geq$ 0.03 ng/ml was associated with culprit lesion CAD with a RR of 1.5 (95% CI 1.1-2.2). However, the study design, troponin assays, and control groups differed between these studies, as did patient inclusion criteria, which may explain the discrepancies. Furthermore, stroke related severe infections, respiratory failure and pulmonary embolism (PE) have been related to myocardial injury due to impaired oxygen supply (44).

Insular areas of the brain play a central role in controlling the autonomic network and the neural outflow of catecholamines to the heart. An exaggerated catecholamine surge due to lesions in these areas could result in a stress cardiomyopathy (10, 16, 45-49), rendering a generalized myocytolysis (50-53), as opposed to a localized infarction due to acute coronary artery thrombus formation. Elevated levels of catecholamines activate calcium channels resulting in a metabolic disturbance with hypercontraction of sarcomeres, a reduced muscle

contraction and a subsequent cardiac dysfunction. An increase in catecholamine levels could also result in platelet activation (54), tachyarrhythmia, hypertensive crisis and coronary vasoconstriction resulting in myocardial injury with elevated levels of plasma troponin. In support of this are several studies revealing data on a higher prevalence of insular lesions in stroke patients with troponin elevation (38, 49, 55). Furthermore, Barber et al (16) reported an association between elevated troponin levels and elevated serum epinephrine, although the levels of epinephrine were modest, and within the reference range of <0.4 nmol/L. Some of the myocardial damage in acute ischemic stroke may thus be due to a direct stroke-induced neurogenic myocardial injury, especially in patients with pre-existing coronary stenosis, contributing to a proportion of the troponin elevations.

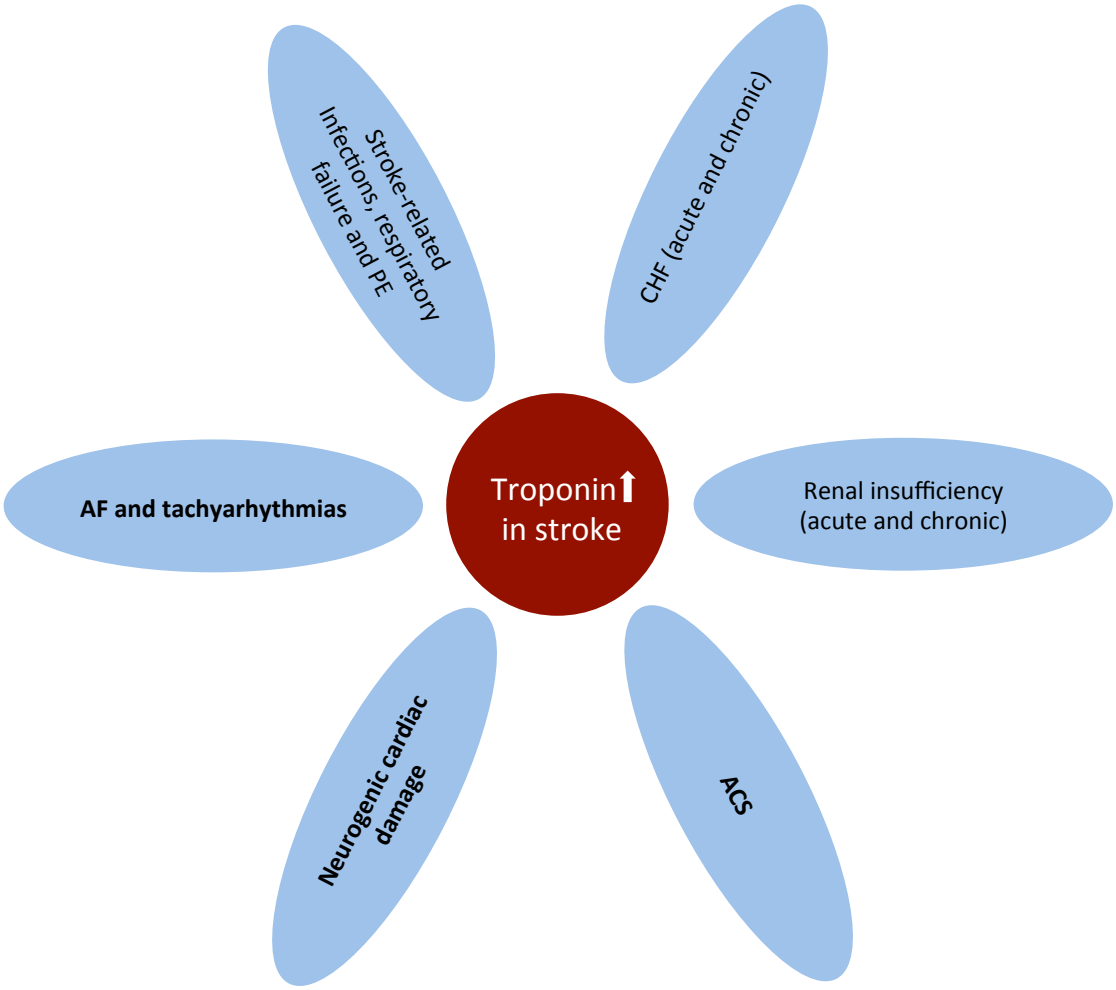

Figure 1. Previously proposed mechanisms behind troponin elevation in patients with acute ischemic stroke.

1.3 CANCER-ASSOCIATED THROMBOSIS

Despite recent data on cancer emerging as a previously underestimated risk factor for ischemic stroke (56-59), the contribution of an underlying malignancy to troponin elevation in these patients remains unrecognized. Due to the unexpected occult malignancy found at autopsy of one of the first included stroke patients with high levels of plasma troponin in **Study II** (described in Paper II), and the subsequent findings of known or occult malignancies in several ischemic stroke patients with troponin elevation, we explored possible pathomechanisms leading to a cancer-induced hypercoagulable state. Following is therefore a brief summary of the current knowledge of cancer-associated thrombosis.

Armand Trousseau was the first to describe the link between cancer and thrombosis in 1865 (60), and venous thromboembolism (VTE) has since then emerged as a well known complication in several malignancies. Cancer patients have been reported to have a 4-7-fold increased risk for VTE (61, 62), and VTE is associated with substantially increased morbidity and mortality in cancer patients (63-65). Moreover, unprovoked VTE may be the earliest sign of cancer (66). The majority of prior data on cancer-associated thrombosis revolves around VTE, while cancer-associated arterial thrombosis, such as ischemic stroke and myocardial infarction, is less investigated. Interestingly, however, an autopsy study over three decades ago, comprising 3,426 cancer patients (excluding intracranial neoplasms), reported cerebrovascular lesions in as many as 14.6% of the patients (67). Recent studies are now reporting a higher prevalence of prior cancer in ischemic stroke patients than in the general population (56), as well as an increased risk of recurrent stroke and cardiovascular mortality among stroke patients with a history of cancer (59). A Swedish large nationwide follow-up study also showed that several malignancies were associated with an increased risk of both ischemic stroke (68) and ACS (69) during the first 6 months after diagnosis. Although common risk factors, such as smoking, predisposes patients to both cancer and ischemic stroke, several recent studies show an overrepresentation of cryptogenic and embolic stroke, with infarcts in multiple vascular territories, as well as elevated d-dimer levels in stroke patients with an underlying cancer (70-72). These findings suggest a causal relationship, such as a cancer-induced hypercoagulable state, rather than merely a coincidence.

The mechanisms by which cancer drives coagulation are not fully clarified, but several pathways have been proposed to play a role, among them tumor cell tissue factor (TF) and tumor-derived tissue factor-positive microparticles (TF+MPs) (73-75). Many tumors express TF (76), a transmembrane glycoprotein that functions as the primary initiator of the

coagulation cascade. Under normal conditions, TF is not detectable in the blood, but tumor-released TF+MPs in the circulation could contribute to a hypercoagulable state through pathological activation of the extrinsic pathway of the coagulation cascade. Soluble P-selectin (77) and platelet activation (78) have also been proposed to play a role, perhaps mediated by mucin-secreting carcinomas (79, 80), contributing to platelet-rich microthrombi. In addition, emerging data now suggests a role of neutrophil activation, with the release of highly pro-coagulant chromatin (81-83), referred to as neutrophil extracellular traps and discussed below, in cancer-associated thrombosis.

1.4 NEUTROPHIL EXTRACELLULAR TRAPS

Upon activation, neutrophils can release extracellular chromatin structures referred to as neutrophil extracellular traps (NETs). In light of the, at the time just published, data on NETs contributing to cancer-associated thrombosis (81), **Study II** explored and found results suggestive of a cancer-induced NET burden in the above mentioned stroke patients with troponin elevation and an underlying cancer. A brief introduction to NETs is therefore also requested.

NETs were first described over a decade ago as an antimicrobial response in which activated neutrophils release their chromatin (nuclear DNA in complex with histones) into the extracellular space (84). Upon neutrophil activation with the NAD phosphate (NADPH) oxidase-dependent production of reactive oxygen species (ROS), antimicrobial granular proteases and the enzyme peptidylarginine deiminase 4 (PAD4) enter the nucleus (85). Once intra-nuclear, PAD4 converts positively charged peptidylarginine residues to uncharged peptidylcitrulline on histone H3, causing a loss of ionic interactions leading to chromatin decondensation (86), the initial step of NETosis**.** The decondensated chromatin is subsequently extruded into the extracellular space (*figure 2*). Coated with antimicrobial granular proteases, such as neutrophil elastase (NE), myeloperoxidase (MPO) and cathepsin G, these web-like chromatin structures, i.e. NETs, were shown to trap and kill microbes (84). This oxidant-dependent event, referred to as “lytic” or “suicidal” NETosis, takes up to 3-4 hours, and requires lytic cell death of the neutrophil. Another distinct form of NETosis, termed “vital NETosis” has also been described, involving budding of microvesicles extruded into the extracellular space where they rupture and expel NETs. This very rapid process takes 5-60 minutes, is oxidant-independent, and does not require neutrophil lysis, with preservation of neutrophil function including phagocytosis and chemotaxis (87).

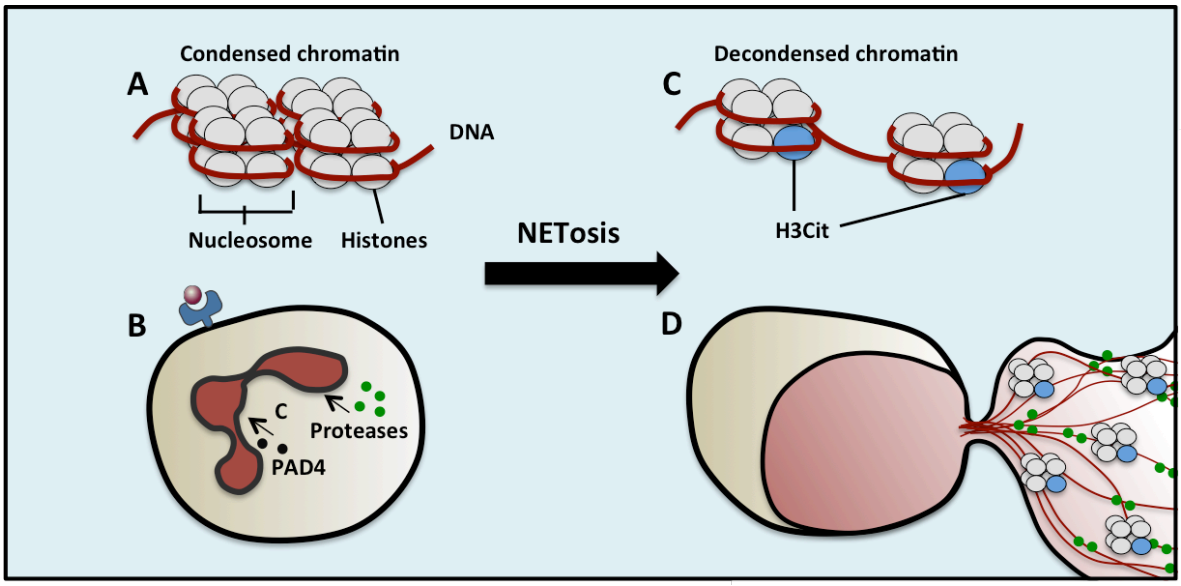

Figure 2. NETosis. **A -** Nucleosomes are tightly packed DNA segments wound in sequence around eight histone protein cores, further organized to form condensed chromatin in the nucleus. **B -** Upon neutrophil activation, antimicrobial granular proteases and peptidylarginine deiminase 4 (PAD4) enter the nucleus, and PAD4 citrullinates the positively charged arginine residues on histone H3 to uncharged citrullines, H3Cit. **C -** Reducing the strong positive charge of histones causes a weakening in the histone-DNA binding, leading to chromatin decondensation. **D -** The decondensated chromatin, comprising DNA, histones and antimicrobial granular proteases (i.e. NETs) is subsequently extruded into the extracellular space. This schematic depicts the "suicidal" form of NETosis.

Despite the beneficial role of NETs in eliminating pathogens as part of the innate immune system, disordered regulation and excessive formation of NETs may be harmful to the host. Indeed, NETs are implicated in the pathophysiology of a growing number of non-infectious conditions such as autoimmune diseases (88, 89), diabetes mellitus (90, 91), pulmonary disease (92) and thrombosis (93). The mechanisms by which NETs promote thrombosis are not entirely known, but NETs have been proposed to provide scaffolds for platelets, red blood cells, TF (94, 95), and plasma proteins promoting and stabilizing thrombi, such as von Willebrand factor (VWF), fibronectin, and fibrinogen (96). Nucleosomes and the NET-associated proteases have also been shown to enhance coagulation by suppressing tissue factor pathway inhibitor (TFPI) (97), and histones have been proposed to activate platelets, trigger VWF secretion (98) and increase plasma thrombin generation by impairing thrombomodulin (99). Furthermore, nucleic acids have been proposed to activate factor XII (100) in the intrinsic pathway, activating coagulation. In light of these pro-thrombotic abilities, NETs have been studied in a variety of thrombotic diseases, such as VTE (96, 98, 101-103), ACS (104-106) and ischemic stroke (107-111).

The role of inflammation in cancer is generally accepted (112), although the interplay between the immune system and cancer is far from fully understood. A role of NETs in cancer biology is now emerging, and recent studies suggest a cancer-induced NETosis contributing to both tumor progression (113-115), metastasis (116-118), thrombosis (81, 83, 115) and multiple organ failure (82) in cancer. The mechanisms by which cancer induces NETosis are under investigation, but cancer-released granulocyte colony-stimulating factor (G-CSF) has been shown to prime neutrophils toward NETosis, and G-CSF neutralizing antibodies have been shown to hamper NETosis (81, 82). These experiments were, however, conducted in murine models of cancer, and little is known of the mechanisms behind cancer-induced NETosis in human.

1.4.1 Detection and quantification of NETs

Despite emerging research on the mechanisms leading to NETosis, there is no golden standard marker for NETs, and available methods for evaluating NETs are hampered by lack of specificity and objective quantification. The majority of studies conducted to assess NETs in various conditions rely largely upon microscopic observations of in vitro stimulation of neutrophils and subsequent NET formation assessing the susceptibility of neutrophils to undergo NETosis, a method that is limited by the difficulties in quantification and lack of objectivity. Other studies have measured plasma levels of NET-associated markers such as cell free DNA (cfDNA), nucleosomes, and the NET-associated enzymes NE and MPO by commercially available enzyme-linked immunosorbent assay (ELISA) kits. However, these markers can be released in events unrelated to NETosis, such as tissue injury, apoptosis and necrosis resulting in cfDNA, and neutrophil and/or macrophage activation releasing MPO and NE without undergoing NETosis. These data should therefore be interpreted with caution. A capture ELISA to quantify complexes of DNA and neutrophil-derived MPO has also recently been established (89, 119, 120). However, MPO is a highly positively charged secreted protein (121), which can bind to the negatively charged cfDNA released in plasma following tissue injury, thus questioning its specificity as a NET marker.

Citrullinated histone H3 (H3Cit) is considered a NET specific marker due to the critical implication of PAD4 and histone citrullination in NET formation (86, 122). Several studies have therefore demonstrated the presence of H3Cit by immunostaining, which, however, still does not surpass the dilemmas of objectivity and quantification. With the intention to increase objectivity and to obtain a quantification of circulating NETs, a novel sandwich ELISA was

developed to measure the NET specific marker H3Cit in plasma in **Study II**. The H3Cit ELISA was then optimized and methodologically validated in **Study III**.

2 AIMS

The overall aim of this thesis was to explore patient characteristics and possible pathomechanisms behind troponin elevation in patients with acute ischemic stroke. As mentioned above, the unexpected findings of known and occult malignancies among the ischemic stroke patients with troponin elevation in **Study II** lead us to focus our investigations on an exploration of cancer-associated arterial thrombosis and a cancer-induced NET burden.

Specific aims:

- To determine patient characteristics of patients with acute stroke and troponin elevation (**Study I**).
- To determine the long-term prognosis of patients with acute stroke and troponin elevation (**Study I**).
- To explore possible mechanisms behind troponin elevation in patients with acute ischemic stroke (**Study II**).
- To elucidate the contribution of NETs in cancer-associated arterial microthrombosis, presenting as ischemic stroke with troponin elevation (**Study II**).
- To further develop and methodologically validate a novel enzyme-linked immunosorbent assay to quantify the levels of the NET specific marker citrullinated histone H3 (H3Cit) in human plasma (**Study III**).

3 MATERIALS AND METHODS

3.1 PATIENTS AND STUDY DESIGN

3.1.1 Study I (paper I)

To determine patient characteristics and five-year prognosis in patients with acute stroke and troponin elevation on admission, a retrospective cohort study was conducted including 247 patients with acute stroke. The study base comprised all consecutive patients diagnosed with acute ischemic stroke or intracerebral bleeding (ICB) admitted to Danderyd Hospital < 7 days of symptom onset between January 1, 2005, and January 1, 2006 (n=725), and was obtained retrospectively from the Swedish national stroke register, Riksstroke (123). Only the first event was included in the study in the event of a recurrent stroke during the study period (n=13). TnI values were obtained from hospital records, and patients without a TnI registered on admission were excluded (n=464). Patients were divided into three groups according to the TnI value on admission; <0.03 μg/L, 0.03-0.11 μg/L and > 0.11 μg/L. Primary endpoint was all-cause mortality within a five-year period.

Demographic data, comorbidities (prior acute ischemic stroke, transient ischemic attack (TIA) or ICB, CHF, CAD, hypertension, diabetes mellitus, and cancer), and medication on admission and at discharge were collected from Riksstroke (123), the Swedish Cancer Registry (124), and hospital records. A diagnosis of hyperlipidemia was considered present if patients received lipid-lowering medication on admission. AF was determined by history or new diagnosis of AF during the hospital stay, and renal insufficiency was defined as plasma creatinine > 120 umol/L on admission. Mortality data, cause of death (divided into four categories: stroke, cardiac, cancer, and other causes) and morbidity (divided into four categories: recurrent stroke, cardiovascular event, cancer, and other events) during the five-year follow-up were obtained from the National Cause of Death Registry (125) and the National Patient Registry (126).

A sub-analysis was conducted comparing the excluded group of patients due to missing TnI on admission with the study group. Age, sex, comorbidity (prior stroke, hypertension, AF and diabetes mellitus), medication on admission and at discharge, and level of consciousness determined by RLS (Reaction Level Scale) were obtained from Riksstroke.

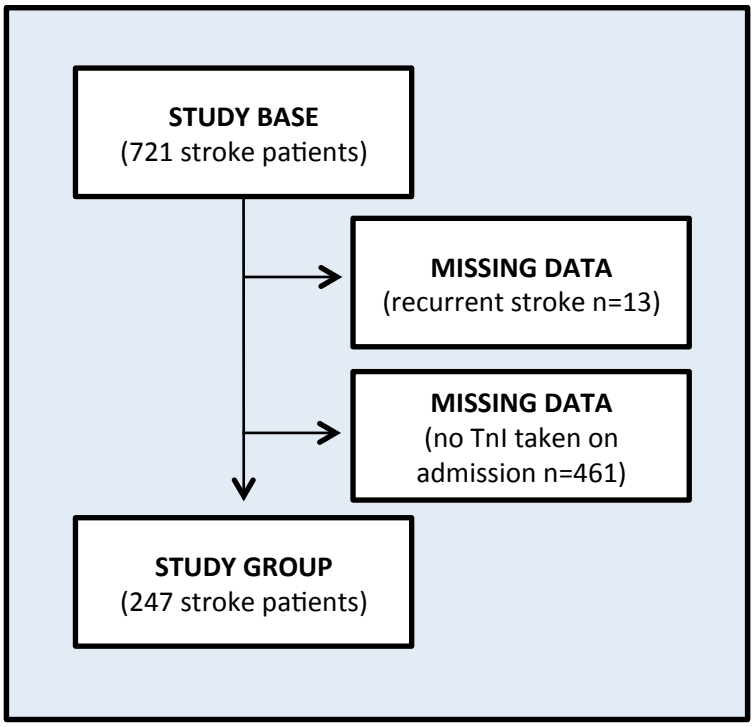

Figure 3. Flowchart of participants in Study I.

3.1.2 Study II (paper II and III)

To explore possible mechanisms behind troponin elevation in patients with acute ischemic stroke, a prospective pilot case-control study was performed including ischemic stroke patients admitted to the stroke unit at Danderyd Hospital, Stockholm, between April 2012 and December 2014. Inclusion criteria were 1) ischemic stroke confirmed by cerebral imaging or ischemic stroke with new focal neurological deficits and 2) symptom onset < 48 hours before admission. Exclusion criteria were acute cardiovascular event (ACS or ischemic stroke) within four weeks of symptom onset. Patients with hsTnT > 40 ng/L (ref value < 15 ng/L) were recruited as case patients (n=12), patients with hsTnT ≤ 15 ng/L were recruited as control patients (n=19), and healthy volunteers (n=10) were recruited as reference for plasma analyses. The cases and controls matched according to sex and age within a five-year interval. Inclusion was restricted to time periods with available research personnel, during which ischemic stroke patients with the highest plasma hsTnT value on admission were selected to the case group. The patients were compared with regard to patient characteristics, clinical investigations (computed tomography (CT) brain imaging, echocardiograms, ECGs), laboratory analyses, including markers of coagulation and NETs, as well as autopsy and histopathological investigations in deceased patients. Demographic data and comorbidity were obtained from medical records and patient history documented on admission.

3.1.3 Study III (paper IV)

Due to the lack of objective and quantitative methods to assess a systemic NET burden, we optimized and methodologically validated a novel ELISA-based assay to quantify the levels of the NET-specific marker H3Cit in human plasma.

Plasma samples were taken from a previously conducted human model of inflammation (127) with the hypothesis that inflammation would induce a systemic NET formation resulting in elevated and detectable levels of H3Cit in plasma, rendering them suitable for an assay validation. For this purpose, we chose samples taken prior to and 3-4 h after receiving intravenous injection of lipopolysaccharide (LPS; 2 ng per kg of body weight *Escherichia coli* endotoxin, Lot H0K354 CAT number 1235503, United States Pharmacopeia, Rockville, MD, USA).

The assay uses an anti-histone antibody as capture antibody and an anti-histone H3 citrulline antibody for detection *(figure 4),* with a standard curve using in vitro PAD4-citrullinated H3Cit (128). The concentrations of the standard curve, incubation times and dilutions of samples were optimized in preliminary experiments.

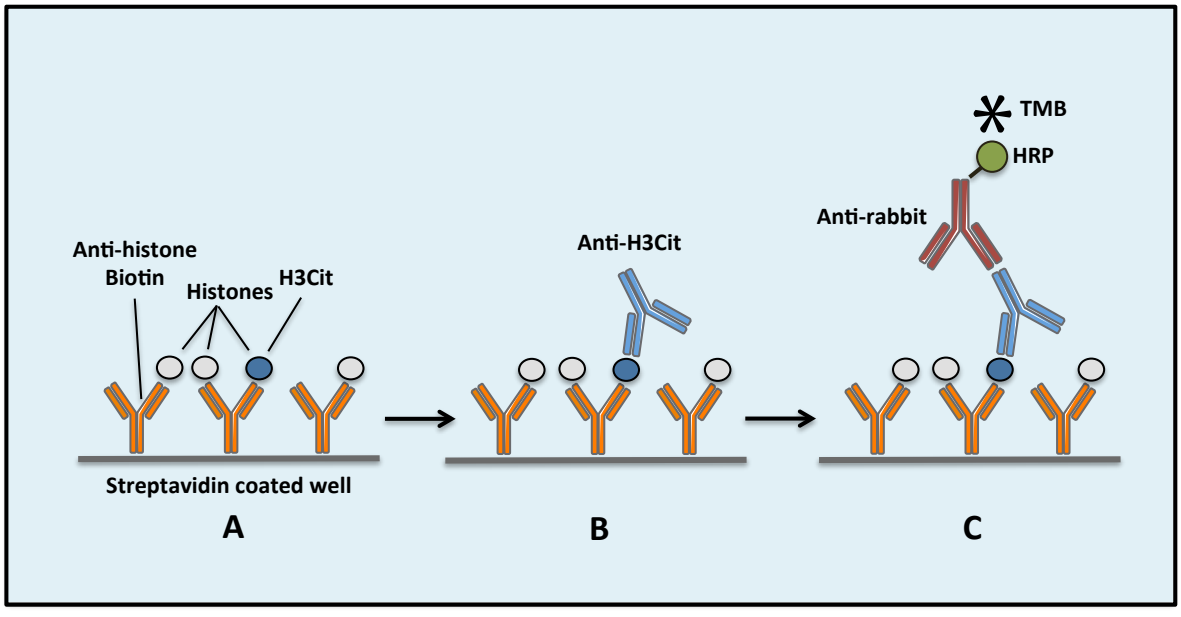

Figure 4. Schematic of the H3Cit ELISA procedure. A - Anti-histone Biotin (the capture antibody) is coated to Streptavidin pre-coated wells during a first incubation. Samples are pipetted into the wells and histones bind to the capture antibody during a second incubation. **B -** After washing, Anti-H3Cit is added to the wells, binding to immobilized citrullinated histone H3 (H3Cit), but not to non-citrullinated histone H3, during a third incubation. **C -** In a fourth incubation, an HRP conjugated anti-rabbit antibody is added and binds to the Anti-H3Cit, after which TMB is added for detection.

The assay was evaluated for *linearity, limit of detection, stability, specificity, effect of the matrix,* and *precision. Trueness* could not be determined as there is no available assay or reference analyte of known concentration for comparison.

To determine the suitable **linear interval** we interpolated the detected O.D. from serial dilutions of H3Cit to different regressions. The 95% confidence interval (95 % CI) was considered, and a linear interval was defined as the linear section of the best-fit standard curve.

The **limit of detection** was approximated from the intersection of the lower asymptote of the upper 95% CI with the 4PL fit of the standard curve.

Stability was assessed by comparing the detector response of three different batches of frozen standard (H3Cit), prepared on three different days, as well as comparing a standard prepared from freshly citrullinated H3Cit with a standard prepared from a frozen aliquot of H3Cit.

A standard curve was prepared with histone H3 incubated under the same conditions as our standard preparation of H3Cit, but without PAD4, rendering non-citrullinated histones. To assesse **specificity** for H3Cit, we compared the detection response to this standard curve with the detection response to our standard curve with H3Cit.

Recovery and the **effect of the matrix** were assessed by spiking known concentrations (625, 312, 156, 78, 38, 19 and 10 ng/ml) of in vitro PAD4-citrullinated H3Cit in plasma samples from four healthy volunteers.

To assess **precision**, the assay was performed on six replicates of eight samples (1-8) within the same assay-run (intra-assay), and on duplicates of the same eight samples in four different assay runs performed on four different days (inter-assay). Precision was expressed by the intra- and inter-assay coefficient of variation (%CV), defined as the ratio between standard deviation and mean value.

Study individuals in **Study II and III** gave written informed consent for the use of their blood samples, and all studies complied with the Declaration of Helsinki. The studies were approved by the ethics review board in Stockholm, Sweden (DNR 2012/1416-31/1 for **Study I**, DNR 2011/1310-31/3, 2014/291-32 and 2014/1898-32 for **Study II** and DNR 2014/1946-31/1, 2015/1533-31/1 for **Study III**).

3.2 CLINICAL INVESTIGATIONS

In **Study I and II**, all patients received CT brain imaging. Stroke etiology was evaluated by a senior stroke physician according to criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) (129) which includes large-artery atherosclerosis, cardioembolism, small artery occlusion, other etiology and undetermined etiology (cryptogenic stroke). In **Study II**, stroke localization and distribution was also determined by a senior neuroradiologist blinded to clinical details. Stroke severity was determined using the National Institute of Health Stroke Scale (NIHSS) by physician on admission.

Twelve-lead ECG on admission was interpreted by a senior cardiologist blinded to clinical data on all patients in **Study I and II**. ECG alterations were defined (according to the modified Minnesota code) as left- or right bundle branch block, T-wave inversion of > 0.1 mV, prolonged QTc (> 0.45 s) and ST segment depression or elevation of > 1 mm, with the exception of ST elevation in V2 or V3, where > 2 mm was required. Transthoracic and/or transesophageal echocardiography and cardiac telemetry were performed on patients in **Study II**.

3.3 LABORATORY DATA

3.3.1 Blood sampling

Plasma samples used in **Study II and III** were prepared from citrated whole blood following immediate centrifugation for 20 minutes at 2000 x *g* in room temperature after which they were stored at -80^{0}C until further analyses.

3.3.2 Laboratory analyses

Plasma concentrations of TnI (**Study I**) were analyzed on admission using fluorimetric immunoassay (Stratus CS STAT Fluorometric Analyser, Dade Behring, Deerfield, IL, USA) and plasma concentrations of hsTnT (**Study II**) were analyzed on admission using the ECLIA electrochemiluminescense immunoassay system (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Routine laboratory data were collected from hospital records (**Study I and II**).

Plasma markers of coagulation and NETs were analysed in **Study II**. Thrombin-antithrombin complex (TAT), soluble P-selectin (sP-selectin), cfDNA, G-CSF, and MPO were analyzed with human TAT ELISA (Enzygnost TAT mikro, Siemens), human sP-selectin/CD62P

Quantikine ELISA (R&D Systems), Quant-iT PicoGreen dsDNA assay (Invitrogen), human G-CSF Quantikine ELISA (R&D Systems) and human myeloperoxidase Quantikine ELISA kit (R&D Systems), according to the manufacturer's instructions. H3Cit was detected using the tailor-made ELISA-based assay further optimized and validated in **Study III**. These analyses were performed in the Wagner Laboratory, Boston Children's Hospital, with the exception of TAT which was analysed at the Department of Clinical Pharmacology, Karolinska University Hospital.

Additional plasma analyses (fibrinogen, d-dimer, cardiolipin antibodies, antinuclear antibodies (ANA), anti-neutrophil cytoplastic antibodies (ANCA), MPO antibodies, GBM (Glomerular Basement Membrane) antibodies, and beta2-glycoprotein antibodies), as well as number and phenotypes of circulating microparticles (MPs) were analyzed in one of the patients in **Study II** (presented in paper II). MPs were analyzed with flow cytometry as described elsewhere (130). Briefly, samples were incubated with lactadherin-FITC (MFG-E8, Haematologic Technologies, Essex Junction, VT, USA) and CD142-PE (TF, Clone HTF-1, BD, NJ, USA). MPs were incubated with anti-CK18 FITC (Fisher Scientific, Gothenburg, Sweden). All samples were incubated in dark for 20 min and later fixated with BD-Cellfix. MPs were gated according to size (<1.0 μm) and the exposure of phosphatidylserin (PS), TF and CK-18.

3.4 HISTOPATHOLOGICAL INVESTIGATIONS

Autopsies and immunohistochemistry were performed at the Division of Pathology, Danderyd Hospital, Stockholm. Specimens obtained at autopsies in **Study II** were stained with standard hematoxylin & eosin, Luxol fast blue for degenerated neural tissue and Ladewigs trichrome for fibrin. Immunohistochemistry was performed on specimens containing thrombi. The antibodies used were anti-histone H3 (citrulline 2+8+17) antibody (Abcam, Cambridge, UK) for H3Cit, CK18 antibody (DAKO, Copenhagen, Denmark) to reveal epithelial tissue, tissue factor polyclonal antibody (Fisher Scientific, Gothenburg, Sweden), and prostate-specific antigen (PSA) antibody (DAKO, Copenhagen, Denmark) to reveal tissue of prostate origin. Confocal immunofluorescence microscopy was performed at the Wagner Laboratory, Boston Children's Hospital, with antigen retrieval in sodium citrate buffer (10 mM, pH 6.0) using microwave after deparaffinization. The sections were permeabilized with 0.1% Triton X-100 on ice for 10 minutes. After blocking with 3% bovine serum albumin (BSA) for one hour at 37°C, slides were incubated overnight at 4°C with

sheep polyclonal anti-VWF (Abcam, ab11713, 1:250), mouse monoclonal anti-human smooth muscle actin (anti-SMA, Dako, M0851, 1:100) and rabbit polyclonal anti-H3Cit (Abcam, ab5103, 1:1000) in antibody dilution buffer (0.3% BSA, 0.05% Tween-20) and then with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:1500) for two hours at room temperature after washes in phosphate-buffered saline. DNA was stained with Hoechst 33342 (1:10000). Images were acquired with Olympus Fluoview software using the Olympus IX 81 confocal microscope.

3.5 STATISTICAL ANALYSES

In **Study I,** a power calculation was based on previous results indicating a 40 % increase in one-year mortality among patients with acute stroke and troponin (TnT) elevation (131) compared to patients with acute stroke without troponin elevation. Assuming an approximate 20% prevalence of troponin elevation in stroke patients, a sample size of 300 patients would detect a difference of 20 % in mortality between the groups, with a power set to 80 % and the two-sided type I error to 5 %. Descriptive statistics were presented as means and proportions. Differences in means were tested by one-way analyses of variance, and differences in proportions were tested by chi-square test. Baseline characteristics were also tested for two levels of TnI: normal (TnI<0.03 mg/l) and elevated (TnI≥0.03 mg/l). Student t test was used for means and z test for proportions. Multiple Cox-regression was used to examine the association between troponin elevation (TnI>0.03 µg/L) and mortality, after adjusting for age, CHF, AF, renal insufficiency, treated hyperlipidemia, and stroke severity. Survival analysis was also performed to compare mortality between the three groups with TnI levels of <0.03 µg/L, 0.03-0.11 µg/L and > 0.11 µg/L. Survival times were censored five years after index stroke, and Kaplan-Meier curves were generated to illustrate the association between troponin level and mortality.

Study II was designed as a descriptive pilot study whereby no power calculation of sample size was conducted. Statistical methods were chosen to fit small numbers of observations and non-normal distributions. Categorical variables were presented as proportions and compared with Fisher's exact test. Continuous variables were presented as medians with interquartile ranges (IQR) and compared with the Mann-Whitney U test. Significance of correlation was analyzed with Spearman's rank correlation.

In **Study III**, O.D. was fitted versus nominal log concentration applying a sigmoidal 4PL regression to the calibration curve. 4PL curves were compared by F-test. The variation of intra- and inter-assay experiments were presented as CV, defined as the ratio of the SD to the mean.

Statistical analyses were performed using IBM SPSS Statistics version 22 **(Study I)**, STATA 12.1 software (**Study II**) and GraphPad Prism 6, GraphPad Software, Inc., La Jolla, CA, USA (**Study III**).

A p-value < 0.05 was considered statistically significant in all studies.

4 RESULTS

4.1 PATIENT CHARACTERISTICS (PAPER I)

Of the 247 patients with acute stroke in **Study I**, 133 patients (54%) presented with TnI < 0.03 µg/L (normal), 74 patients (30%) presented with TnI 0.03-0.11 µg/L (low elevation), and 40 patients (16%) presented with TnI > 0.11 µg/L (high elevation). Age, prior ischemic stroke, renal insufficiency, CHF, and stroke severity were associated with TnI levels > 0.03 µg/L. Surprisingly, there was no significant difference in the rate of prior diagnosis of CAD or AF between the groups. Prior diagnosis of cancer was present in 20.2% of the entire study population, and the prevalence was higher in patients with TnI >0.03 µg/L, although the difference was not statistically significant (*table 1)*.

	cTnI normal	cTnI low elevation	cTnI high elevation	All groups	Normal vs elevated cTnI
	<0.03 µg/L N=133	0.03-0.11 µg/L N=74	>0.11 µg/L N=40	p-value	p-value
Age, years - mean ± SD	74.4 ± 11.2	78.9 ± 10.0	84.0 ± 6.4	<0.001	<0.001
Female- %	48.1	51.4	60.0	0.42	0.37
Prior ischemic stroke- %	21.8	35.1	30.0	0.11	<0.05
Prior TIA- %	6.8	9.5	5.0	0.64	0.73
Prior haemorrhagic stroke- %	0.0	2.7	0.0	0.10	0.13
Chronic heart failure- %	7.5	20.3	15.0	<0.05	<0.05
Coronary artery disease- %	18.8	29.7	22.5	0.20	0.12
Hypertension- %	61.7	48.6	50.0	0.14	<0.05
Atrial fibrillation- %	33.8	43.2	47.5	0.20	0.08
Diabetes mellitus- %	16.5	21.6	10.0	0.28	0.83
Renal insufficiency- %	9.0	13.5	30.0	<0.01	<0.05
Hyperlipidemia- %	25.6	20.3	5.0	<0.05	<0.05
Cancer- %	15.8	25.7	23.1	0.26	0.10
BP systolic- mean ± SD	162 ± 29	167 ± 33	153 ± 36	0.10	0.65
BP diastolic- mean ± SD	89 ± 15	89 ± 17	87 ± 19	0.66	0.61
NIHSS on admission- mean ± SD	5.3 ± 6.1	8.0 ± 8.2	6.6 ± 7.2	0.07	<0.05

Table 1. Patient demographics on admission. Abbreviations: SD, standard deviation; TIA, transient ischemic attack; BP, blood pressure; NIHSS, National Institute of Health Stroke Scale.

There were no differences in medications between the groups, with the exception of lipid-lowering agents, which were less common in both groups with elevated TnI, and beta-adrenoreceptor antagonists (beta-blockers) at discharge, which were significantly more

common in patients with TnI >0.03 µg/L. The rate of beta-blockers on admission did not, however, differ between the groups.

There were no statistically significant differences between the groups with regard to stroke subtype, although there was a trend towards a higher prevalence of cardioembolic stroke if TnI >0.03 µg/L (37.7% vs. 26.3%, p value 0.055).

The frequencies of left- or right bundle branch block, T-wave inversion, or prolonged QTc did not differ between the groups, but ST segment elevation or depression increased with increasing TnI values (9.0% if normal TnI, 14.9 % if low elevation of TnI, and 25.0 % if high elevation of TnI, p-value 0.03). There were, however, few recordings of chest pain on admission among patients in the entire study group (4%), not differing between the groups.

Several routine blood tests were associated with TnI levels (*table 2*). The levels of C-reactive protein, leukocyte count, and serum creatinine on admission increased with increasing TnI, whereas haemoglobin levels decreased with increasing TnI.

	cTnI normal	cTnI low elevation	cTnI high elevation	All groups	Normal vs elevated cTnI
	<0.03 µg/L N=133	0.03-0.11 µg/L N=74	>0.11 µg/L N=40		
C-reactive protein - mean ± SD (ref <5.0 mg/L)	6.2 ± 11.3	15.6 ± 30.1	43.2 ± 60.4	<0.001	<0.001
Leukocyte count - mean ± SD (ref 4.00-11.0 x 10^9/L)	8.4 ± 2.5	10.2 ± 3.8	12.4 ± 4.8	<0.001	<0.001
Creatinine - mean ± SD (ref <100 µmol/L)	85.4 ± 27.9	94.6 ± 37.4	104.5 ± 40.2	<0.05	<0.001
Platelet count - mean ± SD (ref 145-350 10^9/L)	237.2 ± 61.9	252.2 ± 91.3	242.1 ± 97.0	0.71	0.72
Haemoglobin - mean ± SD (ref 134 - 170 g/L)	142.0 ± 14.5	138.1 ± 18.0	135.1 ± 19.4	<0.05	<0.05
Glucose - mean ± SD (ref <7 mmol/L)	7.2 ± 2.4	8.1 ± 3.5	7.8 ± 2.3	0.14	0.06

Table 2. Laboratory data on admission. Abbreviations: SD, standard deviation.

No significant differences were detected between the excluded group (i e missing TnI value on admission) and the study group, with the exception of a higher rate of AF (35.7% vs. 27.2%, p-value 0.02), and a lower level of consciousness (82.1 % vs. 90.2% fully conscious, p value 0.01) in the study group.

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4.2 LONG-TERM MORTALITY (PAPER I)

Among the 247 patients with acute stroke in **Study I**, 141 patients (57 %) died during the five-year follow-up. Over-all mortality increased with increasing TnI values, and the elevated risk for mortality prevailed throughout the 5-year follow-up (*figure 5*).

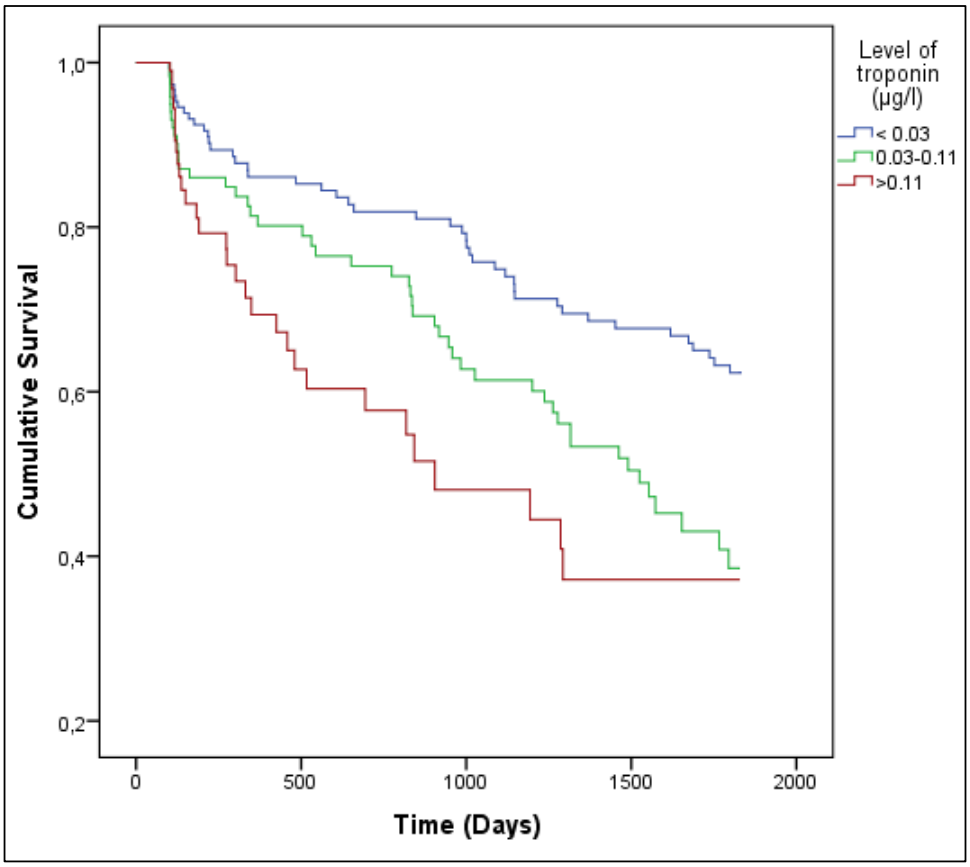

Figure 5. Kaplan-Meier curve depicting cumulative survival after acute stroke. Adjusted for age, chronic heart failure, atrial fibrillation, renal insufficiency, lipid-lowering agents and NIHSS. Stratified on normal, low elevations and high elevations of TnI.

A multivariate Cox proportional hazards model adjusting for age, CHF, AF, renal insufficiency, treatment with lipid-lowering agents, and stroke severity showed that patients with elevated TnI (>0.03 µg/L) had significantly increased mortality over the five-year follow-up compared to patients with normal TnI, with an adjusted HR of 1.90 (95% CI 1.34-2.70) (*table 3*). As expected, age, CHF, renal insufficiency and stroke severity were also independently associated with 5-year mortality.

	Univariate analysis	Multivariate analysis
	HR (95% CI)	HR (95% CI)
TnI >0.03 ug/L	2.65 (1.89-3.72)	1.90 (1.33-2.70)
Age, per year	1.08 (1.07-1.10)	1.06 (1.04-1.08)
Chronic heart failure	2.86 (1.88-4.35)	1.89 (1.21-2.97)
Atrial fibrillation	1.69 (1.22-2.35)	0.85 (0.58-1.24)
Renal insufficiency	2.59 (1.70-3.95)	2.19 (1.42-3.36)
Hyperlipidemia	0.79 (0.60-1.03)	0.96 (0.61-1.51)
NIHSS 0-3	1	1
NIHSS 4-8	1.24 (0.79-1.93)	1.32 (0.84-2.07)
NIHSS 8-	2.41 (1.66-3.49)	1.96 (1.34-2.88)

Table 3. Predictors of all-cause mortality during a five-year follow-up. All baseline variables were entered in the univariate cox regression, only the variables with a significance level of $p<0.05$ were entered in the multivariate cox regression.

There were no significant differences between the groups with regard to cause of death, recurrent stroke, cardiovascular event or new diagnosis of cancer during the follow up.

The excluded group of patients (i e missing TnI value on admission) had a lower mortality during the 5-year follow-up compare to the study group (42.0% vs. 52.8%; p value 0.01).

4.3 AN UNDERLYING CANCER (PAPER II AND III)

4.3.1 The index patient

During the inclusion of patients to **Study II**, a 67-year old man without previous medical history presented with multiple and widely spread cerebral infarctions and markedly elevated levels of plasma hsTnT (420 ng/L on admission, and 530 and 362 ng/L over the following 12 hours). The hsTnT levels rose to 1320 ng/L over the following days, but the patient reported no current or prior chest pain or dyspnea. ECG was normal, but cardiac telemetry showed a very short episode of possible paroxysmal AF as a potential source of cerebral embolism. Repeated TTE showed no signs of infarction, shunts, thrombi, or vegetations, arguing against a concomitant ACS or endocarditis. Ultrasounds of the carotid arteries were normal, as was blood culture obtained on the sustained suspicion of endocarditis, as well as catecholamine levels and blood markers of vasculitis (circulating antibodies against cardiolipin, ANA, ANCA, MPO, GBM, and beta2-glycoprotein). Over the course of the hospital stay, the

patient developed several recurrent and disseminated cerebral infarctions, and died within 11 days of admission.

Macroscopic examination during autopsy showed no thrombotic occlusions or atherosclerosis of the large cerebral or coronary arteries, and no source of emboli in the heart or larger renal or pulmonary arteries. Histopathology, however, revealed an advanced metastatic adenocarcinoma of the prostate. Furthermore, there were disseminated cerebral, pulmonary and myocardial microthrombi *(figure 6)*, which had not been detectable at the macroscopic autopsy.

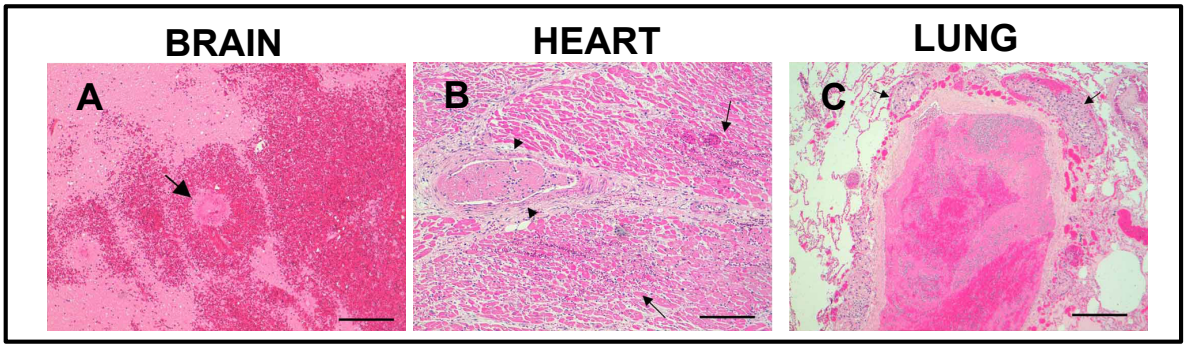

Figure 6. Hematoxylin and eosin staining showing disseminated microvascular arterial thrombosis in the brain, heart, and lung. A - Microthrombus in a small cerebral artery (arrow) with surrounding massive hemorrhagic infarction. Scale bar = 200 μm **B -** Thrombus in a coronary artery (arrowheads) along with areas of acute infarction and granulocyte infiltration (arrows). Scale bar = 200 μm. **C -** Thrombus in a small pulmonary artery. Cancer metastases are seen around the artery (arrows). Scale bar = 500 μm.

To further explore a possible link between the occult cancer and the apparent hypercoagulable state, and in lieu of prior data on mechanisms driving arterial thrombosis in cancer, we sought to find evidence of some of the numerous pathophysiological mechanisms proposed to link cancer with VTE. A series of analyses were performed on stored plasma, thrombi and tumor. Immunohistochemistry of both primary tumor and metastases showed strong staining of TF as well as the epithelial tumor marker CK18 *(figure 7)*. We therefore proceeded to analyze the number of circulating TF and CK18 positive MPs. To our surprise, the number of TF+MPs was markedly lower than those found in a population of 209 ischemic stroke patients without known malignancy; 205 x 10^6 MPs/L vs. 1800 x 10^6 MPs/L (132). There was, however, a large number of circulating MPs positive for CK18 compared with a

patient with ischemic stroke without underlying malignancy; 4377 x 10^9 MPs/L vs. 36 x 10^9 MPs/L. Considering the CK 18-positive tumor tissue, we hypothesized that these MPs could have been tumor-derived. We could not, however, link these results to the hypercoagulable state, as the arterial microthrombi stained strongly for TF, but contrary to what we had expected, negatively for CK 18 *(figure 7)*.

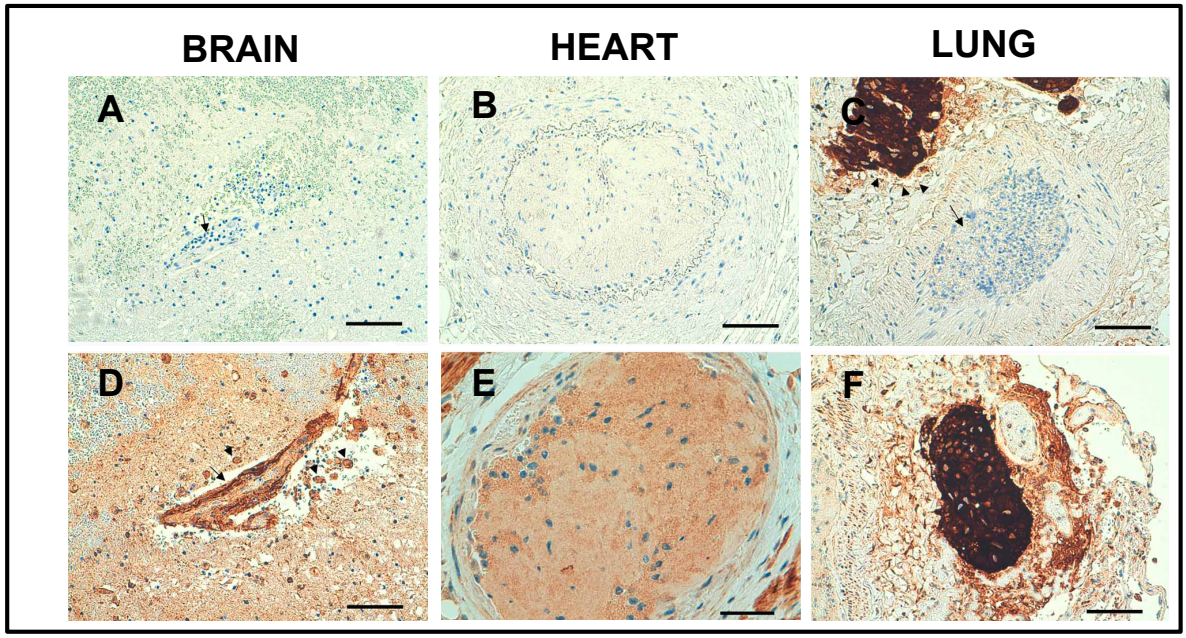

Figure 7. Immunohistochemistry for cytokeratin 18 (CK18) and tissue factor (TF) revealed staining for CK18 in metastases (dark brown) but not thrombi, and staining for TF in both metastases and thrombi (dark brown). **A -**. No CK18 immunoreactivity was detected in a thrombus in a small cerebral artery. Scale bar = 100 μm. **B -** No CK18 immunoreactivity was detected in a thrombus in a coronary artery. Scale bar = 100 μm. **C –** Metastases around a small pulmonary artery staining strongly positive for CK18 (dark brown). Scale bar = 100 μm. **D -** There was some immunoreactivity to TF (dark brown) in a thrombus in a small cerebral artery, but also in the vessel wall (arrow) and in lipid-laden macrophages near the artery (arrowheads). Scale bar = 100 μm. **E –** Positive staining for TF (dark brown) in a thrombus in a coronary artery. Inside the thrombus are also a number of granulocytes with blue stained nuclei. Scale bar = 50 μm. **F -** Strong positive staining for TF (dark brown) in a metastasis in the lung. Scale bar = 100 μm. Image courtesy of Bo Blomgren.

4.3.2 Further indications of cancer-associated microthrombosis

Among the 31 ischemic stroke patients in **Study II**, the hsTnT levels in the case group (n=12) were high; with a mean of 287.8 ng/L and a median of 144.0 ng/L. The mean value of hsTnT in the control group (n=19) was 8.7 ng/L with a median of 9.0 ng/L. Contrary to what we

had expected, there were no significant differences in age, cardiovascular or renal comorbidity burden, or NIHSS score between ischemic stroke patients with and without elevated hsTnT. There was a higher prevalence of undetermined stroke etiology in the group with elevated hsTnT, although the difference did not reach statistical significance (58% vs. 22%, p value 0.052). On evaluation of CT brain imaging, multiple and disseminated cerebral lesions extending single vascular territory was significantly more common in the patients with hsTnT elevations (33.3% vs. 5%, p value 0.03). ECG alterations were present in 8/11 patients with hsTnT elevations and 5/19 patients with normal hsTnT levels, but this difference did not reach statistical significance (p=0.137). Only four patients presented with ST segment deviations suggestive of myocardial ischemia, two of them with normal levels of hsTnT and two of them with elevated levels of hsTnT (53 and 148 ng/L). Abnormal findings on echocardiograms were found in 10/11 patients with hsTnT elevations and 12/19 patients with normal hsTnT levels, not reaching a statistically significant difference (p=0.551), and regional wall motion abnormality was found in 2/11 patients with hsTnT elevations and 2/19 patients with normal hsTnT levels (p=1.000).

However, there was a high portion of active cancer in the group of patients with high hsTnT values (7/12 vs. 1/19 in the control group, p=0.002). The inclusion of patients was based solely on the level of hsTnT, regardless of comorbidity. In fact, four of the eight patients with active cancer were diagnosed with cancer after inclusion in the study, during the hospital stay (n=1) or post-mortem (n=3). Seven of eight primary tumors were adenocarcinomas, but of different origin (*table 4*).

Autopsy was performed on three of the patients with high hsTnT levels and an underlying malignancy, but despite the high levels of hsTnT, macroscopic examination at autopsy revealed only mild atherosclerotic plaque and no occlusions of the coronary arteries. As in our first index patient, however, histopathology revealed multiple and widely spread arterial microthrombi in the brain, heart and lung. Arterial microthrombi surrounded by infarctions were also observed in small renal and splenic arteries in one of the patients.

Patient	Age	Sex	hsTnT ng/L	Cancer type and metastatic spread	Time of cancer diagnosis
1	68	Male	1320	Prostate adenocarcinoma. Metastatic spread to the bladder, lung and bone.	Occult, diagnosed post-mortem
2	62	Female	180	Lung adenocarcinoma. Metastatic spread to the pleura, lymphnodes and liver.	Occult, diagnosed post-mortem
3	83	Male	148	Lung adenocarcinoma. Metastatic spread to the bone.	Diagnosed and pulmonary lobectomy 2 years before ischemic stroke, recurrent diagnosis 4 months prior to ischemic stroke
4	68	Male	694	Hepatocellular adenocarcinoma. Metastatic spread to the gastrointestinal tract and skin.	Diagnosed 5 months prior to ischemic stroke
5	82	Female	53	Pancreatic adenocarcinoma. Metastatic spread to the lungs, peritoneum and liver.	Occult. Diagnosed post-mortem
6	64	Male	9	Prostatic adenocarcinoma. Extraprostatic and perineural spread.	Diagnosed and prostatectomy 10 years before ischemic stroke, recurrent diagnosis < 2 months after ischemic stroke
7	94	Female	72	Breast adenocarcinoma. Spread to the skin.	Diagnosed 5 years before ischemic stroke, recurrent diagnosis with surgery 3 days prior to ischemic stroke
8	85	Female	140	Urothelial carcinoma. Infiltrative spread to surrounding musculature.	Diagnosed 14 months prior to ischemic stroke

Table 4. Troponin levels (hsTnT) and type, metastatic spread and time of cancer diagnosis in ischemic stroke patients with an underlying active cancer.

To further assess whether troponin elevation could be associated with a hypercoagulable state, we determined plasma levels of the coagulation marker TAT and platelet activation marker sP-selectin. Both appeared higher in patients with hsTnT elevation compared with patients with normal hsTnT levels *(figure 8A)*. However, even higher elevations were seen in patients with cancer compared to patients without cancer (median 28.9 with IQR 9.8-47.0 µg/mL vs. median 6.1 with IQR 4.1-10.2 µg/mL, p=0.001, for TAT; median 75.3 with IQR 35.9-100.8 ng/mL vs. median 26.0 with IQR 16.8-39.1 ng/mL, p=0.001, for sP-selectin) (*figure 8B*). TAT and sP-selectin did not differ between patients with and without hsTnT elevation when excluding patients with cancer, linking cancer to the pro-coagulant state *(figure 8C)*.

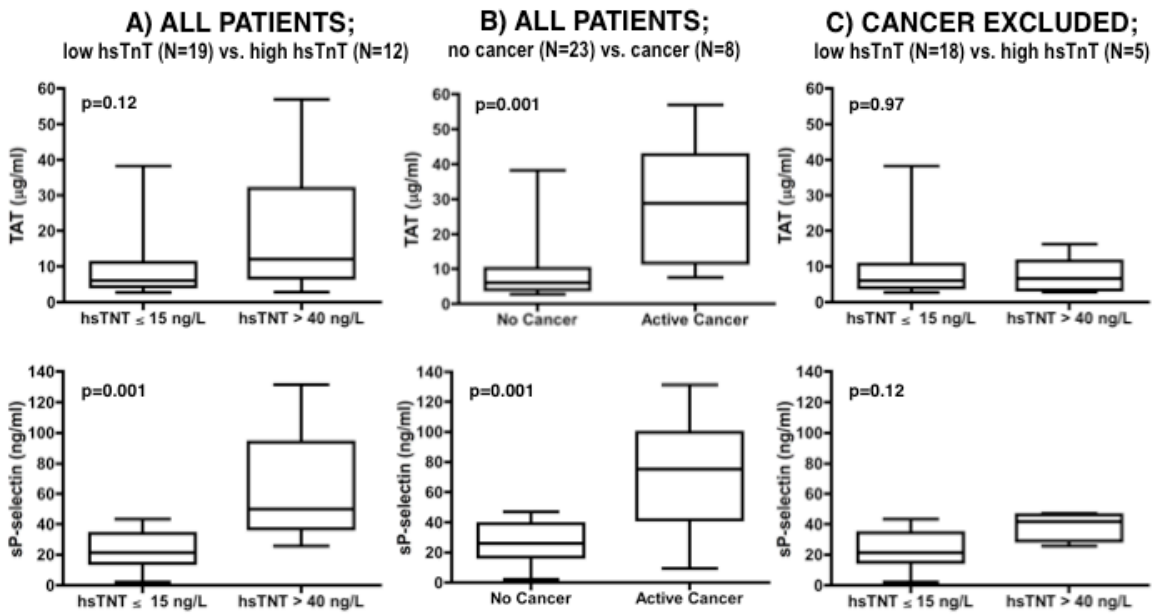

Figure 8. Plasma markers of coagulation and platelet activation. **A -** Plasma markers of coagulation (TAT) and platelet activation (sP-selectin) were significantly higher in stroke patients with hsTnT elevation (n=12) compared to stroke patients without hsTnT elevation (n=19). **B -** Even higher levels were seen in patients with cancer (n=8). **C -** The differences between patients with hsTnT elevation (n=5) and normal levels of hsTnT (n=18) were diminished when excluding patients with cancer, suggesting a link between cancer and coagulation. Mann-Whitney U test was used to determine p-values.

As in **study I**, the patients with elevated hsTnT had a significantly higher mortality than patients with normal levels of hsTnT (58% vs. 5%, p value 0.002), and the short-term mortality was, as expected, even higher in the group with cancer (88% vs. 4%, p value < 0.001).

4.4 A LINK TO NETS (PAPER III)

In light of the previously discussed reports on the link between NETs and cancer-associated thrombosis, we proceeded to assess markers of NETs in plasma and thrombi. Plasma levels of the NET associated markers cfDNA and MPO, as well as the NET specific marker H3Cit were higher in patients with elevated hsTnT compared to patients with normal hsTnT levels, suggesting the presence of a systemic NET burden *(figure 9A)*. As with markers of coagulation and platelet activation, however, the levels of NET markers were even higher

in patients with cancer compared to patients without cancer (median 504.0 with IQR 443.7-562.0 ng/mL vs. median 407.9 with IQR 340.5-451.7 ng/mL, p value 0.04, for cfDNA; median 74.1 with IQR 57.9-146.7 ng/mL vs. median 30.8 with IQR 18.5-58.4 ng/mL, p value 0.01, for MPO, and median 0.22 with IQR 0.12-0.31 O.D. vs. median 0.07 with IQR 0.05-0.08 O.D., p value 0.001, for H3Cit) (*figure 9B*). The differences in circulating H3Cit, cfDNA and MPO between patients with hsTnT elevation and patients with normal hsTnT values were eliminated when excluding patients with cancer, linking cancer to the systemic NET burden *(figure 9C)*.

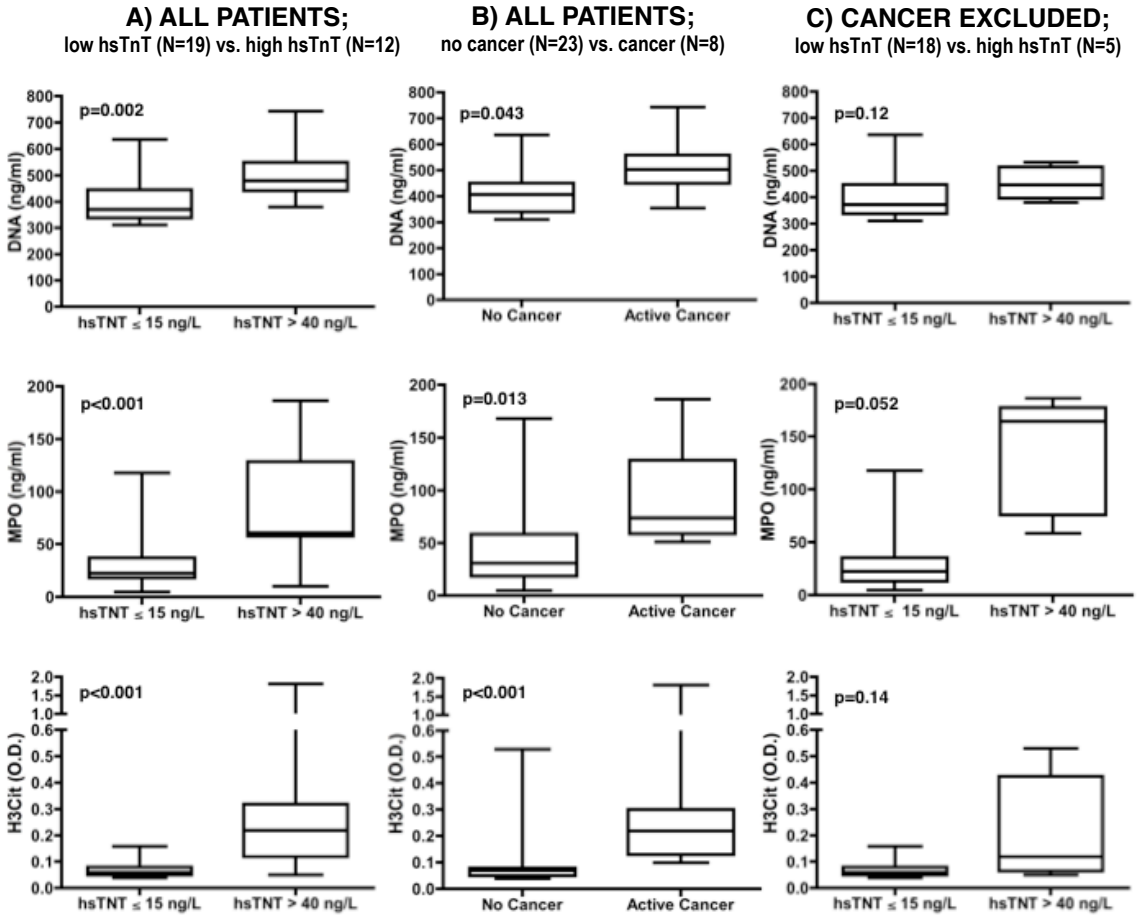

Figure 9. Plasma markers of NETs. A – In line with markers of coagulation and platelet activation, markers of NETs (cfDNA, MPO and H3Cit) were significantly higher in stroke patients with hsTnT elevation (n=12) compared to stroke patients without hsTnT elevation (n=19). **B -** Even higher levels were seen in patients with cancer (n=8). **C -** When excluding patients with cancer, the differences between patients with hsTnT elevation (n=5) and normal levels of hsTnT (n=18) were diminished, suggesting a link between cancer and NETs. Mann-Whitney U test was used to determine p-values.

As mentioned previously, different types of murine cancers have been shown to systemically release G-CSF, priming neutrophils toward NETosis. We therefore assessed

the levels of circulating G-CSF in our patients. A seven-fold increase of G-CSF was seen in plasma of patients with cancer compared to patients without cancer (median 21.0 vs. 3.1 pg/mL, p value 0.02). Leukocyte count was also significantly higher in patients with cancer (median 12.6 vs. 6.8 × 10^9/L, p value 0.002).

Furthermore, there were positive and significant correlations between G-CSF and the NET specific marker H3Cit (r=0.61, p value <0.001) and the NET-associated markers MPO (r=0.55, p value 0.005) and cfDNA (r=0.43, p value 0.02), as well as between H3Cit and markers of coagulation and platelet activation (r= 0.5, p value 0.005 and r=0.61, p value 0.001 for TAT and sP-selectin respectively), linking a systemic cancer-associated NET burden to the hypercoagulable state seen in our patients. As expected, markers of coagulation and platelet activation were significantly higher in stroke patients without known cancer compared to healthy and age-matched controls. Markers of NETs did, however, not differ between stroke patients without known cancer and healthy controls, suggesting the presence of cancer as the likely cause of a systemic NET burden.

These results suggest that high elevations of plasma troponin in ischemic stroke patients may be the result of a NET-induced systemic hypercoagulable state associated with cancer. Interestingly, immunohistochemistry and confocal microscopy supported this hypothesis by showing the presence of NETs (extracellular DNA and H3Cit) and decondensed H3Cit positive cells in multiple cerebral, myocardial and pulmonary thrombi of the three ischemic stroke patients with an underlying malignancy where autopsy was performed *(figure 10)*.

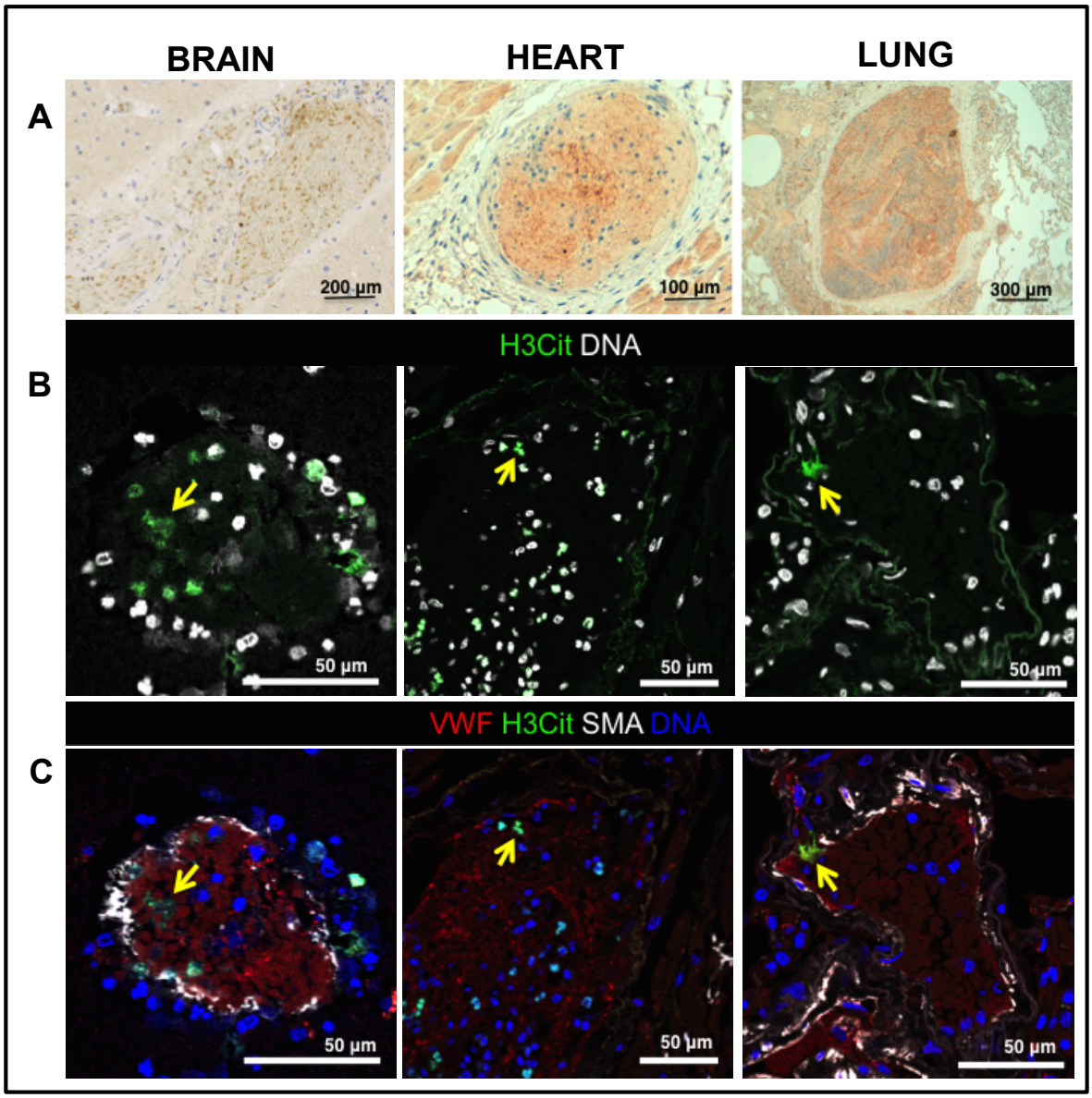

Figure 10. Markers of NETs in microthrombi. A – Immunohistochemistry revealed H3Cit (dark brown) positive cells as well as extracellular H3Cit in microthrombi of the brain, heart and lung. **B, C -** Confocal microscopy showed the co-localization of extracellular H3Cit and DNA, confirming the presence of NETs (arrows) in cerebral, coronary and pulmonary microthrombi. The microthrombi were also rich in von Willebrand factor (VWF). Smooth muscle actin (SMA) staining delineates the vessel wall. Immunohistochemistry image courtesy of Bo Blomgren and confocal microscopy image courtesy of Siu Ling Wong.

4.5 VALIDATION OF A NOVEL H3CIT ELISA (PAPER IV)

The H3Cit ELISA used to measure levels of plasma H3Cit in **Study II** was further developed by introducing a standard curve derived from in vitro citrullinated H3Cit, as described in the method section, and subsequently methodologically validated.

The best-fit curve was a sigmoidal 4PL curve rendering a **linear interval** of the curve between ≈ 0.5 and 3.5 O.D., corresponding to concentrations between ≈ 5 and ≈ 300 ng/mL.

The **limit of detection** was determined by approximating the lowest detectable concentration determined from the curve to ≈ 5 ng/mL, corresponding to the intersection of the lower asymptote of the upper 95% CI with the 4PL fit of the standard curve. The limit of detection with stated probability was therefore set to approximately 5 ng/mL.

The detector response of three different batches of frozen standard (H3Cit), prepared on three different days, was very similar (*figure 11A*). Likewise, there was no significant difference in the detection response from a standard prepared from freshly citrullinated H3Cit compared with a standard prepared from a frozen aliquot of H3Cit (*figure 11B*), revealing a high **stability** and allowing for a good reproducibility.

Although there was a low detection response when large amounts of non-citrullinated histone H3 were present, the detection response was negligible in the linear interval of the assay, confirming a high **specificity** for H3Cit in the linear interval of the assay (*figure 11C*).

We were not able to recover known concentrations of H3Cit in plasma from healthy individuals (*figure 11D*), revealing an **effect of the matrix** (i e plasma).

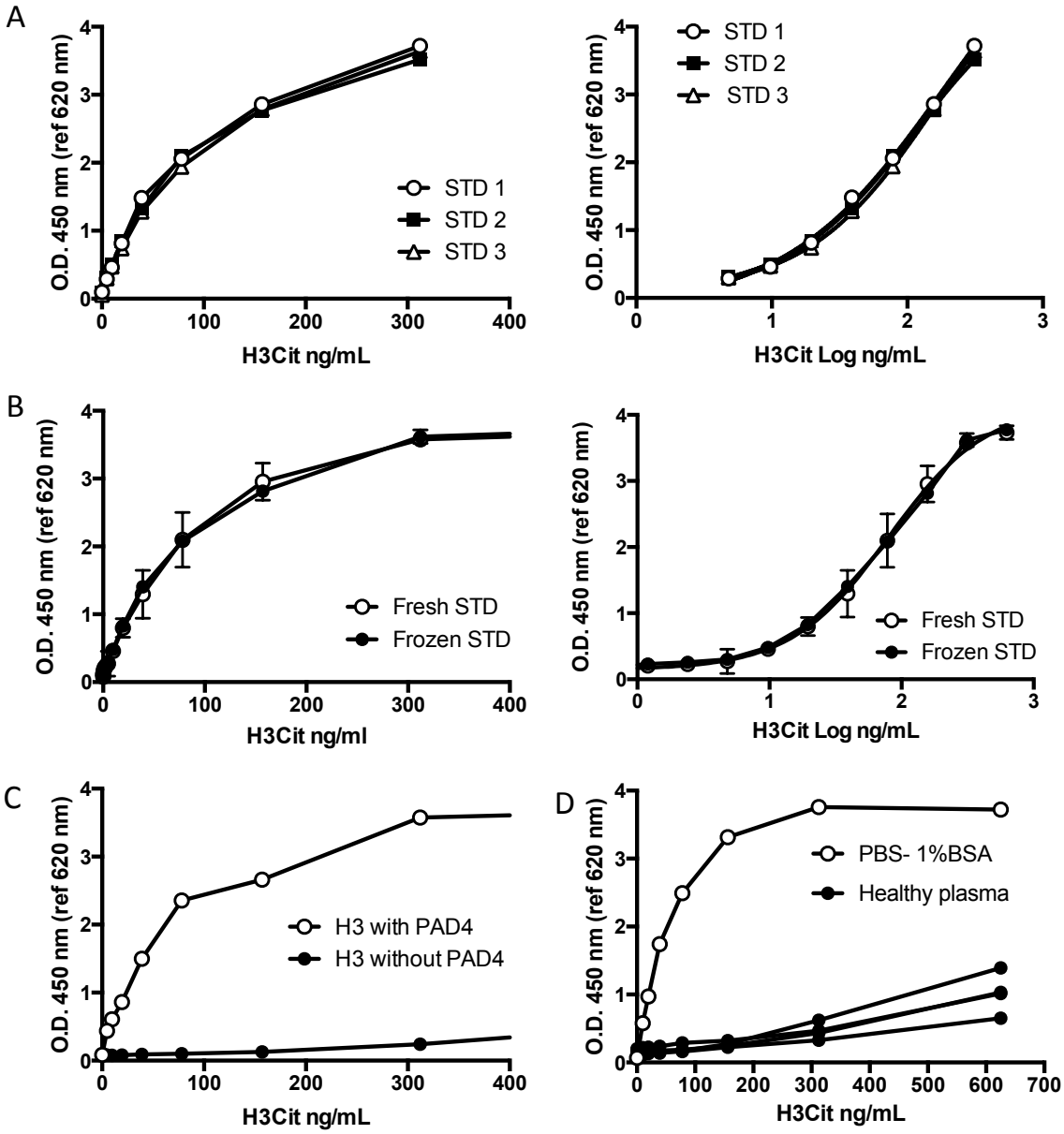

Figure 11. Stability and specificity. A - The detector response when preparing standard curves from three different batches of frozen of PAD4-citrullinated histone H3, prepared on three different days, or **B** - from freshly made or frozen aliquot of H3Cit standards were not significantly different (F (DFn, DFd)= 2.6 (8,9); p= 0.088, and F (DFn, DFd)= 0.2 (4, 52); p= 0.916 respectively). **C** - Data obtained when a standard curve was prepared with histone H3 incubated in the same conditions as our standard preparation of H3Cit, but without PAD4, rendering non-citrullinated histones, representative of three different experiments. There was a low amount of antibody antigen detection when large amounts of non-citrullinated histone H3 were present, but the antibody antigen detection was specific for H3Cit in the linear interval of the assay. **D** - No H3Cit was detected in plasma from healthy volunteers, whereas the spiking of known concentrations of H3Cit into these plasmas diluted 1:2 gave a significantly lower detector response compared to the detector response obtained from the standard diluted in PBS-1% BSA, suggesting an effect of the matrix.

The levels of H3Cit in all samples taken at baseline were under the detection limit of approximately 5 ng/mL, and the levels of H3Cit in all samples taken from the same individuals 3-4 h after LPS injection ranged from 28.7 ng/mL to 93.2 ng/mL. The CV were all <15 %, with the intra-assay variation ranging from 2.13-5.15 % and the inter-assay variation ranging from 5.80-12.55 %, showing a high **precision** with good repeatability and reproducibility of the assay (*table 5*).

Sample	1	2	3	4	5	6	7	8
CV (%) intra-assay (n=6)	5.1	4.5	5.08	2.7	4.35	3.58	2.13	3.1
CV (%) inter-assay (n=4)	11.54	10.27	12.55	8.5	9.6	10.53	5.8	13.5

Table 5. Precision; intra-assay repeatability and four different days inter-assay reproducibility. The precision of the H3Cit ELISA was high, with all intra- and inter-assay coefficients of variation (CV) <15%.

5 DISCUSSION

The release of troponin in ischemic stroke patients can be the result of various, and likely with each other interacting, mechanisms. Prior data, including that from several large and recent studies (12-14, 133), have shown that troponin elevation in ischemic stroke predicts poor short-term outcome. The work of this thesis appends an adverse long-term prognosis in these patients, as well as an association between troponin elevation and ECG changes suggestive of myocardial ischemia. We provide new data suggesting that an underlying malignancy may, by generating a hypercoagulable state leading to concomitant myocardial and cerebral infarction, be a previously unrecognized contributing factor to troponin elevation in ischemic stroke. We also show that neutrophils, presumably primed by an underlying cancer, may contribute to this thrombotic state through the release of NETs. Finally, we suggest and validate a novel method to detect and quantify the NET specific marker H3Cit in human plasma, which may be useful in further studies to confirm these hypotheses.

5.1 PATIENT CHARACTERISTICS

The prevalence of troponin elevation among the stroke patients in **Study I** (46%) was higher than those reported in many of the previous studies (reporting a prevalence of around 10-30%). The majority of previous studies, however, excluded patients with pre-existing cardiac and renal disease, conditions that are known to be associated with troponin elevation (22, 134), which may have underestimated the total prevalence of troponin elevation among ischemic stroke patients. Instead of excluding these patients, we took possible confounding factors into account by adjusting for age, CHF, renal insufficiency, AF, hyperlipidemia and NIHSS score in the prognostic analyses. Worth noted, however, is the large number of patients excluded in **Study I**, due to a missing TnI value registered on admission. The missing group analysis revealed that our study group had a lower level of consciousness and higher 5-year mortality than the excluded group, suggesting that our study group suffered more severe cerebral lesions. They also had a higher rate of prior diagnosis of AF. Considering that stroke severity, prognostic outcome, and AF all have been associated with troponin elevation, a selection bias due to missing TnI value on admission could well have rendered an overestimation of the prevalence of troponin elevation in **Study I**.

As expected, **Study I** revealed an association between troponin elevation and a higher age, comorbidity burden and stroke severity, which is in line with previous data (2, 131, 133, 135, 136). Surprisingly, we did not find a statistically significant increase in the rate of AF among patients with troponin elevation, which is in contradiction to prior data (23, 38). Our data on AF is, however, limited to history of AF, or AF detected from ECG during the hospital stay, and continuous monitoring or prolonged out-patient screening for arrhythmias was not part of the routine workup at the time. Likewise, we did not find a significantly higher rate of cardioembolic stroke in patients with troponin elevation, although there was a trend towards increasing rates with increasing troponin levels. The study was, however, not designed or powered to detect differences in stroke subtype, and a larger sample size may well have revealed a stronger association. Asymptomatic AF convey as high a risk for ischemic stroke as symptomatic AF (137, 138), and given that anticoagulant therapy reduces the risk for recurrent stroke with over 60% (139), a more careful screening for AF in ischemic stroke patients with troponin elevation may be warranted.

Despite the high prevalence of troponin elevation in **Study I**, there were few recordings of chest pain on admission. ST segment deviations suggestive of myocardial ischemia were, however, significantly more common in patients with elevated TnI, in line with previous studies (2, 19, 38, 39, 131, 140), suggesting that silent myocardial ischemia could have contributed to the troponin elevations. Indeed, the prevalence of chest pain may have been underestimated due to verbal impairment in an acute stroke situation. Furthermore, asymptomatic coronary artery stenosis >50% indicating CAD has been shown to be common in stroke patients (26%) (35), and pre-existing CAD may result in a higher vulnerability to myocardial injury. The substantial increase in the rate of beta-blockers at discharge in patients with elevated TnI in our study may reflect a clinical suspicion of CAD, although we lack data on the indications for these subscriptions. Despite the recent contradicting results on coronary vessel status in patients with ischemic stroke and troponin elevation (42, 43), our findings imply that CAD should be considered in ischemic stroke patients with elevated levels of troponin. A major limitation of **Study I** is, however, the lack of serial measurements of TnI, leaving no data on dynamic changes. As previously discussed, stable elevations of troponin may reflect underlying comorbidities such as CHF or renal insufficiency, and distinguishing between patients with stable and dynamic levels of TnI could aid in identifying those with ACS. In the study by Anders et al (37), including 834 ischemic stroke patients, 40 % of the patients with elevated TnI showed dynamic values (> 30% rise or fall) over serial measurements, and 29 of these patients (54%) were diagnosed with myocardial infarction in contrast to none of the patients with stable elevations of TnI. Furthermore, two other recent

studies (13, 141) showed that only dynamic changes in TnT (> 50% and 30% rise or fall respectively) were associated with in-hospital mortality.

In line with recent studies implying that cancer may be an important independent risk factor for ischemic stroke (56-59), the prevalence of prior diagnosis of cancer among all ischemic stroke patients in **Study I** well exceeds the prevalence in an age-matched general population (124). The increasing levels of both C-reactive protein and leukocyte count as well as the decreasing levels of haemoglobin with increasing TnI values in **Study I** also support the idea of an underlying inflammatory process, which may in part be related to an underlying malignancy. Although the prevalence of prior cancer diagnosis increased with increasing TnI values, the difference was not statistically significant, perhaps due to the limited number of events. However, five among the ten patients with the highest values of TnI (ranging from 2-96 μg/L) had an underlying cancer diagnosis, suggesting that high elevations of troponin may be associated with an underlying cancer. Indeed, 7/12 of the patients with high troponin elevations in **Study II** were found to have a known or on admission occult underlying cancer, as opposed to 1/19 in the control group. Notably, these patients were included exclusively on their troponin levels, regardless of underlying comorbidities. Interestingly, these patients did not differ from the patients with normal hsTnT levels with regard to age, cardiovascular or renal comorbidities, or stroke severity (NIHSS score). Furthermore, only 2/11 of the patients with high hsTnT elevations presented with signs of myocardial ischemia on ECG or echocardiograms. Although the sample size was small, this indicates a mechanism leading to high troponin elevations distinct from the ones proposed in previous studies, perhaps linked to a cancer-induced hypercoagulable state, as will be discussed further down.

5.2 PROGNOSIS

In line with extensive previous data (2, 11-14, 19, 131, 142, 143), we found an association between troponin elevation in ischemic stroke patients and a higher risk for mortality. Most of these studies, however, looked at in-hospital and short-term prognosis, with the exception of two recent studies by Jensen et al (131) and Faiz et al (143), with a follow-up of 1.5 years. Despite the adjustment for several possible confounders in **Study I**, troponin elevation was associated with an almost two-fold increase in risk of death, which was sustained over the 5-year follow-up period, revealing novel information of the long-term prognosis in these patients. Successful long-term treatment relies on early risk assessments, and these results indicate the prognostic significance of troponin as a biomarker in stroke patients to identify

those at highest risk for not only short-term and in-hospital mortality but also long-term mortality.

Surprisingly, we did not find any significant differences in the proportion of deaths by cardiac cause between the groups in **Study I**. There was also no difference in the rate of recurrent cardiovascular disease or new diagnosis of cancer. This could, however, be a false negative finding due to the limited number of events in the study.

5.3 CANCER-ASSOCIATED ARTERIAL MICROTHROMBOSIS

As mentioned before, cancer conveys a well-established increased risk for VTE (61-66, 75, 144, 145). Studies performed during recent years have also provided emerging evidence for cancer-associated arterial thrombosis, such as ischemic stroke (56, 57, 59, 68, 70-72, 146-148) and myocardial infarction (69). Although the mechanisms remain unclear, cancer-associated thrombosis seems to involve a hypercoagulable state (74, 75, 78, 79, 149-152), which could have a multiorgan effect. Considering the high prevalence of pre-existing CAD in ischemic stroke patients (35), it could be hypothesized that these patients are vulnerable to suffer both cerebral and myocardial thrombotic events if exposed to a cancer-induced exaggerated hypercoagulable state, presenting as ischemic stroke with troponin elevation.

Indeed, histopathological and plasma analyses and in **Study II** were strongly supportive of a hypercoagulable state in our ischemic stroke patients with high troponin elevations and an underlying malignancy. 4/8 stroke patients with active cancer were diagnosed with cancer at autopsy or within two months after stroke onset. Macroscopic examination during autopsies revealed no thrombotic occlusions of the coronary arteries, and the widespread microvascular thrombosis was revealed first at histopathology. Notably, autopsy including histopathology is very rare in stroke patients, which may contribute to an underestimation of a multiorgan microvascular thrombosis in ischemic stroke patients with an underlying malignancy.

Our results are in keeping with previous studies of ischemic stroke in cancer patients, revealing a principal cerebral lesion pattern of multiple dots extending single vascular territory (70-72, 147, 148, 153), compatible with a thrombotic pathophysiology, as well as an overrepresentation of adenocarcinomas (147, 153). Adenocarcinomas are known to be one of the most pro-thrombotic tumor types with regard to VTE (75, 154, 155), and assuming similar pathomechanisms in arterial thrombosis, this overrepresentation is not

surprising. The increased prevalence of cryptogenic stroke in our patients is also in line with prior studies of ischemic stroke in cancer patients. Interestingly, it was recently reported that cancer patients suffering cryptogenic or embolic stroke have a reduced survival compared with cancer patients with conventional stroke etiology (70). Although further studies are needed to determine the reason for this, it is intriguing to speculate whether this poor prognosis may be a consequence of a widespread arterial microthrombosis resulting in multiple organ failure. Worth noting, however, are the high levels of troponin in our case patients, preventing a generalization to all ischemic stroke patients with troponin elevation as the majority of these patients present with more modest troponin elevations.

The preferred treatment and prophylaxis in cancer-associated thrombosis is low-molecular weight heparin (156, 157), whereas patients with cardiovascular disease and arterial thrombosis, such as non-embolic ischemic stroke and myocardial infarction, commonly receive anti-platelet therapy. Special attention to a possible underlying malignancy in patients with ischemic stroke and troponin elevation may thus not only lead to an earlier diagnosis of an occult cancer, but perhaps also to an optimal treatment of this pro-thrombotic state.

5.4 THE CONTRIBUTION OF NETS

We link the pro-thrombotic state observed in our stroke patients with troponin elevation and an underlying malignancy to NETosis by showing elevated plasma levels of the NET-specific marker H3Cit as well as the NET-associated markers cfDNA and MPO. In further support of this are the positive correlations between the circulating NET markers and markers of coagulation and platelet activation. We also show H3Cit in co-localization with cfDNA, interpreted as NET complexes, in thrombi from a variety of organs in these patients.

Our results are, to the best of our knowledge, the first to present the NET-specific marker H3Cit in plasma as well as in cerebral, myocardial and pulmonary arterial microthrombi in patients with cancer. It is also the first report on NETs in stroke patients with an underlying malignancy. Our plasma analyses show a significant increase in circulating NET markers in our stroke patients with cancer compared to our stroke patients without cancer, and the lack of difference in these markers between stroke patients without cancer and healthy individuals suggests that a systemic NET burden may be associated to the underlying cancer in these

patients. We were not, however, able to perform immunohistochemistry on thrombi in our stroke patients without cancer, as none of these were available for autopsy. We can therefore not rule out the possibility that the NETs found in the thrombi of the stroke patients with underlying cancer were locally released as a consequence of the hypoxic milieu and strong inflammatory response surrounding the infarcted tissue, rather than as an effect of a cancer-induced systemic NET burden. Our results may, nevertheless, reflect not only the role of NETs in cancer-associated arterial thrombosis, but also the role of NETs in cancer biology per se, regardless of thrombosis (113, 114, 117).

In line with prior murine models of cancer implicating the role of cancer-released G-CSF in the induction of NETosis (81, 82, 114), we show elevations of G-CSF in our patients with underlying malignancies, as well as positive correlations between circulating G-CSF and markers of NETs. We cannot, however, be sure of the origin of G-CSF in our patients, nor the causality between G-CSF and NET formation. We also cannot rule out the possibility of other soluble molecules contributing to the priming of neutrophils toward NETosis in our patients. For instance, IL-8 has been shown to play a role in tumor progression (158, 159), as well as in NET formation in cancer (160) and other inflammatory conditions (161).

Recent years have provided evidence that possible NET-inhibitors, such as PAD4 inhibitors and DNase (98, 162, 163), may alleviate the prothrombotic effects of NETs as well as inhibit tumorigenesis and metastasis (164, 165). Interestingly, heparins have also been shown to prevent NET-induced thrombosis by inhibiting platelet binding and aggregation as well as promoting the release of histones through destabilization of chromatin (96), perhaps explaining, at least partially, the superior effect of heparins in cancer-associated thrombosis. Taken together, these studies imply that markers of NETs may come to serve as both diagnostic markers and therapeutic targets in cancer and cancer-associated thrombosis.

5.5 QUANTIFICATION OF NETS

Although over a decade has passed since the discovery of NETs, and despite emerging interest in the field, there is still no golden standard marker or method to detect and quantify NETs in plasma. As a result of this, various markers and methods have been implemented to demonstrate the presence of NETs, making it difficult to interpret and compare results between studies. Furthermore, and as discussed previously, many of these methods are hampered by lack of specificity, objectivity and quantification. **Study III** establishes an assay allowing for a highly objective, fast and reliable quantification of the

NET specific marker H3Cit in human plasma. The methodological validation revealed a high specificity for H3Cit, as well as a high stability of the custom-made standard, allowing for a good reproducibility, with CVs all < 15%.

We were, however, not able to assess trueness of the concentrations, lacking a reference analyte of known concentration or an established method for comparison. The concentration of H3Cit in the standard curve is, furthermore, an estimation based on the assumption that all histones are citrullinated (assuming the optimal enzymatic activity of PAD4), and an underestimation of the concentrations in our samples can thereby not be ruled out. We were not able to recover known concentrations of H3Cit spiked in healthy plasma, presumably due to the instability of free histones in plasma (166), as opposed to the H3Cit quantified in our plasma samples, which are hypothesized to be protected by surrounding DNA as part of nucleosomes in NET complexes. Histones have, in their free form, been shown to have a very rapid degradation in plasma, with a half-life of 4.6 m (166). It is therefore possible that the amount of H3Cit quantified by this assay is in fact the amount of H3Cit protected by the NET complex, excluding a possible portion of free H3Cit in plasma. Although the portion of H3Cit bound to DNA in NET complexes is the endpoint of interest in quantifying NETs, the effect of the matrix does impose a limitation to the assay validation.

Despite the above mentioned validation obstacles, however, the assay represents a first step for standardization and an objective and specific quantification of NETs in human plasma. Inasmuch, standardizations and validations, as performed herein, are a necessity in order to compare and interpret data between studies and across labs. To this note, other promising assays to assess NET burden are under development and validation, such as image-based flow cytometry (167) and flow cytometric detection of key NET components (168). Further studies will illustrate whether this H3Cit ELISA, or any of the other methods, alone or in combination, may be helpful in the quest for the clinical relevance of NET formation in various conditions, such as cancer and thrombosis.

5.6 GENERAL REMARKS

In line with previous studies, we show an association between troponin elevation in ischemic stroke and ECG changes suggestive of myocardial ischemia, as well as contribute to prior data on the adverse short-term prognosis in these patients by providing novel data on an almost 2-fold increased risk of 5-year mortality. These results support prior studies indicating the need for cardiologic work-up and more aggressive prevention measures in these patients.

We also provide novel data suggesting that an underlying cancer-induced pro-thrombotic state may contribute to the adverse prognosis in these patients, suggesting that an underlying cancer should be considered in patients with high levels of plasma troponin. We show that neutrophils, assumed to be primed by an underlying cancer, may contribute to this thrombotic state through the release of NETs. Finally, we suggest and validate a novel method to detect and quantify the NET specific marker H3Cit in human plasma, which may be useful in further studies to confirm these hypotheses. Indeed, due to the limited sample sizes in our studies, our results must be regarded as such; hypotheses laying the ground for further studies. We therefore encourage replication of these results. If confirmed, these clinical observations may motivate further research into the pathomechanisms behind cancer-associated arterial thrombosis, including the contribution of NETs, and aid in the quest for new diagnostic and prognostic markers as well as therapeutic agents.

6 CONCLUSIONS

- Patients with stroke and troponin elevation are on average older, and have more severe strokes and comorbidities, than patients with stroke without troponin elevation. They also present with a higher incidence of ECG changes suggestive of myocardial ischemia.
- Troponin elevation in patients with acute stroke, even when adjusted for several possible confounders, is associated with an almost two-fold increased risk of five-year mortality.
- Cancer-associated arterial microthrombosis leading to concomitant cerebral and myocardial ischemia may be an underestimated pathomechanism behind high levels of plasma troponin in ischemic stroke patients.
- NETs may be a source of arterial microthrombosis in cancer patients, presenting as ischemic stroke with troponin elevation.
- The levels of the NET specific marker citrullinated histone H3 (H3Cit) can be quantified in human plasma with a novel enzyme-linked immunosorbent assay, allowing for a high specificity, precision and stability.

7 FUTURE PERSPECTIVES

The prognostic significance of troponin elevation in the setting of acute stroke is justified beyond doubt in prior studies along with the data from this thesis. Further studies are, however, needed to distinguish between the different pathomechanisms behind a myocardial injury in acute stroke. A cancer-induced hypercoagulative state resulting in concomitant cerebral and myocardial microthrombosis, presented in this thesis as acute stroke with troponin elevation, warrants further validation in larger cohorts. In a wider perspective, cancer-associated arterial thrombosis comprises much more than cerebral and myocardial ischemia, and this mechanism should be studied in other settings as well. The contribution of NETs, and the possibility of NET markers as prognostic markers or targets for novel therapeutic strategies, are appealing, but also need further validation. Indeed, these markers may prove to be of importance in not only a wider range of cancer-associated thrombotic conditions, but also in cancer biology per se.

8 SVENSK SAMMANFATTNING

Troponin, ett protein specifikt för skada i hjärtmuskulaturen, är vanligt förekommande i blodet hos patienter som drabbats av en akut stroke. Trots att ett flertal studier talar för en sämre kort-tids prognos för dessa patienter är den kliniska signifikansen av förhöjda troponin-nivåer vid akut stroke fortfarande kontroversiell, och den bakomliggande orsaken är ofta okänd. Syftet med denna avhandling var att fastställa kliniska karakteristika och lång-tids prognos hos dessa patienter, samt att utforska möjliga bakomliggande mekanismer.

I en retrospektiv kohortanalys av 247 strokepatienter (Studie I), fann vi ett klart samband mellan troponin-förhöjning hos strokepatienter och hög ålder, stor samsjuklighet och svårighetsgrad av stroke. Strokepatienter med troponin-förhöjning hade också vid insjuknandet en högre förekomst av EKG förändringar talande för en hjärtpåverkan. En långtidsuppföljning visade vidare att dessa patienter hade en nära dubbelt så hög risk att dö inom 5 år.

I en fall-kontroll studie (Studie II) fann vi en hög förekomst av bakomliggande cancer hos strokepatienter med höga nivåer av troponin i blodet. Blodprovsanalyser talade starkt för ett underliggande hyperkoagulativt tillstånd hos dessa patienter, d v s en ökad benägenhet att bilda blodproppar i olika organ. Histopatologiska vävnads-undersökningar visade också små, arteriella blodproppar, s k mikrotromboser, i flertalet vitala organ, såsom hjärta och hjärna. Neutrofil-aktivering, med utsläpp av så kallade *neutrophil extracellular traps* (NETs) i blodet har visats bidra till venös tromboembolism vid cancer. Vi ville därför undersöka huruvida NETs skulle kunna vara bidragande orsak även till arteriell trombotisering vid cancer, med tromber i såväl hjärna som hjärta. Liksom blodprovs-analyser talande för ett hyperkoagulativt tillstånd, var också markörer för NETs kraftigt förhöjda i blodet hos strokepatienter med höga nivåer av troponin och en bakomliggande cancer. Histopatologiska undersökningar talade också för en bidragande NETs-bildning med immunodetektion av NETs markörer i de arteriella tromboserna. För att fastställa nivåerna av NETs i plasma hos dessa patienter, utvecklades en ny metod för att mäta det NET-specifika proteinet H3Cit. För att underlätta vidare användning av denna metod i framtida studier, gjordes en standardisering och metodologisk validering (Studie III), vilken visade på hög specificitet, precision och stabilitet.

Sammantaget talar dessa resultat för att strokepatienter med troponin förhöjning i blodet bör utredas med avseende på hjärtat. Resultaten talar också för att ett underliggande cancer-associerat hyperkoagulativt tillstånd bör övervägas vid höga nivåer av troponin. Slutligen visar de på ett samband mellan ett cancer-associerat hyperkoagulativt tillstånd och NETs, vilket förespråkar vidare studier för att undersöka huruvida NETs markörer i blodet skulle kunna vara av värde vid diagnostik av och prognos för cancer-associerad arteriell trombos. För detta syfte förslår vi en metod för att mäta det NET-specifika proteinet H3Cit i plasma.

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