Published in final edited form as:

Thromb Res. 2016 March ; 139: 56–64. doi:10.1016/j.thromres.2016.01.009.

NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation

Charlotte Thålin, MD¹, Melanie Demers, Ph.D.^{2,3}, Bo Blomgren, Ph.D.⁴, Siu Ling Wong, Ph.D.^{2,3}, Magnus von Arbin, Ph.D.¹, Anders von Heijne, M.D.⁵, Ann Charlotte Laska, Ph.D.¹, Håkan Wallén, Ph.D.⁶, Denisa D. Wagner, Ph.D.^{2,3,7}, and Sara Aspberg, Ph.D.⁶

¹ Department of Clinical Sciences, Danderyd Hospital, Division of Internal Medicine, Karolinska Institutet, Stockholm, Sweden

² Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, Massachusetts, USA

³ Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA

⁴ Department of Clinical Sciences, Danderyd Hospital, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden

⁵ Department of Clinical Sciences, Danderyd Hospital, Department of Radiology, Karolinska Institutet, Stockholm, Sweden

⁶ Department of Clinical Sciences, Danderyd Hospital, Division of Cardiovascular Medicine, Karolinska Institutet, Stockholm, Sweden

⁷ Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts, USA

Abstract

INTRODUCTION—Large elevations of high sensitive Troponin T (hsTnT) in ischemic stroke patients is associated with a poor outcome. In a pilot study we found a high prevalence of malignancies among these patients. Since neutrophil extracellular traps (NETs) have been linked to cancer-associated thrombosis, we hypothesized that the concomitant cerebral and myocardial ischemia could be the result of a NET-induced hypercoagulable state.

MATERIALS AND METHODS—Clinical assessments, plasma analyses and autopsies with histopathology (in cases of in-hospital mortality) were performed on ischemic stroke patients with high elevations of hsTnT (n=12) and normal hsTnT (n=19).

RESULTS—Patients with hsTnT elevation had an unexpectedly higher prevalence of cancer (p=0.002), half of which were diagnosed post-mortem. Autopsies of these patients revealed

Corresponding author: Denisa D. Wagner, Program in Cellular and Molecular Medicine, Boston Children's Hospital, 3 Blackfan Circle, 3rd Floor, Boston, MA 02115, denisa.wagner@childrens.harvard.edu, Ph: 617-713-8300; Fax: 617-713-8333.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURE OF CONFLICTS OF INTEREST

Disclosures: None

widespread myocardial, cerebral and pulmonary microthrombosis with H3Cit in thrombi. A pro-coagulant state and an increase of the NET specific marker citrullinated histone H3 (H3Cit) was found in plasma of patients with elevated hsTnT compared to patients with normal levels ($p<0.001$). Plasma analyses in cancer patients showed even higher H3Cit levels ($p<0.001$), and an increase in granulocyte colony-stimulating factor, known to prime neutrophils towards NETosis. H3Cit correlated positively with thrombin-antithrombin complex ($p=0.004$) and soluble P-selectin ($p<0.001$), further linking NETosis to the prothrombotic state.

CONCLUSIONS—The high prevalence of known or occult cancer in our study suggests that cancer-associated arterial microthrombosis may be underestimated. By linking the thrombosis to NETs, we suggest markers of NETosis that could aid in revealing cancer in arterial microthrombosis as well as arterial microthrombosis in cancer.

Keywords

Cancer-associated microvascular thrombosis; Neutrophil extracellular traps; Troponin elevation; Ischemic stroke

INTRODUCTION

Cerebrovascular and cardiovascular thrombosis is a leading cause of death. Ischemic stroke can be associated with a variety of cardiac changes, including plasma elevations of cardiac enzymes such as troponins. Previous studies have shown that troponin elevation in ischemic stroke is associated with an overall increased risk of poor outcome and mortality ¹. The underlying pathophysiology of troponin elevation in ischemic stroke is still unclear. A number of possible mechanisms have however been proposed, such as concomitant acute coronary syndrome (ACS) ², neurologically induced myocardial injury due to sympathoadrenal activation ³, atrial fibrillation, congestive heart failure (CHF), renal insufficiency and severe infection ^{4,5}.

There is now growing evidence of neutrophil extracellular trap (NET) burden in a variety of thrombotic diseases: among them ischemic stroke ⁶, ACS ^{7,8} and cancer-associated thrombosis ⁹⁻¹¹. NETs were first described in 2004 ¹² as a mechanism for trapping and killing of bacteria by the innate immune system. Upon activation, neutrophils release chromatin (DNA and histones) coated with antimicrobial granular proteins such as myeloperoxidase (MPO). Prior to releasing NETs, the protein citrullinating enzyme peptidyl arginine deiminase 4 (PAD4) enters the nucleus and citrullinates histones initiating chromatin decondensation. Citrullinated histone H3 (H3Cit) is thereby considered a NET specific marker. NETs have been found to promote coagulation by activation of platelets and coagulation factors as well as providing a scaffold for platelets and red blood cells, promoting thrombus formation ^{13,14}.

In an attempt to better understand the mechanisms leading to troponin elevation in ischemic stroke patients, a pilot-study was conducted and ischemic stroke patients were selected in a case-control design on the basis of highly elevated or normal levels of plasma high sensitivity Troponin T (hsTnT). The presence of comorbidities, such as ACS, CHF, atrial fibrillation and renal insufficiency, did not differ between the groups with and without

troponin elevation, ruling out the implication of these previously suggested mechanisms. Instead, we found an unexpectedly high prevalence of malignancies among the patients with large hsTnT-elevations. Autopsy and histopathological investigation performed on three patients with elevated hsTnT and malignancies revealed widespread arterial H3Cit-positive microthrombosis. We hypothesized that high elevations of plasma troponin in ischemic stroke patients could be the result of a cancer-associated NET-induced pro-coagulant state leading to concomitant cerebral and myocardial ischemia. In light of a recent report on cancer-associated granulocyte colony-stimulating factor (G-CSF) priming neutrophils towards NETosis in mouse models ⁹, we sought to examine the contribution of circulating G-CSF in NET formation. We report elevated levels of circulating G-CSF in these patients, as well as positive correlations between circulating G-CSF, markers of coagulation, and NETs, linking a cancer-induced systemic NET burden to widespread arterial microthrombosis presenting as stroke with troponin elevation.

METHODS

Study population and design

A prospective, observational case-control study including 31 patients with ischemic stroke admitted to the stroke unit at Danderyd Hospital, Stockholm, between April 2012 and December 2014. Patients with ischemic stroke and hsTnT > 40 ng/L (ref value < 15 ng/L) were recruited as case patients (n=12) and patients with ischemic stroke and hsTnT ≤ 15 ng/L were recruited as control patients (n=19). They were matched according to sex and age within a five-year interval. Inclusion criteria for both groups 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 for both groups were acute cardiovascular event (ACS or ischemic stroke) within four weeks of symptom onset. Plasma concentration of hsTnT was analyzed on admission using the ECLIA electrochemiluminescence immunoassay system (Roche Diagnostics Scandinavia AB). The assay performance for the cut-off values (≤ 15 ng/L for normal values and >40 ng/L for elevation) is in accordance with current guidelines. 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. Healthy volunteers (n=10), matched on sex and age within a five-year interval, were recruited as reference for plasma analyses.

Demographic data and comorbidity were obtained from medical records and patient history documented on admission. Active cancer was defined as diagnosis of, or treatment for, cancer within the prior six months, known recurrent or metastatic disease, or diagnosis within two months after stroke onset.

CT brain imaging was performed on admission and stroke localization and distribution was determined by a senior neuroradiologist blinded to clinical details. Stroke severity was determined using the National Institute of Health Stroke Scale (NIHSS) by certified raters.

Plasma analyses

Blood samples were drawn within two days of admission with patients in bed in the supine position 30 minutes prior to blood sampling. Plasma samples were prepared from citrated whole blood following immediate centrifugation for 20 minutes at 2000 g after which they were stored at -80°C until further analyses. Plasma analyses were performed at the Wagner Laboratory, Boston Children's Hospital. At time of analyses, samples were thawed once. Thrombin-antithrombin complex (TAT), soluble P-selectin (sP-selectin), cell free DNA (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 a tailor-made capture ELISA method. Briefly, the microplate modules of the cell death detection ELISA were coated with the anti-histone antibody (Component 1) overnight and blocked with the incubation buffer (Roche). Plasma samples were incubated for one hour, washed with PBS-Tween 0.05%, followed by incubation with rabbit anti-histone H3 (citrulline 2+8+17) antibodies (Abcam) and anti rabbit-horseradish peroxidase-conjugated antibodies (Biorad). The samples were analyzed side-by-side on the same plate, and the inter-individual differences in duplicates were negligible (mean difference 0.007 O.D.).

Autopsy and histopathology

Specimens obtained at autopsy 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 antibody used was anti-histone H3 (citrulline 2+8+17) antibody (Abcam) for H3Cit. Confocal immunofluorescence microscopy at the Wagner Laboratory, Boston Children's Hospital, was performed 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-von Willebrand factor (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.

The study complied with the Declaration of Helsinki and the protocol was approved by the Stockholm local ethics committee (dnr 2011/1310-31/3 and 2014/442-31/4). Written informed consent was obtained from each study participant or a family member.

Statistics

Statistical methods were chosen to fit small numbers of observations and non-normal distributions. Categorical variables are presented as proportions and compared with the Fisher's exact test. Continuous variables are 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. All statistical analyses were performed using STATA 12.1 software (STATA, Texas, USA) and a p-value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics, clinical assessment and histopathological findings

In order to identify the cause of elevated troponin levels in ischemic stroke patients, study patients were selected in a case-control design on the basis of highly elevated or normal levels of plasma hsTnT. During time periods of study inclusion, eligible patients with the highest plasma hsTnT were therefore selected, rendering a high mean of hsTnT in the case group; 287.8 ng/L with a median of 144.0 ng/L. The mean value of hsTnT in the control group was 8.7 ng/L with a median of 9.0 ng/L.

There were no significant differences in patient characteristics and NIHSS score between ischemic stroke patients with and without elevated hsTnT, except for the prevalence of active cancer (n=8), which was significantly higher in the group with elevated hsTnT compared to the group with normal values of hsTnT; 7/12 vs. 1/19, p=0.002 (Table 1). Four of these eight patients had a known diagnosis of active cancer on admission; three patients were diagnosed with active cancer post-mortem and one within two months after stroke onset. Seven of eight primary tumors were adenocarcinomas, but of different origin (Table 2).

Multiple and disseminated cerebral lesions extending single vascular territory detectable on CT brain imaging were significantly more common in this group compared to patients without active cancer (50 % vs. 4 %, p=0.010). Macroscopic examination during autopsy of three of the ischemic stroke patients with high troponin elevations showed no thrombotic occlusions of the coronary arteries. Furthermore, only mild atherosclerotic plaque formation was seen. Histopathology, however, revealed disseminated focal areas of myocardial damage at different stages accompanied by widespread microvascular thrombosis. Abundant and widely spread arterial microthrombi were found in the brain, heart and in the pulmonary arterial tree (Fig 1 A), suggesting a systemic pro-thrombotic state rather than thrombus formation on ruptured atherosclerotic plaques or embolizations. In addition, thrombi with concomitant infarctions were observed in small renal and splenic arteries in one of the three patients where an autopsy was performed.

Interestingly, immunohistochemistry (Fig 1B) and confocal microscopy (Fig 1C and D) revealed 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. This suggests the potential implication of NETs in the microthrombosis associated with high troponin levels in ischemic stroke patients.

Plasma markers of coagulation, platelet activation and NETs

To assess whether troponin elevation could be associated to a pro-coagulant state, we determined plasma levels of TAT and sP-selectin (Fig 2A). TAT levels appeared higher in

patients with elevated hsTnT compared to patients with normal hsTnT levels, although the differences did not reach statistical significance (median 12.1 with IQR 6.7-33.2 µg/mL vs. median 6.1 with IQR 4.4-11.2 µg/mL, $p=0.12$). sP-selectin was significantly higher in patients with elevated hsTnT levels (median 50.1 with IQR 34.7-95.8 ng/mL vs. median 21.4 with IQR 14.4-33.9 ng/mL, $p=0.001$). Moreover, increased plasma levels of the NET associated markers cfDNA (median 481.3 with IQR 439.9-552.1 ng/mL vs. median 371.7 with IQR 339.7-446.4 ng/mL, $p=0.002$) and MPO (median 60.7 with IQR 57.4-146.7 ng/mL vs. median 22.7 with IQR 18.3-37.3 ng/mL, $p<0.001$) as well as a three-fold increase of the NET specific marker H3Cit (median 0.22 with IQR 0.12-0.32 O.D. vs. median 0.06 with IQR 0.05-0.08 O.D., $p<0.001$) were also found in patients with elevated hsTnT compared to patients with normal hsTnT levels, suggesting the presence of NETs.

Since the ischemic stroke patients with elevated troponin had a high prevalence of cancer and a NET-induced coagulant state has been reported to play a role in cancer-associated thrombosis⁹, we compared the levels of these markers in patients with and without active cancer. Significant and even higher elevations of both TAT and sP-selectin were seen in patients with cancer compared to patients without cancer (TAT: median 28.9 with IQR 9.8-47.0 µg/mL vs. median 6.1 with IQR 4.1-10.2 µg/mL, $p=0.001$, sP-selectin: median 75.3 with IQR 35.9-100.8 ng/mL vs. median 26.0 with IQR 16.8-39.1 ng/mL, $p=0.001$) (Fig 2B). Interestingly, similar elevations of cfDNA (median 504.0 with IQR 443.7-562.0 ng/mL vs. median 407.9 with IQR 340.5-451.7 ng/mL, $p=0.04$), MPO (median 74.1 with IQR 57.9-146.7 ng/mL vs. median 30.8 with IQR 18.5-58.4 ng/mL, $p=0.01$) and plasma H3Cit (median 0.22 with IQR 0.12-0.31 O.D. vs. median 0.07 with IQR 0.05-0.08 O.D., $p<0.001$) were seen in patients with active cancer vs. no cancer. When excluding patients with cancer (Fig 2C), TAT and sP-selectin did not differ between patients with and without hsTnT elevation ($p=0.97$ and 0.12 respectively), thus linking cancer to the pro-coagulant state. In accordance with markers of coagulation and platelet activation, the differences in circulating cfDNA, MPO and H3Cit between patients with hsTnT elevation and patients with normal hsTnT values were eliminated when excluding patients with cancer ($p=0.12$, 0.052 and 0.14 respectively) (Fig 2C).

Furthermore, elevations of H3Cit correlated positively with elevations of cfDNA, MPO, TAT, and sP-selectin (Fig 3A). Of note, one of the stroke patients with troponin elevation and cancer had very high levels of H3Cit, as depicted in Fig 2 and 3. The differences in H3Cit levels between cancer patients and non-cancer patients (Fig 2B) were, however, still large and significant when excluding this patient from analyses (median 0.22 with IQR 0.12-0.28 vs. median 0.07 with IQR 0.05-0.08, $p<0.005$). Likewise, the correlations between elevations of H3Cit and elevations of cfDNA, MPO, TAT and sP-selectin remained significantly positive when excluding this patient ($r=0.74$, $p<0.001$; $r=0.64$, $p<0.001$; $r=0.45$, $p<0.05$; $r=0.57$, $p=0.001$, respectively) (Fig 3). These results suggest that troponin elevation in ischemic stroke patients may be the result of a NET-induced systemic pro-coagulant state associated with cancer.

Circulating G-CSF

Cancer has been shown to prime neutrophils towards NETosis through the release of G-CSF⁹. In light of the high prevalence of cancer, and its association with a pro-thrombotic state in our study, we determined the levels of circulating G-CSF and leukocyte count in order to assess a link between G-CSF and NET burden. 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, $p=0.016$). Leukocyte count was also significantly higher in patients with cancer (median 12.6 vs. $6.8 \times 10^9/L$, $p=0.002$). Furthermore, there were positive correlations between levels of G-CSF and levels of H3Cit, MPO, TAT and sP-selectin (Fig 3B).

When comparing the whole study group, comprising all ischemic stroke patients ($n=31$), with sex- and age matched healthy controls ($n=10$), markers of coagulation and platelet activation were significantly higher in ischemic stroke patients (median 7.2 vs. 4.3 $\mu g/mL$, $p=0.002$ and 31.6 vs. 21.0 $\mu g/mL$, $p=0.011$ for TAT and sP-selectin, respectively). There was also an elevation of circulating cfDNA in ischemic stroke patients compared to healthy controls (median 413.7 vs. 314.8 ng/mL, $p<0.001$). G-CSF, H3Cit and MPO, however, did not differ between ischemic stroke patients and healthy controls (median 9.5 vs. 8.1 pg/mL, $p=0.56$; median 0.08 vs. 0.07 O.D., $p=0.56$ and median 21.0 vs. 37.0, $p=0.092$ for G-CSF, H3Cit and MPO respectively), suggesting that the presence of cancer is likely the cause of G-CSF release and consequent priming toward NETosis.

DISCUSSION

In an attempt to evaluate the cause of large elevations of troponin levels in ischemic stroke patients, we now report the implication of NET-associated arterial microthrombosis potentially due to cancer. Plasma analyses and histopathological investigations were strongly supportive of a systemic pro-thrombotic state. We link this pro-thrombotic state to NETosis by showing elevated levels of the NET-specific marker H3Cit in plasma as well as in thrombi from a variety of organs. The presence of a NET-induced pro-coagulant state was strongly associated with underlying cancer in the ischemic stroke patients with elevated troponin. Apart from our case report of one of the patients in this study¹⁰, H3Cit has, as far as we know, not previously been described in plasma or thrombi of ischemic stroke patients. Although the mortality was high among the stroke patients with cancer, the histones detected in plasma were citrullinated, implying that they did indeed originate from NETs and not merely histone release due to cell death in critically ill patients. This link is further supported by the positive correlations between H3Cit and MPO, an enzyme which is most abundantly expressed in neutrophils, and decorate NETs upon release. Furthermore, although elevations of both TAT and sP-selectin are established findings in cancer, the positive correlations between these markers and H3Cit further support a procoagulant state to which NETosis contributes, since NETs have been shown to be procoagulant and pro-inflammatory^{8,13}. Of note, sP-selectin was recently shown to promote NETosis in mice¹⁵. The high prevalence of cancer, elevations of circulating G-CSF and leukocytes, as well as the positive correlation between levels of G-CSF and H3Cit in these patients furthermore indicate a possible cancer-associated NET burden. Notably, exclusion of patients with cancer resulted in nonsignificant differences in markers of coagulation and NETosis

between patients with and without hsTnT elevation. Cancer-induced NETosis may thus have contributed to the devastating, and in severe cases lethal, widespread arterial microthrombosis. Interestingly, macroscopic examination during autopsies revealed no thrombotic occlusions of the coronary arteries, and the widespread microvascular thrombosis was revealed first at histopathology. Autopsy including histopathology is very rare in stroke patients, and the multiorgan microvascular thrombosis resulting from a cancer associated pro-coagulant state may be underestimated in ischemic stroke patients with an underlying malignancy.

Cancer-induced arterial thrombosis

Venous thromboembolism (VTE) is a well-established complication in a variety of malignancies, where a four to six-fold increased risk of VTE has been reported^{16,17}. Tumor-shed microvesicles exposing tissue factor, selectin-mucin interactions in mucin secreting adenocarcinomas, cytokines, and increase in leukocytes and platelet counts have all been proposed to play a role¹⁸⁻²². The pathophysiology and extent of cancer associated arterial thrombosis is, however, less investigated. Interestingly, in 1985, an autopsy study comprising 3,426 cancer patients (excluding intracranial neoplasms) reported cerebrovascular lesions in 14.6% of the patients²³. A recent and large epidemiological study also reported higher cancer prevalence in a stroke population than in an age-matched general population²⁴. Furthermore, an increased risk of myocardial infarction²⁵ or stroke²⁶ has been reported in patients with cancer, as well as an increased risk of cardiovascular mortality in stroke patients with prior cancer diagnosis²⁷, suggesting that cancer-associated arterial thrombosis may be underestimated. Four of the eight patients with active cancer in our study were diagnosed with cancer at autopsy or within two months after stroke onset. Armand Trousseau was the first to describe the connection between cancer and VTE in 1865²⁸, reporting unexpected thrombosis as the forewarning of an occult malignancy. The high mortality of stroke patients without autopsy examinations may contribute to an underestimated occurrence of cancer associated ischemic stroke.

NETosis in arterial thrombosis

Elevations of nucleosomes and cfDNA have repeatedly been shown to correlate to infarct area and adverse outcome in patients with ischemic stroke and myocardial infarction²⁹⁻³³. Being non-specific markers of tissue injury, however, the origin of cfDNA and nucleosomes is not clear as they could be released from necrotic tissues following injury and vessel wall damage. Recently, however, reports have shown H3Cit in infarcted areas in mouse models of myocardial⁸ and cerebral infarction⁶. The strong inflammatory response and hypoxic milieu surrounding an infarcted tissue could in this setting activate and prime neutrophils towards NETosis, inducing a local NET burden at the site of the infarction. Once released, NETs could subsequently contribute to thrombus growth and stabilization³³. In contrast to this, our present findings of a three-fold increase in circulating H3Cit in our stroke patients with active cancer compared to stroke patients without cancer, and the lack of elevated circulating H3Cit in our stroke patients compared to healthy controls, suggests a systemic NETosis initiating thrombus formation in these patients, rather than NETosis being a consequence of the infarction. The presence of H3Cit and H3Cit-positive cells, likely neutrophils, in thrombi also supports the idea of a systemic NETosis.

Cancer-induced NETosis

The marker of NETosis (H3Cit) have been found in plasma of cancer patients with acute microangiopathies¹¹. The link between NETosis and cancer was also recently demonstrated by Demers and colleagues in a murine model of chronic myelogenous leukemia in which malignant and non-malignant neutrophils were more prone to NET formation⁹. In the same study, spontaneous pulmonary thrombosis was observed in a murine mammary cancer model, correlating with high levels of cfDNA and H3Cit in plasma. In both models, tumor-associated G-CSF was shown to prime neutrophils towards NETosis. G-CSF stimulates bone marrow to produce granulocytes and increases survival and proliferation of mature neutrophils in the bloodstream³⁴. It is normally synthesized by leukocytes and endothelial cells, but is also overexpressed in a variety of cancer cells where it has been shown to skew hematopoiesis toward a myeloid lineage as well as promote the production of atypical T-cell suppressive neutrophils^{35,36}. We cannot be sure of the origin of G-CSF in our study patients. However, the seven-fold increase in circulating G-CSF levels in our patients with cancer, and the lack of elevations of circulating G-CSF in the rest of our stroke patients compared to healthy controls, suggests that G-CSF indeed is cancer related. Furthermore, the positive correlation between elevations of circulating G-CSF and H3Cit and MPO as well as between G-CSF and markers of coagulation and platelet activation supports a link between G-CSF, NETosis and coagulation, as well as a link between cancer and arterial thrombosis.

Limitations

There are certain limitations worth noting, such as the small sample size in our study. Due to the sample size, we cannot determine the prevalence of a cancer induced NET burden in this patient group. However, the differences between the groups were large and of high statistical significance, and the data strongly suggests that NETs may be a previously unrecognized and important contributing factor to arterial microthrombosis in cancer. Further, the patients were selected on troponin levels and cancer diagnosis did not influence the inclusion. In fact, the cancer diagnosis was unknown in half of the cancer patients at inclusion, being diagnosed with cancer after stroke onset or at autopsy, as stated in Table 2. We clearly show markers of NETs in cerebral, myocardial and pulmonary thrombi in patients with cancer. We cannot exclude the possibility that microthrombi observed in the small pulmonary arteries are emboli from thrombi in veins e.g. in the lower extremities. The main findings on autopsies were, however, thrombi in small arteries of several different organs strongly suggesting an arterial thrombotic diathesis in the stroke patients with cancer. Inclusion of a larger number of patients is needed to further validate our results.

Another limitation is that we could not perform immunohistopathology of thrombi in patients without cancer. None of these patients died and the thrombi were thus not available for analysis. Notably, there was a clear selection in including patients with the highest troponin levels. These results cannot be generalized to the ischemic stroke population with troponin elevation, as the majority of these patients have more modest elevations of troponin, probably due to previously suggested mechanisms such as myocardial comorbidity. However, our results strongly suggest a novel mechanism behind large elevations of troponin in ischemic stroke patients.

CONCLUSIONS

Taken together, we present new findings suggesting a cancer-induced systemic NET burden resulting in concomitant widespread cerebral and myocardial microthrombosis presenting as ischemic stroke with high elevations of plasma troponin. Elevated plasma troponin in ischemic stroke patients deserves special attention, and the presence of occult malignancy should be considered, not only to enable earlier diagnosis and possibly elimination of a causative tumor, but also to enable initiation of treatment of the pro-thrombotic state. Low-molecular-weight heparin (LMWH) is currently the preferred treatment and prophylaxis of cancer associated pro-thrombotic events, whereas oral anticoagulants have been less effective³⁷. Interestingly, heparins have also been shown to prevent NET-induced platelet binding and aggregation as well as promote the release of histones through destabilization of chromatin¹⁴. Antiplatelet agents are, however, dominating in ischemic stroke, with the exception of cardioembolic stroke where oral anticoagulants is the treatment of choice. Furthermore, recent pre-clinical studies have demonstrated the possibility of alleviating the pro-thrombotic effects of NETosis with new therapeutic agents such as DNase^{8,38,39} and PAD4 inhibitors⁴⁰.

In a broader perspective, markers of a pro-thrombotic state, such as TAT and sP-selectin, and markers of NETosis, such as H3Cit, could aid in revealing cancer in patients with arterial thrombosis. These markers, as well as G-CSF, could also be useful in screening for susceptibility to arterial thrombosis in cancer patients. Further studies are needed to explore the extent of NET burden in cancer-associated arterial thrombosis, as well as the benefit of LMWH or new anti-NET therapeutic agents to alleviate the pro-thrombotic effects of NETs.

ACKNOWLEDGMENTS

We thank Stephen Cifuni, lab manager at the Wagner Laboratory, for assistance with the transfer and handling of samples and specimens, Annika Lundström, MD, for helpful discussions, and Eva Isaksson, Berit Eriksson and Pernilla Becker, research nurses at the Stroke Unit, Danderyd University Hospital.

SOURCES OF FUNDING

This work was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health Grant R01HL102101 (Wagner) and Fund 176 and Coagulation Research Fund 245 of Karolinska Institutet (Thalin).

ABBREVIATIONS

ACS	acute coronary syndrome
CHF	congestive heart failure
NETs	neutrophil extracellular traps
PAD4	peptidylarginine deiminase 4
H3Cit	Citrullinated histone H3
hsTnT	high sensitivity Troponin T
G-CSF	granulocyte colony-stimulating factor

NIHSS	National Institute of Health Stroke Scale
TAT	thrombin-antithrombin complex
sP-selectin	soluble P-selectin
cfDNA	cell free DNA
VTE	Venous thromboembolism
LMWH	low-molecular-weight heparin

REFERENCES

1. Kerr G, Ray G, Wu O, Stott DJ, Langhorne P. Elevated Troponin after Stroke: A systematic review. *Cerebrovasc Dis.* 2009; 28(3):220–6. [PubMed: 19571535]
2. Fure B, Bruun Wyller T, Thommessen B. Electrocardiographic and troponin T changes in acute ischemic stroke. *J Intern Med.* 2006; 259(6):592–7. [PubMed: 16704560]
3. Barber M, Morton JJ, MacFarlane PW, Barlow N, Roditi G, Stott DJ. Elevated troponin levels are associated with sympathoadrenal activation in acute ischaemic stroke. *Cerebrovasc Dis.* 2007; 23(4):260–6. [PubMed: 17199083]
4. Mahajan VS, Jarolim P. How to interpret elevated cardiac troponin levels. *Circulation.* Nov 22; 2011 124(21):2350–4. [PubMed: 22105197]
5. Scheitz J, Nolte C, Laufs U, Endres M. Application and Interpretation of High-Sensitivity Cardiac Troponin Assays in Patients With Acute Ischemic Stroke. *Stroke.* 2015; 46:1132–40. [PubMed: 25737317]
6. Perez-de-Puig I, Miró F, Ferrer Ferrer M, Gelpi E, Pedragosa J, et al. Neutrophil recruitment to the brain in mouse and human ischemic stroke. *Acta Neuropathol.* 2015; 129(2):239–57. [PubMed: 25548073]
7. Mangold A, Alias S, Scherz T, Hofbauer T, Jakowitsch J, Panzenböck A, et al. Coronary Neutrophil Extracellular Trap Burden and DNase Activity in ST-Elevation Acute Coronary Syndrome are Predictors of ST-Segment Resolution and Infarct Size. *Circ Res.* 2015; 116(7):1182–92. [PubMed: 25547404]
8. Savchenko A, Borissoff J, Martinod K, De Meyer SF, Gallant M, Erpenbeck L, et al. VWF-mediated leukocyte recruitment with chromatin decondensation by PAD4 increases myocardial ischemia/reperfusion injury in mice. *Blood.* 2014; 123(1):141–8. [PubMed: 24200682]
9. Demers M, Krause D, Schatzberg D, Martinod K, Voorhees JR, Fuchs TA, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. *Proc Natl Acad Sci USA.* 2012; 109(32):13076–81. [PubMed: 22826226]
10. Thalín C, Blomgren B, Mobarrez F, Lundström A, Laska AC, von Arbin M, et al. Trousseau's syndrome, a Previously Unrecognized Condition in Acute Ischemic Stroke Associated with Myocardial Injury. *Journal of Investigative Medicine High Impact Case Reports.* 2014 DOI: 10.1177/2324709614539283.
11. Demers M, Wagner DD. NETosis: a new factor in tumor progression and cancer associated thrombosis. *Semin Thromb Hemost.* 2014; 40(3):277–83. [PubMed: 24590420]
12. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* 2004; 303(5663):1532–5. [PubMed: 15001782]
13. Martinod K, Wagner D. Thrombosis: tangled up in NETs. *Blood.* 2014; 123:2768–76. [PubMed: 24366358]
14. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD Jr, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA.* 2010; 107(36):15880–15885. [PubMed: 20798043]
15. Etulain J, Martinod K, Wong SL, Cifuni SM, Schattner M, Wagner DD. P-selectin promotes neutrophil extracellular trap formation in mice. *Blood.* 2015; 126(2):242–6. [PubMed: 25979951]

16. Walker AJ, Card TR, West J, Crooks C, Grainge MJ. Incidence of venous thromboembolism in patients with cancer - a cohort study using linked United Kingdom databases. *Eur J Cancer*. 2013; 49(6):1404–13. [PubMed: 23146958]
17. Blom JW, Vandershoot JP, Oostindier MJ, Osanto S, Fan der Meer FJ, Rosendaal FR. Incidence of venous thrombosis in a large cohort of 66 329 cancer patients: results of a record linkage study. *J Thromb Haemost*. 2006; 4(3):529–35. [PubMed: 16460435]
18. Varki A. Trousseau's syndrome: multiple definitions and multiple mechanisms. *Blood*. 2007; 110(6):1723–9. [PubMed: 17496204]
19. Geddings JE, Mackman N. Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. *Blood*. 2013; 122(11):1873–80. [PubMed: 23798713]
20. Ay C, Dunkler D, Marosi C, Chiriac A-L, Vormittag R, Simanek R, et al. Prediction of venous thromboembolism in cancer patients. *Blood*. 2010; 116(24):5377–82. [PubMed: 20829374]
21. Connolly G, Phipps R, Francis C. Platelets and Cancer-Associated Thrombosis. *Semin Oncol*. 2014; 41(3):302–10. [PubMed: 25023346]
22. Königsbrügge O, Pabinger I, Ay C. Risk factors for venous thromboembolism in cancer: novel findings from the Vienna Cancer and Thrombosis Study (CATS). *Thrombosis Research*. 2014; 133:S39–43. [PubMed: 24862144]
23. Graus F, Rogers LR, Posner JB. Cerebrovascular complications in patients with cancer. *Medicine (Baltimore)*. 1985; 64(1):16–35. [PubMed: 3965856]
24. Selvik H, Thomassen L, Logallo N, Naess H. Prior Cancer in Patients with Ischemic Stroke: The Bergen NORSTROKE Study. *J Stroke Cerebrovasc Dis*. 2014; 23(5):919–25. [PubMed: 24075585]
25. Zoller B, Ji J, Sundquist J, Sundquist K. Risk of coronary heart disease in patients with cancer: A nationwide follow-up study from Sweden. *Eur J Cancer*. 2012; 48(1):121–8. [PubMed: 22023886]
26. Zoller B, Ji J, Sundquist J, Sundquist K. Risk of haemorrhagic and ischaemic stroke in patients with cancer: A nationwide follow-up study from Sweden. *Eur J Cancer*. 2012; 48(12):1875–83. [PubMed: 22296948]
27. Lau KK, Wong YK, Teo KC, Chang RS, Hon SF, Chan KH, et al. Stroke Patients with a Past History of Cancer Are at Increased Risk of Recurrent Stroke and Cardiovascular Mortality. *PLoS ONE*. 2014; 9(2) DOI:10.1371/journal.pone.0088283.
28. Trousseau A. Phlegmasia alba dolens. Lectures on clinical medicine, delivered at the Hotel-Dieu, Paris. 1865; 5:281–332.
29. Geiger S, Holdenrieder S, Stieber P, Hamann GF, Bruening R, Ma J, et al. Nucleosomes in Serum of Patients with Early Cerebral Stroke. *Cerebrovasc Dis*. 2006; 21(1-2):32–37. [PubMed: 16282687]
30. Tsai NW, Lin TK, Chen SD, Chang WN, Wang HC, Yang TM, et al. The value of serial plasma nuclear and mitochondrial DNA levels in patients with acute ischemic stroke. *Clinica Chimica Acta*. 2011; 412(5-6):476–9.
31. Lam NYL, Rainer TH, Wong LKS, Lam W, Lo YMD. Plasma DNA as a prognostic marker for stroke patients with negative neuroimaging within the first 24 h of symptom onset. *Resuscitation*. 2006; 68(1):71–8. [PubMed: 16325316]
32. Shimony A, Zahger D, Gilutz H, Goldstein H, Orlov G, Merkin M, et al. Cell free DNA detected by a novel method in acute ST-elevation myocardial infarction patients. *Acute Cardiac Care*. 2010; 12(3):109–111. [PubMed: 20712451]
33. De Boer OJ, Li X, Teeling P, Mackaay C, Ploegmakers HJ, van der Loos CM, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost*. 2013; 109(2):290–7. [PubMed: 23238559]
34. Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, et al. Human Granulocyte Colony-Stimulating Factor: Biologic Activities and Receptor Characterization on Hematopoietic Cells and Small Cell Lung Cancer Cell Lines. *Blood*. 1990; 75(4):851–7. [PubMed: 1689190]
35. Casbon AJ, Reynaud D, Park C, Khuc E, Gan DD, Schepers K, et al. Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. *Proc Natl Acad Sci USA*. 2015; 112(6) DOI:10.1073/pnas.1424927112.

36. Templeton AJ, McNamara MG, Seruga B, Vera-Badillo FE, Aneja P, Ocana A, et al. Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Solid Tumors: A Systematic Review and Meta-Analysis. *JNCI*. 2014; 106(6) DOI:10.1093/jnci/dju124.
37. Lee AY. Thrombosis in Cancer: An Update on Prevention, Treatment, and Survival Benefits of Anticoagulants. *Hematology Am Soc Hematol Educ Program*. 2010; 2010:144–9. [PubMed: 21239784]
38. De Meyer S, Suidan G, Fuchs T, Monestier M, Wagner D. Extracellular Chromatin Is an Important Mediator of Ischemic Stroke in Mice. *Arterioscler Thromb Vasc Biol*. 2012; 32(8):1884–91. [PubMed: 22628431]
39. Brill A, Fuchs TA, Savchenko A, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost*. 2012; 10(1):136–44. [PubMed: 22044575]
40. Lewis H, Liddle J, Coote J, Atkinson SJ, Barker MD, Bax BD, et al. Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nature Chemical Biology*. 2015; 11(3): 189–91. [PubMed: 25622091]

HIGHLIGHTS

- Cancer-associated arterial microthrombosis may be underestimated
- This hypercoagulable state may be linked to neutrophil extracellular traps
- Markers of NETs could aid in diagnosing cancer-associated arterial microthrombosis
- Markers of NETs could aid in diagnosing cancer in arterial microthrombosis

Fig 1. Histopathological investigations of cerebral, myocardial and pulmonary arterial microvascular thrombosis

A. Hematoxylin and eosin staining revealed microthrombosis in a small cerebral artery, in a small intramuscular coronary artery as well as in a small pulmonary artery. Small focal infarction was observed around the coronary microthrombi. Metastatic spread of prostate adenocarcinoma (small arrows) surrounding the pulmonary artery (arrow) was also present. B. Immunohistochemistry of H3Cit showed the presence of NETs in microthrombi of the brain, heart and lung. H3Cit positive cells as well as extracellular H3Cit was observed in all microthrombi. Granulocytes (blue nuclei stain) were also observed inside and around the

coronary thrombus. C, D. Confocal microscopy revealed extracellular H3Cit which colocalised with DNA and confirmed the presence of NETs (arrows) in the cerebral, coronary and pulmonary microthrombi. The microthrombi were rich in von Willebrand factor (VWF). Smooth muscle actin (SMA) staining delineates the vessel wall.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 2. Plasma markers of coagulation, platelet activation and NETosis

A. Plasma markers of coagulation, platelet activation and NETosis were significantly higher in stroke patients with hsTnT elevation (n=12) compared to stroke patients without hsTnT elevation (n=20). B. Comparison of these plasma markers between ischemic stroke patients with and without cancer. Even higher levels were seen in patients with cancer (n=8). C. Exclusion of patients with cancer in the group of ischemic stroke patients with troponin elevation. When excluding patients with cancer, the differences between patients with hsTnT elevation (n=5) and normal levels of hsTnT (n=19) were diminished, suggesting a

link between NETosis, coagulation and cancer. Mann-Whitney U test was used to determine p-values.

Fig 3. Markers of NETosis correlate with neutrophil activation and a pro-thrombotic state
 A. A positive correlation between the NET biomarker H3Cit and cfDNA, MPO, TAT, and soluble P-selectin further suggested a NET-induced pro-coagulant state. B. G-CSF also revealed a positive correlation with H3Cit, MPO, TAT and soluble P-selectin, strengthening the conclusion that the priming toward NETosis may drive the pro-thrombotic state. Notably, these correlations all remained significant when excluding the patient with the highest level of H3Cit (A. $r=0.74$, $p<0.001$ for the correlation between H3Cit and cfDNA; $r=0.64$, $p<0.001$ for the correlation between H3Cit and MPO, $r=0.45$, $p=0.013$ for the

correlation between H3Cit and TAT; $r=0.57$, $p=0.001$ for the correlation between H3Cit and sP-selectin. B. $r=0.57$, $p=0.001$ for the correlation between G-CSF and H3Cit; $r=0.51$, $p=0.004$ for the correlation between G-CSF and MPO, $r=0.38$, $p=0.038$ for the correlation between G-CSF and TAT; and $r=0.39$, $p=0.033$ for the correlation between G-CSF and sP-selectin. C. $r=0.64$, $p<0.001$ for the correlation between H3Cit and MPO). Spearman's rank correlation was used for significance of correlations.

Table 1

Patient characteristics and 2-month mortality of patients with hsTnT 15 ng/L vs. patients with hsTnT>40 ng/L and patients with no cancer vs. patients with active cancer.

	hsTnT 15 ng/L (N=19)	hsTnT > 40 ng/L (N=12)	p-value	No cancer (N=23)	Active cancer (N=8)	p-value
Age, years- median (IQR)	75 (15)	78 (17)	0.687	75 (14)	75 (20)	0.440
Female- no. (%)	11 (58)	8 (67)	0.717	15 (65)	4 (50)	0.676
Prior ischemic stroke/TIA- no. (%)	6 (32)	5 (42)	0.705	9 (39)	2 (25)	0.676
Chronic heart failure- no. (%)	2 (11)	2 (17)	0.630	3 (13)	1 (13)	1.000
Coronary artery disease- no. (%)	4 (21)	1 (8)	0.624	3 (13)	2 (25)	0.583
Hypertension- no. (%)	11 (58)	6 (50)	0.724	12 (52)	5 (63)	0.698
Atrial fibrillation- no. (%)	4 (21)	4 (33)	0.676	6 (26)	2 (25)	1.000
Diabetes mellitus- no. (%)	0	2 (17)	0.142	1 (4)	1 (13)	0.456
Renal insufficiency- no. (%)	2 (11)	3 (25)	0.350	3 (13)	2 (25)	0.583
Hyperlipidemia- no. (%)	8 (42)	4 (33)	0.717	8 (35)	4 (50)	0.676
Active systemic cancer- no. (%)	1 (5)	7 (58)	0.002			
NIHSS on admission- median (IQR)	4.0 (2)	6.5 (5)	0.194	4 (2)	6.5 (14.5)	0.083
Mortality < 2 months- no. (%)	1 (5)	7 (58)	0.002	1 (4)	7 (88)	<0.001

IQR, interquartile range; TIA, transient ischemic attack; NIHSS, National Institute of Health Stroke Scale. Fisher's exact test for categorical data, Mann-Whitney U test for continuous data.

Table 2

hsTnT level, cancer type, metastatic spread and time of cancer diagnosis in patients with ischemic stroke and active cancer.

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, lymph nodes 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