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Neutrophil Extracellular Trapping Role in Cancer, Metastases and Cancer Related Thrombosis: A Narrative Review of the Current Evidence Base

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Abstract

Purpose of Review NETs formation is a newly discovered, reactive oxygen species dependent regulated process**,** whereby neutrophils degranulate and extrude genetic material, after engulfing various infectious or neoplastic antigens, culminating in a measurable serologic footprint. Recent research has highlighted the involvement of NETs in cancer and cancer-related pathologies. We review the role of NETs formation in cancer biology, prognosis and potential therapeutic modulators.

Recent findings Elevated NETs levels are associated with cancer metastasis and may be modified by some anaesthetic-analgesic techniques during tumour resection surgery. It promotes tumour cell migration, angiogenesis and hypercoagulability. Although there are potential anti-NETs formation therapeutics available, their role has not been formally assessed in cancer patients.

Summary Limited available evidence suggests an association between elevated NETs expression and cancer metastasis, but its validity as a prognostic indicator for cancer-related outcomes is inconclusive. Further observational and interventional studies are warranted to comprehend the potential prognostic and therapeutic role of NETs in cancer.

Keywords Neutrophil Extracellular Trapping • NETosis • NETs • White blood cells • Neutrophils • Cancer • Metastases • Thrombosis

Introduction

Neutrophil Extracellular Traps (NETs) formation is a reactive oxygen species (ROS) dependent regulated process**,** whereby neutrophils degranulate, releasing intracellular and nuclear contents which form an extracellular snare with a role in bacterial or neoplastic cell trapping and killing. This process bequeaths a serologic footprint which may be detected many years later. The term NETosis has been previously used for describing this phenomenon. Recently, NETosis was defined as being a ROS dependent regulated cell death, specific to cells of haematopoietic origin and associated with NETs release [1]. It is recommended to avoid using the term NETosis in those instances where there is no evidence of cell death associated with NETs formation. The term NETs extrusion may also be used for NETs formation without cell death. These two terms will be used interchangeably for the reminding of this article.

NETs are rich in cell nuclear components (e.g. DNA, histone proteins). They also contain granular and cytoplasmatic proteins or enzymes (myeloperoxidase (MPO), neutrophil elastase (NE)) [2] and antimicrobial and proinflammatory proteins [3]. A defining characteristic of NETs generation is the translocation of genetic material from the nucleus to the extracellular environment [4].

Although NETs formation was initially described over 20 years ago, it has only emerged as a current topic of interest since 2014 [5, 6]. Since then, it has been identified as a factor in many physiological and pathological processes like immunity, sepsis, inflammation, cancer metastasis, cancer related thrombosis and more recently in SARS-CoV 2 infection [7-11].

In this review we summarize the current understanding of the multifactorial role of NETs in tumour cells biology, metastatic development and VTE occurrence. We also review pharmacological agents, including anaesthesia technique, with a potential role in NETs modulation.

Mechanisms of NETs formations

Classically, neutrophils are recognized as the mainstay of the innate immune system, delivering chemotaxis, endothelial adherence, phagocytosis, oxidative burst, and toxic granule dependent microbial killing [4]. Important steps of NETs formation include chromatin decondensation, nuclear membrane rupture, nucleoplasm spillage into the cytoplasm, cell membrane destruction and cell death have been described. This has been described both in vitro and in vivo.

Suicidal NETs formation is NADPH oxidase (NOX)-dependent and involves chromatin decondensation, nuclear envelope disintegration, plasma membrane perforation, cell lysis and tumour cell or bacteria fixation to the DNA structures [12, 13]. Subsequent work, revealed the role of ROS, NE and MPO in NETs release [2]. The ROS triggers the release of

NE and MPO from the cytoplasmic granules and promotes the MPO-mediated proteolytic activity of NE. The NE and MPO are translocated to the nucleus, which facilitates histone degradation chromatin decondensation [2].

This contrasts with ‘’vital NETosis’’, a more rapid process in which neutrophils became anuclear cells still capable of survival and of engulfing antigens [4, 14]. Currently three types of vital NETs formation have been described (Table 1).

Suicidal and vital NETs formation terminology is being gradually replaced by NOX-dependent and NOX-independent NET. NOX-dependent or NOX-independent pathway is activated in response to specific triggers. Lipopolysaccharide (LPS) and bacteria stimulates the NOX-dependent pathway leading to nucleus breakdown [2] and activation of a specific set of kinases (ERK1, ERK2, Akt, P38, Src) [15].

Bacterial or neoplastic cell products and uric acid crystals activate the NOX-independent pathway, leading to formation of mitochondrial ROS [16]. This acts on a number of kinases. The activation of each pathway’s kinases leads to transcriptional firing, which enables chromatin decondensation [6]. In the NOX-independent pathway peptidyl arginine deaminase type 4 **(**PAD4)-mediate the production of chromatin networks by the cancer cells themselves [17, 18]). There is also evidence that NETs formation can take place in a PAD4 independent fashion [19] particularly in the NOX-dependent pathway[16, 20].

Measuring NETs expression

The production of NETs creates a footprint, by which NETs formation can be detected. The various components of NETs (e.g. histones, DNA, enzymes) and the molecules involved in NETs expression (e.g. PAD-4) serve as potential targets for measuring this process. NETs expression can be quantified directly by microscopy [21], flow-cytometry or real time imaging or indirectly, using the enzyme-linked immunosorbent assay (ELISA) technique mainly for serological measurements [22]. MPO, NE, citrullinated histone 3 (citH3), among others, represent measurable components or markers of NETs [23] [24] [25], but may not be specific: For example NE and MPO can be released during degranulation without NETs formation [26].

NETs and cancer

Elevated NETs levels are associated with the diagnosis of cancer [4, 10, 15, 25]. NETs contribute to primary tumour growth [9], metastasis [27] and cancer related thrombosis [28] through various mechanisms (Figure 1). NETs associated enzymes are involved in cancer growth by stimulating phosphatidylinositol-3 kinase (e.g. NE) and matrix destruction (e.g. matrix metallopeptidase 9 (MMP-9)) [29, 30]. In breast cancer cells in vitro, NETs expression upregulates gene expression for several factors related to pro-metastatic properties of breast cancer [31]. In addition, cancer cell biology and NETs formation share

common elements of ROS signalling pathways [32] and reliance on similar kinases (e.g. p38) [15, 33]. For example, stimulation of toll-like receptor 4 (TLR4) receptor (e.g. by LPS) induces NETs formation [34] with a role in host immunity. High mobility group box protein 1 (HMGB1) protein is involved in many steps of the protumorigenic pathway [35] and is produced by neutrophils on degranulation/NETs formation. This protein seems to be one of the NETs associated chemotactic factors responsible for the NETs induced tumour cell invasion [24]. Other proteins released during NETs production like MMP-9, and NE have been shown to be involved in tumour progression, creating an adequate environment for tumour cell seeding and growth [24]. Despite of some evidence that in vitro cancer induced NETs formation is a suicidal process [36], it remains unclear if the in vivo process is vital or suicidal [34].

In an in vitro model of surgery, it was shown that NETs stimulated Kupffer cells to produce proinflammatory cytokines directly associated with cancer progression (interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-alpha), chemokine (C-X-C motif) ligand 10 (CXCL-10)) [24, 37]. This was alleviated by DNase and PAD4 inhibition [24]. Although the full list of stimuli of NETs production in cancer is unknown, there is evidence for circulation of pro-NETs formation factors. Neutrophils from healthy donors were stimulated to produce NETs when incubated with cancer patient's plasma [38].

Tumour cells-neutrophil interplay in NETs formation

Tumour cell-neutrophil-platelets-vascular endothelium interactions are essential for cancer cell survival and the development of conditions which facilitate metastasis. These processes have been recently summarised [34]. Neutrophils are subject to modulation form both circulating tumour cells and factors released by the solid tissue tumours through extracellular vesicles (EV). Some of the identified modulators produced by the primary tumours are tissue factor (TF), granulocyte colony-stimulating factor (G-CSF), HMGB1, IL-8 and ROS. The interaction between tumour factors, activated platelets and endothelium leads to neutrophil activation, NETs formation and adhesion of the neutrophils to the endothelium [39]. In breast cancer, increased G-CSF is associated with increased metastases [40]. In a murine model of breast cancer and insulinoma it was shown that cancer associated heart, kidney and vascular dysfunction which caused increased NETs, was improved by neutrophil depletion, G-CSF inhibition and DNAse administration [41]. In this model, vascular NETs development was mediated by increased levels of intercellular adhesion molecule **1** (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), IL-1Beta, IL6 and chemokine CXCL1. These observations may explain the distant organ disfunction seen in cancer.

Extracellular vesicles are membrane enclosed structures produced by cells (e.g. during cell stress response), and could contain specific cytoplasmic solid and soluble components specific to the parent cell [34]. Tumour EVs can directly trigger NETs formation after they have been phagocytosed by neutrophils. They have been found to be present in patients with breast cancer and to predict therapeutic failure and poor prognosis [42]. Some

of the constituents of EVs (e.g. ILs and G-CSF) are NETs formation mediators [43]. In a murine model (BALB/c mice) injected with 4T1 breast cancer cells, the EVs of 4T1 cells triggered NETs formation and when administered intravenously contributed to accelerated venous thrombosis [44]. In a mouse model of lung tumours post intravenous B16 melanoma cells injection, the authors showed that the tumour cells EVs were ingested by neutrophils [45] which could have pro-NETs generation effects.

In addition, NETs act like ‘’fishing nets’’ capturing circulating tumour cells which generate further endothelial damage facilitating metastasis [15]. Key ligand/neutrophils receptor interactions (e.g. HMBG1-TLR4/RAGE) are essential in this scenario [34] and could develop into therapeutic targets. Although the IL-8-CXCR1/2 interaction is beneficial in acute infection [46], it seems to be detrimental in cancer. In a murine model of lung cancer and concomitant sepsis, intravascularly deposited NETs captured lung cancer cells, and was associated with gross metastatic burden at 2 weeks [47]. The metastatic burden was attenuated by NETs formation inhibitors (e.g. DNAse, neutrophil elastase inhibitor (NEi)). In a murine model of murine colon cancer (CT-26) peritoneal metastasis, decreasing the circulating neutrophils or disrupting NETs formation by DNAse markedly decreased the number of metastases [27]. In the same study, mice and humans were shown to have significant NETs in peritoneal metastases from colon cancer and blocking the integrins abolished NETs induced cancer cell adhesion.

Cancer cell-NETs formation occurs by two pathways: Pro-tumorigenic effects of NETs and cancer cells’ NETs inducing capacity. In a study involving patients with pancreatobiliary malignancy and the pancreatic cancer cell line AsPC-1, cells themselves and their conditioned medium were strong promoters of NETs formation. In this study, ROS inhibition did not block NETs formation but prostaglandin E1 (PGE-1) did, suggesting a role of cyclical adenosine monophosphate (AMP) in NETs formation.[48]. It was shown that HMGB1 protein is released from NETs and serves as a ligand on the TLR-4 receptor on the surface of cancer cells [24, 25, 49].

The age of the neutrophil is relevant for both NETs formation and cancer biology [50]. Interestingly, the more immature neutrophils have a reduced NETs formation activity via reduced ROS production and increased mitotic activity [51].

NETs and cancer associated thrombosis

Endothelial cells and platelets require prior activation before triggering NETs formation [52]. Platelets can be activated by the histones in NETs structure [53] and can directly stimulate NETs formation, interacting with the neutrophil via the P-selectin/P-selectin glycoprotein ligand 1 [34]. NETs are rich in citrullinated histone 3 (citH3), NE and MPO, all triggering further platelet activation and contributing to the thromboembolic phenomenon associated with cancer. In addition to the previous known mechanisms of platelet activation, an in vitro study using AsPC-1 pancreatic cell line suggested platelet activation may be driven

by the tumour cells directly via tissue factor (TF) expression [54], which could potentially be a constituent of the tumour EVs [34]. HMBG1 factor and TLR4 receptor are used by the activated human platelets to trigger NETs formation [52]. Furthermore, DNA activates the intrinsic coagulation pathway [55]. Jung et al proposed that the DNA contained in NETs could be the trigger for NETs associated thrombin generation and subsequent cancer related thrombotic phenomena [25].

NETs have been identified in patients with thrombotic phenomena (i.e. stroke). In a prospective, observational case-control study, patients were divided into two groups based on the serum troponin levels [56]. High sensitivity troponin (hsTnT) was associated with a higher risk of malignancy. Autopsies in these patients showed widespread micro thrombosis containing citH3, a biomarker for NETs. A procoagulant state with increased NETs serum levels was seen in patients with elevated hsTnT compared to patients with normal levels ($p<0.001$). CitH3 was also correlated with thrombin-antithrombin complex and soluble P-selectin providing a further link between NETs and the prothrombotic state in cancer patients.

A similar prothrombotic state was found in a study of patients with pancreatobiliary malignancy, who had a high level of circulating NETs markers (histone-DNA complexes and cfDNA) and hypercoagulability markers [25]. In addition, hypercoagulability markers were higher in patients with more advanced cancer (stage IV vs stage I/II). In the same study, using the AsPC-1 pancreatic cancer cell line the investigators also documented that antithrombin significantly blocks NETs formation.

NETs as a prognostic marker

Plasma taken from treatment-naive patients with lung or upper GI adenocarcinoma and compared to the plasma of healthy individuals showed elevation in NETs expression (measured as MPO) in the cancer patient cohort compared to the control group ($p=0.03$). This study also showed significant elevation in NETs levels in patients with advanced cancer (stage III & IV esophagogastric, stage II & III lung) compared to patients with a less advanced diagnosis (stage I & II esophagogastric and stage I lung) ($p=0.01$) [57]. NETs expression was also higher in patients with early-stage head and neck cancer compared to healthy control participants of the same age [58]. As part of the same study, it was shown that neutrophils of these cancer patients released more NETs compared to the healthy control cohort. The increased NETs expression in cancer patients and the increased release of NETs from neutrophils in vitro, support the hypothesis that NETs may be a biomarker for cancer.

Although most evidence points towards a deleterious effect of NETs in cancer, a multi-omics study on ovarian cancer patients found that NETs formation corelated with better outcome [23]. Increased CRP is typically associated with poor prognosis in this type of cancer, while the S100A8 protein is released by neutrophils upon local NETs generation. The S100A8/CRP abundance ratio significantly stratified patients and the increase in the ration correlated with correlated favourable survival.

The opposite was observed in patients with colorectal cancer, with an in vitro increase in NETs expression in response to stimulation of systemic neutrophils. This was associated with more post-operative complications (Clavien-Dindo classification ≥ 3) and prolonged hospital stay (>5 days). [21]

NETs formation is been predictive for decreased disease-free survival. In a cohort of 50 colorectal patients undergoing curative liver metastases resection, it was observed that increased postoperative NETs formation was associated with a >4-fold reduction in disease-free survival [24]. Similar findings occurred in a murine model (C57BL/6J WT mice or PAD4−/− mice) of surgical stress employing liver ischemia-reperfusion, where NETs expression was associated with accelerated development of metastatic disease (MC38 or MC38/Luc cancer cells) [24]. These effects were mitigated by inhibiting NETs formation with DNAse (68% reduction in hepatic tumour burden) or PAD-4 inhibition.

In a study of 60 patients with various adenocarcinomas, squamosa cell carcinomas, melanomas, and glioblastomas, there was a 3-fold increase in the median plasma citH3 (NETs biomarker) levels in patients with advanced cancer compared with age-matched healthy individuals or severely ill patients without cancer [59]. Further, plasma levels of IL-8, IL-6, TNF alpha, IL-1beta and GCSF (all involved in NETs production) were all elevated in cancer patients. Levels of citH3 above the 75th percentile were associated with 2-fold short increased short-term mortality. In addition, these patients had a significantly higher numbers of peripheral neutrophils, neutrophils positive for intracellular citH3, plasma MPO, MPO-DNA complexes, suggestive of neutrophil activation and NETs extrusion. In a systematic review of 30 studies where the authors examined the relationship between perioperative inflammation, NETs expression and metastasis in colorectal cancer, the presence of a preoperative systemic inflammatory state but not sepsis was associated with increased cancer recurrence [60].

Cancer cells can hijack neutrophil function, inducing NETs formation independently of direct tumour cells-neutrophil interaction (via G-CSF priming) and could be a marker of tumour aggression. In an in vitro study, NETs were found in 16/20 of the primary breast tumours and in 13/19 metastatic lung lesions. Lung metastases of triple negative breast cancer had the highest level of NETs expression when compared to luminal breast cancer. [36] Interestingly these in vitro experiments revealed a lytic (i.e. suicidal) NETs formation pathway and concluded that NETs do no promote tumour cells extravasation, but rather mediates the expansion of already disseminated cells

NETs promote angiogenesis

NETs are promoters of inflammation via NE and MPO, which could damage the endothelium, triggering further IL-8, ROS and platelet activation, in turn further stimulating neutrophil recruitment and NETs release [34]. In addition, in vitro, histones are stimulators of angiogenesis when incubated with endothelial cells [25]. As they are a major component of

NETs, it is reasonable to conclude NETs can promote angiogenesis too. The entire effect was blocked by histone chelating agents (e.g., heparin, polysialic acid (PSA).

Does anaesthesia technique and the perioperative surgical stress impact on NETs formation?

The effects of surgical stress and anaesthetic technique on NETs expression has been assessed in in vivo and clinically, with intriguing results.

In an observational cohort study evaluating metastatic potential of surgical stress induced NETs formation, liver ischaemia-reperfusion (I/R) injury increased NETs expression. Serum was obtained day 1 post-operatively for patients who underwent a hepatectomy for metastatic colorectal cancer. NETs expression (measured by serum MPO-DNA complexes) was significantly increased in patients who underwent major liver resection (3 or more segments removed) with unavoidable liver I/R injury when compared to patients who underwent smaller resection or to healthy controls. Patients with elevated NETs levels post-operatively, experienced a 4-fold reduction in disease-free survival when compared to the cohorts with lower NETs expression (95% CI: 1.39-12.81, p=0.01). In the same study, using a murine model of liver I/R injury and MC38 cancer cells, the ischaemic group showed increased serum citH3, when compared to controls. After 2 weeks, the liver I/R mice showed greater hepatic metastases compared with mice who did not receive liver I/R injury. [24]

The impact of anaesthetic technique on perioperative NETs expression was tested in the first randomised controlled clinical trial in this field on women undergoing primary breast tumour resection [61]. Patients were randomly assigned to receive one of the following 4 anaesthetic techniques: inhalational sevoflurane (S), sevoflurane plus IV lidocaine (SL), intravenous propofol (P) or propofol plus IV lidocaine (PL). Although there was no difference between the S and P groups, the addition of lidocaine significantly decreased citH3 expression (109 ± 23 vs 125 ± 22 ng ml^{-1}, p=0.01 for SL and S and 98 ± 14 vs 130 ± 32 ng ml^{-1}, p=0.007, for PL and P respectively). MPO, another NETs formation biomarker was also decreased by lidocaine. Lidocaine further decreased expression of MMP3 but not MMP9, regardless of which anaesthetic technique was used. Therefore, an inhalational versus intravenous anaesthetic technique has little effect on NETs expression but the addition of lidocaine to either technique significantly reduced these markers, consistent with the hypothesis that intravenous lidocaine during cancer surgery of curative intent might reduce recurrence. However, in a parallel group RCT [62], which evaluated the effects of different anaesthetic techniques among women undergoing breast cancer surgery, NETs expression was not influenced by anaesthetic technique. Forty women partaking in a larger clinical trial (NCT-00418457), undergoing breast cancer surgery were randomly assigned to receive volatile general anaesthesia (GA) or propofol GA plus paravertebral regional anaesthesia (PPA). NETs levels were measured using the MPO and citH3 biomarkers. There was no

difference between the two groups in either MPO concentration (10.5 ± 6.6 vs 11.5 ± 4.7, ng ml^{-1}, p=0.60) or citH3 concentration (3.6 ± 2.3 vs 4.0 ± 5.9, ng ml^{-1}, p=0.80) respectively.

NETs formation modulators

The perioperative period represents a window of opportunity for metastasis. The therapeutic options during this phase should not interfere with immunity, wound healing, and decrease host-defence [24]. A key aspect of anti-NETs therapies is represented by appropriate patient selection. For example, expression of some pro-metastatic factors (e.g. G-CSF) by some tumours and the presence of NETs in those tumours has been proposed as a screening tool to identify patients who may benefit from anti-NETs therapies [36]. Although drugs which affect NETs formation are clinically available (e.g. dornase alpha, lidocaine, PGE-1 etc.), the concept of NETs inhibition in cancer is not currently widely supported. The potential NETs formation inhibitors have been summarised in Table 2.

Conclusion

Since its discovery, NETs have been shown to be strongly associated with increased cancer progression with potential value as a prognostic indicator and a marker for increased risk of cancer-related pathologies such as metastasis and thrombus formation. The limited observational nature of most of the studies and the paucity of clinical trials precludes a causal link being made. Two recent RCTs investigating the impact of anaesthetic technique showed that while addition of lidocaine infusion decreased NETs formation, regional anaesthesia had no effect. Whether modulation of NETs perioperatively translates into improved longer term oncologic outcomes warrants further evaluation in observational and interventional studies.

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Compliance with ethical standards

Conflict of Interest The authors have no conflict of interest to declare.

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Human and animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Supplementary material Table 1S: Summary of relevant articles

A. Perioperative insults

CANCER DIAGNOSIS PROCEDURES
Spontaneous and procedural induced CTCs, tumour factors and EV release.

RADIO/CHEMOTHERAPY
Impaired immunity, poor host defence

SURGERY
Immune cells activation, blood transfusion induced immune modulation, tissue hypoxia, pain stimuli, CTCs, tumour factors and EV release.

ANAESTHETIC DRUGS
Opioid induced immunosuppression, volatile agents induced cancer cell proliferation.

Inflammation AND Infection

B. NETs formation

Activated Platelets

PAF[£]

Tumour cells

Endothelial cells

ROS

HMGB1[%]

TF

Glutamine Released under hypoxic conditions

G-CSF*

IL-8

Activates

LPS[$]

S. aureus

Neutrophil activation via calcium influx, IL-8 and PGE2 secretions

P-SEL mediated EC/N binding

TLR 2

TLR 4/RAGE

G-CSFR

CXCR 1/2

P-SEL mediated P/N binding

PSGL-1

Neutrophils

SIGLEC 5/6, LAIR 1, SIRL 1

During NETs formation, the neutrophils adhere to the endothelium promoting activation

Platelet activation and capture; VTE promotion

NETs formation

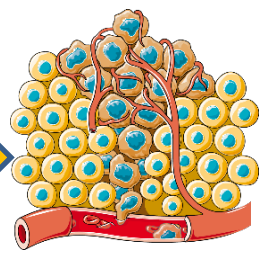
CTCs capture => metastases => Endothelia cell injury

citH3

Endothelial cells damage; Inflammation promotion via IL-8

NE

MPO



Local and Metastatic Disease

Legend:

Red arrows, endothelial cell pathways.
Yellow arrows, platelets pathways.
Green arrows, tumour cell pathway.
+, stimulates.
- , inhibits NETs formation.
A, acts upon.
R, releases.
Blue boxes, neutrophil receptors.

Fig. 1. Schematic representation of the NETs formation modulators and the development of the pre-metastatic niche in the perioperative setting. [15, 23, 34, 63-65]

EV, external vesicles; CTCs, circulating tumour cells; VTE, venous thromboembolism; P, platelet; N, neutrophil; EC, endothelial cell; HMGB1, high mobility group box 1; TF, tissue factor; G-CSF, granulocyte colony-stimulating factor; IL-8, interleukin 8; LPS, Lipopolysaccharides; P-SEL, selectin P; PSGL-1, selectin P receptor 1; CXCR1/2, CXC chemokine receptor ½; TLR4, toll-like receptor 4; RAGE, receptor for advanced glycation end products; TLR 2, toll-like receptor 4; SIGLEC 5/6, Sialic acid-binding immunoglobulin-type lectins 5/6; SIRL1, signal inhibitory receptor on leucocytes 1; LAIR1 leucocyte-associated immunoglobulin-like receptor 1; *, correlates with the metastatic potential of some cancers; $, dormant cancer cell activator; %, synergistic effect with LPS to exacerbate infection response; £, induces tumour cell proliferation.

Table 1. NETs formation subtypes		
Subtype	**Manuscript**	**Triggers**
NETs containing mitochondrial DNA	Yousefi et al [66] 2009	Granulocyte/macrophage colony-stimulating factor (G-CSF), toll-like receptor 4 (TLR4) or complement factor 5a (C5a) receptor stimulation via an ROS mechanism.
NETs (NOX-independent)	Pilsczek et al [67] 2010	S. aureus.
NETs (NOX-independent)	Yipp et al [14] 2012	Lipopolysaccharide via TLR-4 receptor on platelets. Requires platelets/neutrophils crosstalk.

Table 2. NETs formation inhibitors				
Substance	**Manuscript**	**Experimental Model**	**Findings**	**Comments**
Anthracyclines (e.g. epirubicin, daunorubicin, doxorubicin, and idarubicin)	Khan et al [68]	Human neutrophils, in vitro.	Both NOX-dependent and NOX-independent NETs expression was blocked.	Anthracyclines effect may be mediated by alteration of transcription initiation.
Anti-GCSF antibodies	Park et al [36, 69]	Murine model. 4T1 breast cancer cells.	Reduced cancer cell induced NETs expression and metastases.	In humans these could be difficult as GCSF is commonly used as a treatment of neutropenic sepsis in cancer patients [36].
Anti-HMGB1 antibodies	Tohme et al [24]	In vitro. MC 38 colorectal cancer cells.	NETs mediated tumour cell proliferation, invasion and migration was significantly decreased ($p<0.001$).	Leads to MAP kinase pathway inhibition.
Antithrombin III	Ishikawa et al [70]	Murine model of LPS induced endotoxemia.	Reduced mortality, lung neutrophil infiltration, expression of NETs, HMGB1 protein and CXCL1 and 2.	In addition to the anticoagulation effects, antithrombin III has also anti-inflammatory effects.
	Jung et al [25]	In vitro, AsPC-1 pancreatic cancer cells.	Significant inhibition of AsPC-1-induced NET formation.	
Diethylcarbamazine	Segoviano-Ramirez et al [71]	In vitro. PMNs from healthy and type 2 diabetes melitus patients (DM2).	NETs formation was significantly inhibited both in healthy ($p \leq 0:000001$) and DM2 ($p \leq 0:000477$) volunteers.	Although a drug used as an anti-parasitic, this could be useful in NETs expression modulation in diabetic wound healing and cancer potentially.
DNase	Jung et al [25]	In vitro, AsPC-1 pancreatic cancer cells.	Significant inhibition of AsPC-1 NETs promoted cancer cell migration.	Among all other modulators, DNase has the largest use in various experimental models and is already in clinic for CF treatment. It is likely that it may be among the first drugs to be

	Tohme et al [24]	In vitro and Murine model. MC 38 colorectal cancer cells.	Significant inhibition of tumour adhesion, the development and growth of metastatic disease.	used in cancer for outcome control. Preliminary in vitro and animal results suggest DNAse reduces NETs formation, with an effects of tumour cell adhesion, tumour burden and metastasis [24, 36].
	Park et al [36]	Murine model. 4T1 breast cancer cells.	DNase coated nanoparticles decreased lung metastatic burden.	
	Kolaczkowska et al [72]	Murine model of methicillin-resistant Staphylococcus aureus bacteraemia. PAD4 and NE negative mice.	DNase failed to remove most histones form the vessel wall and only partially reduced the endothelia injury. Inhibition of NETs formation (in murine models of PAD 4 and NE deficiency) also prevented collateral host tissue damage.	
Gasdermin D inhibitor LDC7559	Sollberger et all [73]	In vitro, human neutrophils.	Gasdermine D is required for NETs expression and acts upstream of NE. LDC7559 reduced histone H3 protein processing.	Gasdermin D is a pore forming protein involved in the release of granular proteins required for NETs expression and facilitates NE translocation to the nucleus. Also forms membrane pores releasing the NETs in the extracellular space.
Heparin and polysialic acid (PSA)	Jung et al [25]	In vitro, AsPC-1 pancreatic cancer cells.	Blocked histone induced angiogenesis.	These two substances have in common a highly negative electric charge which binds to positively charged histones, leading to blockage of NETs induced cancer cell migration, angiogenesis [25, 74].
HMG-CoA reductase inhibitors	Sapey et al [75]	In vitro. Neutrophils from patients receiving high dose simvastatin for community	Significant reduction in NETs expression and NE activity at day 4. No difference in	The result of these studies looking at the NETs role in response to bacteria are very different. Keeping in mind the different experimental models, translation of anti-NETs therapies from bench to bedside

		acquired pneumonia.	mortality and length of stay.	may prove difficult and may not be associated with a clinical effect. This could be the case in cancer too.
	Chow et al [76]	In vitro. Human and mice PMNs, cultured with various statins: mevastatin, simvastatin, lovastatin and Fluvastatin. The NETs formation was quantified in response to bacterial exposure.	Statins boosted the production of anti-bacterial DNA-based extracellular traps by human and murine neutrophil ($p < 0.005$)	
Lidocaine	Galos et al [61]	Patients undergoing primary breast cancer resection.	Lidocaine infusion decreased expression of citH3 and MMP-3.	This study offers the premises for a large trial regarding the impact of lidocaine on the metastasis.
NADPH oxidase 2 (NOX2) inhibitors (e.g. apocyanin)	Park et al [36]	In vitro. Murine model. 4T1 breast cancer cells.	NOX2 inhibition reduced NET formation and neutrophil-promoted cancer cell invasion.	NADPH oxidase is a critical enzyme as shown already in the NETs production process. Although inhibitors against it are available and represent a potential tool for blocking NETs production, it is not feasible as this enzyme is involved in critical processes like: bacterial killing, phagocytosis, degranulation [36].
PAD4 inhibition (e.g. YW4-03, Cl-amidine)	Tohme et al [24]	In vitro and Murine model. MC 38 colorectal cancer cells. PAD4 deficient mice.	In vitro PAD4 inhibitors or the use of PAD4 deficient neutrophils block the adhesion of tumour cells to a monolayer of NETs expressing neutrophils. Significantly reduced the tumour cell growth, $p<0.0001$.	PAD-4 inhibition has been associated with preventing NETs formation in animal models, but it effect on metastasis will be difficult to predic due to the short half-lives of the current commercially available inhibitors [36].

			Levels of cit-H3 in the tumours after three weeks were absent in PAD4−/− mice and mice receiving YW4-03.	
	Park et al [36]	Murine model. 4T1 breast cancer cells.	It decreased NETs formation (P=0.02). No effect on tumour cell migration.	
PGE1	Jung et al [25]	In vitro, AsPC-1 pancreatic cancer cells.	Inhibited cancer cell induced NETs formation, by decreasing the intracellular calcium content.	PGE 1 has been used clinically for neonatal duct depended disease, sexual dysfunction, critical limb ischemia due to its anti-inflammatory, vasodilative and anti-platelets effects [77].
Ph	De Souza et al [78]	In vitro. Human neutrophils.	Raising the pH promotes intracellular calcium influx which leads to increases PAD4 activity, citrullination of histone, histone cleavage and NET formation.	Both extra and intracellular alkaline Ph has been linked with cancer promotion [79]. Also, Ph sensitive channels could become a therapeutic target in some cancers [80]. The alkaline pH modulates the NETs formation as enzymes like PAD4 and NE prefer an alkaline environment and processes like mitochondrial ROS and PAD4-mediate citrullination are augmented by alkaline pH with an impact on both NOX dependent and independent processes [78, 81].

Supplementary material

Table 1S: Summary of relevant articles

Author, Title, Journal, Year	Objectives	Design, Population, Sample size	Methodology	Findings	Strengths & Limitations
Galos EV et al [61] Neutrophil extracellular trapping and angiogenesis biomarkers after intravenous or inhalation anaesthesia with or without intravenous lidocaine for breast cancer surgery: a prospective, randomised trial British Journal of Anaesthesia, 2020 October	Examine the effects of 4 different anaesthetic techniques on NETs formation expression in breast cancer surgery in women.	RCT, Women undergoing breast cancer surgery of curative intent N=120	Women were randomly assigned to receive one of 4 anaesthetic techniques: sevoflurane (S), sevoflurane plus i.v. lidocaine (SL), propofol (P), and propofol plus i.v. lidocaine (PL). Venous blood was taken prior to induction of anaesthesia and 20-28 hrs post-op. MPO & CitH3 concentrations were measured by enzyme-linked immunosorbent assay and represented NETosis expression.	Perioperative use of lidocaine decreased MPO concentrations (8.5 [3.4] vs 10.8 [1.8] ng ml^{-1}, P=0.03 for SL and S and 8.6 [3.1] vs 11.6 [2.5] ng ml^{-1}, P=0.01 for PL and P, respectively) And CitH3 concentrations as well (109 [23] vs 125 [22] ng ml^{-1}, P=0.01 for SL and S and 98 [14] vs 130 [32] mg ml^{-1}, P=0.007, for PL and P, respectively)	Strengths • RCT design • Large sample size • Clear inclusion criteria and cohorts • 2 highly sensitive tests for NETosis expression used • Recent, up to date research • Blinding of scientist conducting translational work
Aghamelu O et al [62] Serum NETosis expression and recurrence risk after regional or volatile anaesthesia during breast cancer surgery: A pilot, prospective, randomised single-blind clinical trial Acta Anaesthesiologica Scandinavica 2020 Nov	To examine the effects of 2 different anaesthetic techniques on the serum expression of NETs formation in women undergoing breast cancer surgery	RCT, undergoing breast cancer surgery of curative intent n=40	Women were randomly assigned to receive one of 2 anaesthetic techniques: General anaesthesia or propofol general anaesthesia combined with paravertebral regional anaesthesia. Venous blood was taken pre and within 24 hours post operatively. MPO & CitH3 concentrations were measured by enzyme-linked immunosorbent assay and represented NETs formation expression.	There was no difference in either postoperative MPO in GA vs PPA (10.5$\pm$6.6 vs 11.5$\pm$4.7 ng ml^{-1}, p=0.60) or in postoperative CitH3 in GA vs PPA (3.6$\pm$2.3 vs 4.0$\pm$5.9, p=0.80).	Strengths • RCT design • Clear inclusion and exclusion criteria • Power calculation completed for sample size to detect a significant difference • 2 highly sensitive tests for NETs formation expression used • Recent, up to date research • Blinding of scientist conducting translational work

Rayes R. et al [57] Primary tumours induce neutrophil extracellular traps with targetable metastasis-promoting effects JCI insight, 2019	To investigate whether elevated NETs levels are seen in high-mortality associated malignancies & that the level of NETs formation is associated with disease stage	Observational cohort study Patients with lung or upper GI adenocarcinoma who had not previously received treatment (n=60) & a healthy control group (n=15)	Serum was collected from patients and analysed. MPO concentrations were measured by enzyme-linked immunosorbent assay.	Elevated NETs expression in cancer patients compared to the healthy control group (p=0.03). Elevated NETs levels in patients with advanced upper GI and lung cancer, compared with patients with a less advanced cancer (p=0.01)	Strengths • Recent, up to date research • Blinding of scientist conducting translational work Limitations • Only 1 biomarker for NETs used • Failure to outline clear inclusion, exclusion criteria
Richardson JJR et al **[21]** Neutrophil Extracellular Trap Production in Patients with Colorectal Cancer In Vitro International Journal of Inflammation, 2017	To investigate NETs expression in colorectal cancer patients compared to healthy controls.	Observational cohort study Patients with colorectal cancer (n=45) & control group of healthy, well-matched volunteers (n=20)	Neutrophils were isolated from a venous blood sample from both cohorts and were then analysed under different stimuli (no stimulant, IL-8, LPS). Cancer characteristics & patient outcomes were compared to their respective NETs levels. NETs expression was measured via fluorescent microscopy.	Serum of patients with colorectal cancer showed increased NETs expression compared to healthy controls in response to no stimulus (9,735 AFU versus 11347 AFU, p = 0.0209), IL-8 (8,644 AFU versus 11,915 AFU, p = 0.0032), and LPS (10,576 AFU versus 12,473 AFU, p = 0.0428). Increased NETs expression was seen in patients who developed significant post-operative complications*** (11,760 AFU versus 18,340 AFU, p = 0.0242) and who had a prolonged hospital recovery (9,008 AFU versus 12,530 AFU, p = 0.0476)	Strengths • Several stimulants tested for NETs expression, increases sensitivity of tests • Sample size • Blinding of scientist conducting translational work
Park J et al [36] Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps Science Translational Medicine, 2016	To assess whether breast cancer cell induced NETs formation promotes the invasion and formation of metastatic lung disease in in vitro & in vivo models.	Observational Study Murine Models N=8 mice Cell samples of human primary breast cancer & metastatic lung cancer. N=20	Murine 4T1 (metastatic tumour) and 4T07 (non-metastatic tumour) breast cancer cells were transplanted into murine models (n=4 in each). Neutrophils were measured using Ly6G immunostaining. MPO & CitH3 concentrations were measured by enzyme-linked immunosorbent assay and represented NETs expression. Immunofluorescence staining of the primary breast tumour and the metastatic lung lesions was performed and analysed, in order to measure metastasis	An increased number of neutrophils were expressed in the 4T1 metastatic tumour compared to the non-metastatic 4T07 tumour (p=0.0009). NETs formation was found to be present in 16/20 of the primary tumours and in 13/19 metastatic lung lesions.	Strengths • 2 biomarkers to detect NETs used, increased sensitivity • Blinding of scientist conducting translational work Limitations • Failure to outline clear inclusion, exclusion criteria

Thålin, C et al [56] NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation. Thrombosis Research, 2016	To observe whether the myocardial and cerebral thrombosis were the result of a cancer associated NETs induced hypercoagulable state	A prospective, observational case-control study patients presenting with an ischaemic stroke, who subsequently died n=31	Patients were analysed according to those with a large serum elevation of high sensitive Troponin T (hsTnT- elevated levels associated with malignancy) (n=12), and those who had normal levels (n=19). Autopsies were completed and showed widespread microthrombosis containing CitH3, a biomarker for NETs.	Increased serum NETs in patients with elevated hsTnT compared to patients with normal levels ($p<0.001$). CitH3 level was also correlated with thrombin-antithrombin complex ($p=0.004$) and soluble P-selectin ($p<0.001$).	Strengths • Blinding of scientist conducting translational work Limitations • Failure to outline clear inclusion, exclusion criteria
Jung H. et al **[25]** Cancer cell–induced neutrophil extracellular traps promote both hypercoagulability and cancer progression PLOS One, 2019	To investigate cancer induced NETs formation & whether NETs expression promotes thrombus formation and cancer progression	Observational cohort study Patients with pancreatobiliary malignancy (n=62) & healthy control (n=30)	Peripheral blood sample was taken from participants. Neutrophils were isolated and analysed. Histone- DNA complex detected by ELISA measured NETs expression. To measure coagulability, ETP was measured using thrombin assay and hypercoagulability markers were measured with a Zymuphen MP-Activity kit.	There was increased NETs expression in the cancer cohort compared to healthy controls ($P < 0.05$). Similarly, there was significantly increased ETP & hypercoagulability markers in the cancer patient cohort. ($P<0.05$)	Strengths • Recent, up to date research • Blinding of scientist conducting translational work Limitations • Failure to outline clear inclusion, exclusion criteria
Hisada, Y et al [28] Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumours. *Haematologica*, 2019	To investigate the levels of NETs in mice bearing pancreatic cancer & the contribution of NETs fromation to venous thrombosis	Observational cohort study Mice injected with Human pancreatic cancer cell line BxPc-3 and a control cohort of mice (n= 9 to 20 mice in each cohort)	Human pancreatic cancer cell line BxPc-3 and grown in the pancreas of male mice. CitH3 was used to detect NETs expression. Thrombus formation was induced by IVC stasis in both cohorts. CitH3 and Ly6G (neutrophil marker) levels, Immunofluorescence and electron microscopy were used to detect contents of thrombi.	Increased NETs levels were seen in tumour bearing mice compared to control cohort ($P<0.05$). Thrombi from tumour bearing mice had higher NETs and neutrophil contents compared to control group ($P<0.05$)	Strengths • 2 biomarkers used for NETs detection, increases sensitivity • Blinding of scientist conducting translational work Limitations • Failure to outline clear inclusion, exclusion criteria
Tohme, S et al **[24]** Neutrophil extracellular traps promote the development and progression of liver metastases after surgical stress HPB, 2017	To determine whether NETs expression plays a role in tumour metastasis & development in a murine model of surgical stress which was induced by liver ischemia-reperfusion.	observational cohort study patients who underwent: major hepatectomy (35), smaller hepatectomy (15), healthy control group (20) Total: n=70 2 even cohorts of murine models with MC38 induced colon cancer	Serum was taken day 1 post-op for patients who underwent a hepatectomy for metastatic colorectal cancer. NETs expression, measured by detecting MPO-DNA complexes using ELISA. One cohort of mice was subjected to surgical stress by liver ischaemia for 60 minutes before reperfusion, the other cohort did not undergo I/R. A splenectomy was performed and analysed for NETs expression using ELISA to detect CitH3.	Patients with elevated NETs levels post-operatively, were 4 times less likely to have a disease-free survival* compared to the cohorts with lower NETs expression (95%CI: 1.39-12.81, $p=0.01$). Mice subjected to liver I/R showed greater hepatic metastases compared with mice who were not exposed to hepatic I/R	Strengths • Blinding of scientist conducting translational work • Sample size Limitations • Failure to outline clear inclusion, exclusion criteria

Arpinati, L et al **[82]** NETosis in cancer: a critical analysis of the impact of cancer on neutrophil extracellular trap (NET) release in lung cancer patients vs. mice Cancer Immunology, Immunotherapy, 2020	To investigate the predisposition of isolated neutrophils to undergo NETs expression in the presence of lung cancer, comparing human patients with two mouse models	Observational cohort study Isolated neutrophils from healthy patients (n=9) and from patients with LLC (n=9) and mice injected with LLC cell cultures and a control mice cohort Total n=18	Neutrophils were isolated from cancer patients and healthy donors and the extent of NETs release was measured by microscopic detected of stained anti CitH3 and anti MPO antibodies. Spontaneous neutrophil NETs expression and PMA-induced NETs expression activation was measured. Neutrophils were isolated from serum of cancer induced mice and control mice and analysed in the same manner.	Neutrophils from cancer patients and healthy donors displayed similar amounts of spontaneous NETs (9.3 ± 1.29% and 8.7 ± 0.79% respectively) and similar amounts of PMA induced NETs (33.5 ± 2.31% and 40.2 ± 2.73% respectively). NETs activation in both mice cohorts was much lower overall, but LLC bearing mice had significantly higher spontaneous NETs activity compared to controls (3.67 ± 0.21% and 0.64 ± 0.26% respectively) and under PMA induced NETs (4.17 ± 0.29% 2.2 ± 0.22%, respectively). These results show differences in NETs activation in LLC bearing humans and mice and these differences should be taken into consideration during future investigations.	Strengths • Recent research • 2 biomarkers used for NETs detection, increases sensitivity • Blinding of scientist conducting translational work Limitations • low population size and no power calculation to show significant change • Failure to outline clear inclusion, exclusion criteria

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Papers of special interest, published in the last 3 years have been marked as:
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