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Neutrophil extracellular traps as potential therapeutic targets to prevent tumor-induced organ failure and metastasis

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Abstract

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Cancer does not only affect surrounding tissues, but also leads to complications at distant sites. The reason for this is that tumors secrete factors and prime cells that travel through the body, making cancer a systemic disease. In fact, cancer death is mostly caused by systemic complications such as metastasis, organ failure or thrombosis. However, surprisingly little is known about the effect of cancer on distant organs that are not sites of tumor growth.

Neutrophil extracellular traps (NETs) are web-like structures consisting of neutrophil DNA and granular proteins that are released from the neutrophil in response to a stimulus. NETs are not only formed in bacterial infections but also in noninfectious inflammatory disorders like cancer. While NETs can be beneficial for the host in severe infections they come with a cost. NETs are pro-thrombotic, cause damage to the vessel-lining endothelial cells, are involved in metastasis formation and impair organ function. The removal of NETs by DNase I an enzyme that breaks the NET backbone can therefore be of use as a therapy for certain conditions.

The aim of this thesis was to investigate the mechanisms leading to systemic effects of cancer and potential therapeutic strategies with a special focus on NETs.

In paper I we investigated how the presence of a primary tumor affected heart function. We conclude that tumor-induced NETs contribute to inflammation and myocardial strain in malignant disease and identify NETs as potential therapeutic targets in cancer patients to prevent cardiac inflammation and dysfunction.

In paper II we investigated if the long-term removal of NETs by the use of a murine DNase I expressing adeno-associated virus vector was possible and how this affected organ function and metastasis formation in tumor-bearing mice. We conclude that long-term, species-specific expression of DNase I is possible. Elevated DNase I expression improved kidney function and reduced the proportion of mice with metastasis.

In paper III we investigated how cancer and cancer-associated NETs affect endothelial gene expression in distant organs. We found that the number of differentially expressed genes increased with tumor size and that it was higher in metastatic organs. In response to a tumor, the kidney vasculature showed signs of renal hepatization, a state of kidney stress. In conclusion, this study gives a global overview of the gene expression changes that take place in the vasculature of distant organs in individuals with cancer and the potential role of NETs.

Keywords: Neutrophil extracellular traps; NETs; cancer; metastasis; inflammation; organ dysfunction; thrombosis

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To my family

Thesis defence

This thesis will be publicly defended on the 4th of October 2023 at 9:15 in B41, Biomedical Centre (BMC), Husargatan 3, Uppsala, Sweden.

Faculty Examiner (Opponent)

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  INSERM, Paris, France

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- Associate Professor **Christopher Sjöwall**
  Linköping University, Sweden

Chairperson

- Professor **Andor Pivarcsi**
  Uppsala University, Sweden

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Cedervall, J.*, **Herre, M.*,** Dragomir, A., Rabelo-Melo, F., Svensson, A., Thålin, C., Rosell, A., Hjalmar, V., Wallén, H., Lindman, H., Pejler, G., Hagström, E., Hultström, M., Larsson, A., & Olsson, A. K. (2022). Neutrophil extracellular traps promote cancer-associated inflammation and myocardial stress. *Oncoimmunology, 11*(1), 2049487. ***equal contribution**

II. **Herre, M.,** Vemuri, K., Cedervall, J., Nissl, S., Saupe F., Micallef J., Lindman H., Maguire CA., Tetz G., Tetz V., Olsson, A. K. (2023). AAV-mouse DNase I sustains long-term DNase I expression *in vivo* and suppresses breast cancer metastasis. *Manuscript.*

III. **Herre, M.,** Cedervall, J., Vemuri, K., Lugano, R., Holland, E. C., Dimberg, A. and Olsson, A. K. (2023). Global gene expression analysis of distant organ vasculature in mice with cancer. *Manuscript.*

Other articles co-authored but not included in this thesis:

I. **Herre, M.,** Cedervall, J., Mackman, N., & Olsson, A. K. (2023). Neutrophil extracellular traps in the pathology of cancer and other inflammatory diseases. *Physiol Rev, 103*(1), 277-312.

II. Femel, J., van Hooren, L., **Herre, M.,** Cedervall, J., Saupe, F., Huijbers, E. J. M., Verboogen, D. R. J., Reichel, M., Thijssen, V. L., Griffioen, A. W., Hellman, L., Dimberg, A., & Olsson, A. K. (2022). Vaccination against galectin-1 promotes cytotoxic T-cell infiltration in melanoma and reduces tumor burden. *Cancer Immunol Immunother, 71*(8), 2029-2040.

III. Zhang, Y., Manouchehri Doulabi, E., **Herre, M.,** Cedervall, J., Qiao, Q., Miao, Z., Hamidi, A., Hellman, L., Kamali-Moghaddam, M., & Olsson, A. K. (2022). Platelet-Derived PDGFB Promotes Recruitment of Cancer-Associated Fibroblasts, Deposition of Extracellular Matrix and Tgfβ Signaling in the Tumor Microenvironment. *Cancers (Basel), 14*(8).

IV. Yau, A. C. Y., Globisch, M. A., Onyeogaziri, F. C., Conze, L. L., Smith, R., Jauhiainen, S., Corada, M., Orsenigo, F., Huang, H., **Herre, M.,** Olsson, A. K., Malinverno, M., Sundell, V., Rezai Jahromi, B., Niemelä, M., Laakso, A., Garlanda, C., Mantovani, A., Lampugnani, M. G., Dejana, E., & Magnusson, P. U. (2022). Inflammation and neutrophil extracellular traps in cerebral cavernous malformation. *Cell Mol Life Sci, 79*(4), 206.

V. Zhang, Y., Cedervall, J., Hamidi, A., **Herre, M.,** Viitaniemi, K., D'Amico, G., Miao, Z., Unnithan, R. V. M., Vaccaro, A., van Hooren, L., Georganaki, M., Thulin, Å., Qiao, Q., Andrae, J., Siegbahn, A., Heldin, C. H., Alitalo, K., Betsholtz, C., Dimberg, A., & Olsson, A. K. (2020). Platelet-Specific PDGFB Ablation Impairs Tumor Vessel Integrity and Promotes Metastasis. *Cancer Res, 80*(16), 3345-3358.

VI. Vaccaro, A.*, van Hooren, L.*, **Herre, M.*,** de Alves Pereira, B., Saupe, F., Liu Conze, L., Zhang, L., Lugano, R., Hellman, L., Dimberg, A., Olsson, A. K. (2023). Neutralization of pleiotrophin by vaccination improves vascular function and temozolomide efficacy in glioma. *Submitted.* ***equal contribution**

Contents

Abbreviations

AAV	Adeno-associated virus
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ARF	Acute renal failure
AST	Aspartate aminotransferase
BNP	B-type natriuretic peptide
C3	Complement component 3
C5a	Complement component 5a
CAF	Cancer-associated fibroblast
CAT	Cancer-associated thrombosis
CCDC25	Coiled-coil domain containing 25
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CRP	C-reactive protein
CTC	Circulating tumor cells
DEG	Differentially expressed genes
DVT	Deep vein thrombosis
ECM	Extracellular matrix
eGFP	Enhanced green fluorescence protein
GDF-15	Growth/differentiation factor-15
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GSDMD	Gasdermin D
GSEA	Gene set enrichment analysis
H3Cit	Citrullinated histone H3
HMGB1	High mobility group box-1
hs-TnT	High-sensitive troponin T
I/R	Ischemia-reperfusion
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
LMSCs	Lung mesenchymal stromal cells
LPS	Lipopolysaccharide
MMP9	Matrix metalloproteinase 9
MMTV	Mouse mammary tumor virus
MPO	Myeloperoxidase
MPs	Microparticles
NADPH	Nicotinamide adenine dinucleotide phosphate

NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NGAL	Neutrophil gelatinase-associated lipocalin
NT-proBNP	N-terminal pro b-type natriuretic peptide
PAD2	Peptidyl-arginine deiminase type-2
PAD4	Peptidyl-arginine deiminase type-4
PMA	Phorbol myrisate acetate
PyMT	Polyoma middle T oncoprotein
RIP	Rat insulin gene promoter
ROS	Reactive oxygen species
S.aureus	Staphylococcus aureus
sTNFR1	Soluble tumor necrosis factor receptor-1
tACPA	Anti-citrullinated protein antibody
Tag	Simian virus 40 T-antigen
TAT	Thrombin-antithrombin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
TSP-1	Thrombospondin-1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

Introduction

Cancer

The human body is a carefully controlled, complex system. In order to keep this system working as an entity, damaged parts have to be recognized and subsequently repaired or replaced. This is important on a macroscopic level where for example bleeding wounds have to be closed to guarantee blood supply to all organs. On a molecular level these repair mechanisms are not as obvious, however they take place on a regular basis: when cells become older or accumulate defects, they are either removed from the system or need to be repaired.

Cancer development starts when these control mechanisms of our body fail. Cancer cells acquire different kinds of characteristics and develop properties that distinguish them from healthy cells. These newly acquired characteristics were described as the hallmarks of cancer by Hanahan and Weinberg in 2000, and were later revised in 2011 [1, 2]. The eight hallmarks that were introduced are: the evasion of cell death, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, tissue invasion and metastasis, limitless replicative potential, deregulation of cellular energetics, genome instability, avoidance of immune destruction and tumor-promoting inflammation [2]. All hallmarks describe acquired characteristics of the cancer cells or tumor-induced changes in cells belonging to the tumor microenvironment (TME) like endothelial cells, cancer-associated fibroblasts (CAFs) or immune cells, which are closely associated with the primary tumor and its metastatic foci.

The following paragraph will outline that cancer is a disease that is not only limited to the tumor cells and the closely associated TME, but that cancer rather is a systemic disease affecting organs that are not direct sites for tumor growth, and therefore often are considered healthy.

Cancer – A systemic disease

Cancer originates from a local disease in which normal cells accumulate defects that enable them to circumvent checkpoints and allowing them to form a primary tumor mass. However, cancer often develops into a systemic disease affecting not only the tissue or organ in which the primary tumor grows, but also distant parts of the body to which tumor cells spread or organs that are

free of tumor cells but that are still affected by the cancer. There are many examples how cancer affects distant sites by hijacking other, potentially healthy cells or by secreting soluble factors that travel to distant sites.

In fact, the majority of cancer-related deaths is not due to problems caused at the primary tumor site, but due to systemic complications. Invasion and metastasis, one of the hallmarks of cancer, is the major cause of cancer mortality and is estimated to be responsible for up to 90% of cancer deaths [1, 3, 4]. Metastatic disease starts when cancer cells disseminate from the primary tumor and intravasate into blood- or lymphatic vessels. Having arrived at the metastatic site, the tumor cells extravasate, start to grow, proliferate and form new colonies [5]. Tumor cells hijack a number of cells to support them: macrophages have been shown to upregulate certain proteases that degrade the extracellular matrix (ECM) and facilitate the intravasation of tumor cells through the endothelial cell layer into the vessels [2, 5]. Other metastasis promoting cells are platelets and neutrophils. Platelets bind to tumor cells in the circulation, protect the cells from shear stress and the recognition by natural killer cells that would otherwise detect the downregulated MHC class I on the tumor cell surface and support the extravasation process [6-8]. Neutrophils were shown to for example form clusters with circulating tumor cells that expanded their metastatic potential [9]. Another strategy of neutrophils to increase metastasis formation is neutrophil extracellular trap (NET) formation, which will be discussed in more detail later.

The second leading cause of cancer death is cancer-associated thrombosis. The relationship between cancer and thrombosis formation was already discovered in the mid 19th-century by Armand Trousseau [10]. The MEGA study, a large population-based case-control study revealed that the overall risk for cancer patients to develop venous thrombosis is 7-fold higher than for individuals without cancer [11]. There are various reasons for the increased thrombotic risk in cancer patients: one reason is the abnormal tumor vasculature, which is characterized by a discontinuous endothelial layer, high blood vessel permeability, impaired perfusion and alterations in the basement membrane such as perforation and decreased thickness. The abnormal tumor vasculature can lead to the exposure of subendothelial matrix including collagen and tissue factor (TF) to blood components like platelets and factors of the coagulation cascade that in turn induce a pro-coagulant state [12, 13]. TF, which is the initiator of the extrinsic coagulation pathway and which induces the activation of thrombin and formation of a fibrin clot, is also expressed by the cancer cells themselves. Cancer cells further release soluble factors like inflammatory cytokines such as interleukin-1 beta (IL-1β) or tumor necrosis factor alpha (TNF-α) and proangiogenic factors such as vascular endothelial growth factor (VEGF), that contribute to the emergence of the procoagulant phenotype in endothelial cells and leukocytes. Tumor cells also express cell surface adhesion molecules and factors causing platelet activation and aggregation [14, 15]. Also neutrophils releasing neutrophil extracellular traps have been found

to be involved in the cancer-induced coagulopathy [16]. The involvement of NETs in cancer-associated thrombosis formation will be discussed in detail later.

A third systemic complication in cancer patients is dysfunction of peripheral organs, i.e. organs that are not sites of primary or metastatic tumor growth. It is estimated that the mortality in cancer patients suffering from acute renal failure (ARF) is at least 30%. Treatment of ARF in general is challenging, but it also further complicates cancer treatment since dose adjustments of chemotherapeutic agents often become necessary to avoid renal toxicity caused by ARF-induced altered pharmacokinetics. This can lead to cancer patients being under- respectively overdosed [17]. In our group we have investigated kidney function in a breast cancer mouse model and in mice developing pancreatic neuroendocrine tumors. Our studies reveal that mice with cancer had an impaired kidney vascular function and this observation correlated with tumor size [18]. Tumor-bearing mice further showed signs of renal insufficiency with increased plasma creatinine levels and increased neutrophil gelatinase-associated lipocalin (NGAL) protein levels in the urine [19]. Not only the kidneys but also the heart can be affected by the presence of a tumor. Already in 1968 it was found that cancer patients had a smaller heart mass and reduced ventricular wall thickness than healthy controls [20]. More recent studies describe that post-mortem heart tissue of cancer patients shows extensive fibrosis [21]. It is however well known that cancer treatment itself can have toxic effects on peripheral organs and cause organ malfunction. Biomarkers characteristic for organ dysfunction therefore have to be measured before the initiation of cancer treatment to draw reliable conclusions about tumor-induced organ dysfunction. A study performed in 2015 showed that cancer patients have elevated levels of biomarkers associated with cardiac disease like N-terminal pro b-type natriuretic peptide (NT-proBNP) and high-sensitive troponin T (hs-TnT) already before the start of anticancer therapy [22]. In our group we found that tumor-bearing mice have increased levels of neutrophils and a reduced vascular perfusion in the heart compared to healthy littermates [18]. We also found that tumor-bearing mice express increased amounts of biomarkers that indicate cardiac strain and inflammation. Further, we investigated the situation in human cancer patients and observed elevated levels of biomarkers related to cardiovascular disease [23]. NETs also here seem to play an important role, which will be discussed in more detail later.

In this thesis work I investigated the systemic effects of cancer including metastasis, thrombosis and organ dysfunction with a special focus on the involvement of NETs.

The Neutrophil

Neutrophils are cells of the innate immune system and the most abundant leukocytes in human blood, constituting 50-70 % of the total leukocyte population. In mice, however, neutrophils constitute only 10-30 % of the white blood cells, which is important to consider when working on mouse models [24]. Neutrophils have a for them characteristic segmented nuclear shape and a size of 7-10 μm diameter. They are produced in the bone marrow from a myeloid progenitor cell, from where they are released into the circulation. Their life-span in the circulation was traditionally estimated to be 5-10 hours [25], however this common view was challenged by a slightly controversial study that found the life-span to be 5.4 days instead [26]. In response to inflammation or cytokine signalling the neutrophil life span and amount can increase several-fold [27]. Their importance in the immune system can be seen by looking at patients with congenital neutropenia that suffer from severe immunodeficiency [28].

The cytoplasm of the neutrophil contains at least four types of granules: Primary granules, also called azurophilic granules, that contain myeloperoxidase (MPO), cathepsin G, neutrophil elastase (NE), proteinase 3 and defensins, proteins that can directly eliminate microbes. Secondary granules, also called specific granules that contain for example lactoferrin. Tertiary granules, also called gelatinase granules that contain extracellular matrix (ECM) degrading proteins like matrix metalloproteinase 9 (MMP9). Secretory granules, that contain various cytokines [29].

Stimuli derived from damaged tissue or invading pathogens lead to the recruitment of neutrophils both from the bone marrow and the circulation to the site of infection as one of the first immune cells. Upon infection or inflammation neutrophil recruitment follows a multistep cascade: tethering, rolling and adhesion to the endothelium, crawling and finally transmigration into the tissue [29]. In response to activation, the neutrophil has three effector functions: 1) Phagocytosis: After being opsonized, the microbes are engulfed by the neutrophil. Primary and secondary granules of the neutrophil fuse with the phagosome and reactive oxygen species (ROS) produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are pumped into the phagosome, which contributes to microbial killing. 2) Degranulation of neutrophil granules and their antimicrobial factors into the surrounding tissue. 3) Neutrophil extracellular trap formation, which will be explained in detail in the following paragraph [29, 30]. Neutrophils are not only responsible for the degradation of invading pathogens; they also play an important role in sterile inflammation in which they contribute to tissue healing by removing necrotic cells and releasing growth factors needed for tissue regeneration. However, neutrophils can also contribute to tissue damage by releasing granular contents and by neutrophil extracellular trap formation [31].

Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) are neutrophil-derived extracellular structures of decondensed chromatin that are decorated with cytoplasmic and granular proteins **(Figure 1)**.

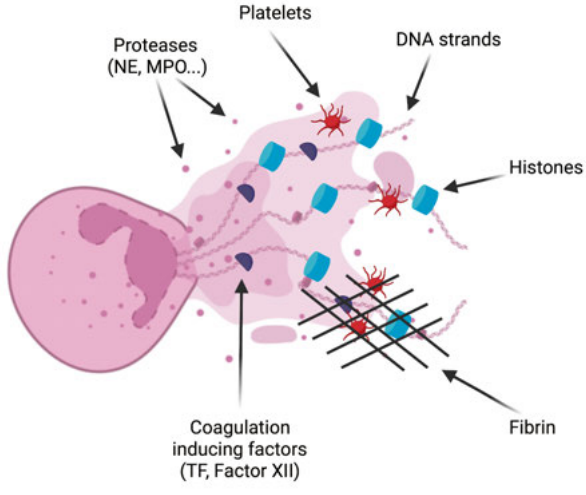

Figure 1: *Schematic illustration of neutrophil extracellular traps (NETs)*. Upon stimulation with various stimuli, neutrophils release their chromatin content together with granular proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). NETs are prothrombotic and platelets as well as fibrin can be found in the NET scaffold. Other coagulation factors like e.g. tissue factor (TF) and factor XII interact with NETs. Image from Herre et al. [30]

Their protein repertoire mainly consists of antimicrobials and proteases making the NETs efficient infection fighters. NETs were for the very first time detected in 2004 by Brinkmann et al. [32]. In their study they showed that upon stimulation with interleukin-8 (IL-8), phorbol myristate acetate (PMA), or lipopolysaccharide (LPS), neutrophils released their chromatin content together with proteins from the neutrophil granules. They further found that the presence of NETs increased the effectiveness of bacterial killing by causing a high local concentration of antimicrobials and by reducing the spread of the systemic bacteria by ensnaring them into the chromatin. Degradation of the NETs by DNase treatment significantly reduced the bacterial killing [32]. In the following years the field on NET research exploded with one publication in 2004 and more than 700 publications in 2022. Three different mechanisms of NET formation have been described until now **(Figure 2)**.

First, lytic NET formation, which is the most studied mechanism. Not only Brinkman et al., who stimulated the neutrophils with IL-8, PMA and LPS

observed lysis of the netting neutrophil, but also Fuchs et al. in 2007 defined lytic NET formation as a mechanism of active cell death [32, 33]. They observed the initiation of NET formation with the disruption of actin dynamics, depolarization of the neutrophil, disassembly of the nuclear envelope, decondensation and release of the chromatin into the cytoplasm where it mixed with cytoplasmic and granular proteins and finally rupture of the plasma membrane and release of the NETs into the extracellular space. Upon stimulation with IL-8, PMA or LPS delobulation happened 60 minutes after neutrophil stimulation. After another 60 minutes the nuclear membrane formed vesicles and after an additional hour chromatin decondensation and nuclear envelope rupture occurred [33]. A recent study performed by Thiam et al. described a similar sequence of events using both human and mouse neutrophils and stimulating them with LPS, ionomycin or Candida Albicans [34]. High-resolution time-lapse microscopy revealed shedding of microvesicles from the neutrophil membrane after which chromatin decondensation occurred. 30 minutes to 2 hours after neutrophil stimulation nuclear rounding happened and this was followed by neutrophil cell death up to five hours after the initial stimulus. All stimuli were lethal for the neutrophils, no viable NET formation could be detected. These findings were conserved between the species [34]. Lytic NET formation was found to be ROS-dependent. ROS produced by NADPH oxidase activated MPO that in turn triggered the activation of NE and its translocation from the neutrophil granules to the nucleus where it participates in chromatin decondensation [33, 35, 36].

In contrast to lytic NET formation after which the netting neutrophil dies, also viable NET formation resulting in the formation of NETs and an anucleated cytoblast has been observed. In 2012 Yipp et al. described NET formation in response to Gram-positive skin infection [37]. In this study they observed that neutrophils that had undergone NET formation were still able to migrate and phagocytose bacteria. In contrast to lytic NET formation, which takes several hours, viable NET formation occurred more rapidly, within minutes [38]. The kinetics of viable NET formation in response to S. aureus infection were described by Pilsczek et al. [39]. During the first 25 minutes a dilation between the inner- and outer nuclear membrane, termed blebbing, was observed. This was followed by formation of vesicles filled with DNA, budding off from the nucleus into the cytoplasm and subsequent breakdown of the nuclear envelope, which was most often seen 1 hour after neutrophil stimulation. The vesicles were then released into the extracellular space where they lysed and released their content. At the same time neutrophil cytoplasmic granules were also released and mixed with the released DNA in the extracellular space. This first hour of NET formation in response to S aureus treatment was found to be independent of NADPH oxidase and ROS production.

A third mechanism of NET formation is the formation of mitochondrial NETs in which the DNA released originates from mitochondria rather than the nucleus. In 2009 Yousefi et al. demonstrated that neutrophil priming for

20 minutes with granulocyte/macrophage colony-stimulating factor (GM-CSF) and subsequent toll-like receptor 4 (TLR4) or complement factor 5a (C5a) stimulation for 15 minutes led to the formation of NETs and viable neutrophils [40]. Interestingly, the NET structures contained granular proteins such as MPO and NE but nuclear proteins were not detectable. Also, the DNA was of mitochondrial rather than nuclear origin. In contrast to the observation that viable NET formation that contained nuclear DNA was independent of ROS formation, treatment with a ROS inhibitor or the use of neutrophils from patients with chronic granulomatous disease that are unable to produce ROS, stopped the formation of NETs. So far, mitochondrial NETs have not only been detected in neutrophils stimulated with LPS or C5a [40], but also in patients with traumatic injury and subsequent surgery [41] and patients with anaplastic thyroid cancer in which NETs were induced by tumor-secreted IL-8 [42]. Further, the formation of NETs containing both nuclear and mitochondrial DNA has been detected. However, in these studies the major part of DNA originated from the nucleus [43, 44]. Which type of NET formation is triggered depends for example on the stimuli and the host species in which the NETs form.

During the last decade it has become increasingly clear that NETs are not only formed in severe bacterial infections but also in many non-infectious, inflammatory diseases like autoimmune diseases or most relevant for this thesis, cancer. The first study describing NETs in cancer was published in 2012 [45]. This study showed that tumor-bearing mice had an increased number of circulating neutrophils and that these neutrophils were more prone to form NETs than neutrophils in healthy controls. Only a few months later in 2013 NETs were for the first time detected in human cancer patients [46].

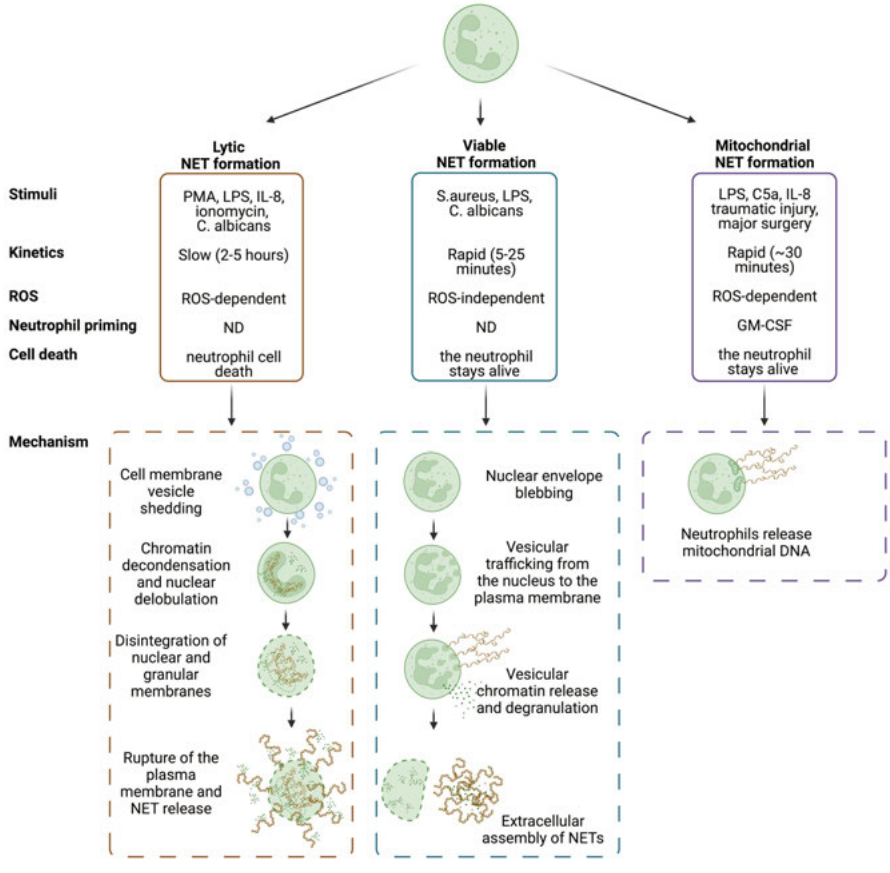

Figure 2: *Schematic illustration showing three mechanisms of NET formation*, i.e. lytic NET formation, viable NET formation and mitochondrial NET formation. The three mechanisms differ regarding their inducing stimuli, kinetics, dependence on reactive oxygen species (ROS) and their cellular mechanisms leading to NET release. Image from Herre et al. [30]

Tumor secreted factors like G-CSF and IL-8 have been identified to be involved in neutrophil priming and subsequent NET formation [45, 47, 48]. NETs have been demonstrated to promote cancer-associated pathologies as will be described in the following sections.

NETs and metastasis formation

NETs and their contribution to metastasis formation has been described in many studies. It has become clear that metastasis promoting NETs can either be induced through post-surgical complications leading to infections and in turn NET release or through tumor-secreted factors that induce the formation

of NETs. The triggers and mechanisms of metastasis promoting NET formation will be discussed in the following paragraph **(Figure 3)**.

In 2013 a study linking post-surgically-induced NETs to metastasis formation was published [49]. Surgical removal of a tumor can be accompanied by post-surgical infection, which is associated with adverse treatment outcomes. The authors showed that upon post-surgical infection caused by cecal ligation and puncture, NET formation was induced. NETs were found in the hepatic sinusoidal spaces and pulmonary capillaries. It was further shown that systemic injection of different tumor cells one day after the induction of surgical-infection lead to an increase in liver metastasis. NET removal with DNase I or a neutrophil elastase inhibitor decreased metastasis formation. They concluded that systemic NETs physically trapped the circulating tumor cells leading to increased metastasis formation [49]. The same group further investigated the mechanism of interaction between circulating tumor cells and NETs. They found that β1-integrin was expressed both on cancer cells and NETs and that this factor was important for the adhesion of tumor cells [50]. Relatively recently an additional NET-associated molecule, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), was identified to be involved in the interaction of NETs and tumor cells upon postoperative complications in a model of colon carcinoma. CEACAM1 knockout led to decreased cancer cell adhesion, migration and metastasis [51]. Even though surgery is an important tool for the treatment of cancer, there is a risk for not only post-surgical infections as described above, but also surgical stress. Tohme et al. described in 2016 that upon liver ischemia-reperfusion (I/R) injury they detected increased levels of NETs, which was associated with increased metastasis formation and metastatic growth after splenic injection of mouse colorectal cancer cells. Inhibition of NETs with an inhibitor for protein-arginine deiminase type-4 (PAD4), an enzyme involved in NET formation, or NET removal with DNase I alleviated the formation of metastases. It was suggested that activation of toll-like receptor 9 (TLR9) in cancer cells by NET extracellular DNA and NET-released high mobility group box (HMGB)-1 induced the metastatic progression. They further found that increased NET formation after curative liver resection in colorectal cancer patients correlated with a decreased disease-free survival [52]. In a following study, the authors showed that I/R injury followed by colorectal cancer cell injection into the tail vein led to an increase in distant metastasis formation in the lung compared to sham treated mice [53]. The authors further investigated the cause for the increased distant metastasis and found that it was platelet dependent. Tumor cells that had formed aggregates with platelets were more prone to be captured by NETs than platelet-free cancer cells [53].

Besides the induction of NET formation by surgical infection or stress, NETs can also be induced by tumor-secreted factors.

In 2016 it was shown that injection of metastatic breast cancer cells in the tail vein of mice induced the formation of NETs shortly after the injection

showing that tumor cells can induce the formation of NETs in the absence of infection or surgical stress [54]. The tumor cells associated with the NETs in the lung and mice developed less metastases after NET removal with DNase-coated nanoparticles. G-CSF secreted from the tumor cells was found to be responsible for the induction of NET formation. They further investigated the presence of NETs in human breast cancer patients and found an association of the presence of NETs with aggressive, triple-negative breast cancer [54]. Besides G-CSF, cathepsin C secreted from tumor cells was shown to stimulate the formation of NETs and subsequent metastasis formation [55]. It was demonstrated that the previously reported metastasis-suppressive and anti-angiogenic ECM protein thrombospondin-1 (TSP-1) was degraded by NETs which promoted lung metastasis [56]. NETs have also been found to participate in the formation of a premetastatic niche in the lungs of mice that develop breast cancer. Yang et al examined a specific subset of neutrophils called tumor-associated aged neutrophils or Naged, in the metastatic lung of mice developing breast cancer [57]. Naged were found to use SIRT1 as key transcription factor, which induced mitochondrial permeability and subsequent formation of mitochondrial NETs. Tumor cells interacted with Naged via their NETs in the metastatic lung [57]. In another study it was found that lung mesenchymal stromal cells (LMSCs) at a premetastatic stage upregulate complement 3 (C3), which induced neutrophil recruitment and NET formation. This in turn promoted the formation of breast cancer lung metastasis. They further investigated if these findings could also be seen in breast cancer patients. Breast cancer patients with metastatic disease had increased serum C3 levels and the C3 expression was located to the stroma of metastatic tissue. This suggests that C3 could also play a role in lung metastasis in human breast cancer patients [58]. It was further suggested that the DNA backbone of the NETs does not only physically trap the circulating tumor cells, but that it in addition functions as a chemotactic stimulus attracting cancer cells to the site of NET formation via coiled-coil domain containing 25 (CCDC25), a transmembrane protein of the cancer cells. These findings suggest a more active role of NETs in the induction of metastasis formation [59].

In 2018 a group of scientists further showed that NETs could awaken dormant, already extravasated tumor cells. They showed that the NET components NE and MMP9 cleaved the ECM protein laminin, uncovering a residue that bound to and activated integrins on the cancer cells, leading to cancer cell proliferation [60].

In our group, we have shown that endothelial cells become activated and express increased levels of adhesion molecules in tumor-bearing mice in the absence of infection and surgical stress. Removal of NETs restored the expression of these molecules to levels seen in healthy individuals. One can speculate that the NET-induced endothelial activation could facilitate the extravasation and invasion of tumor cells in organs distant from the primary tumor i.e. at potential metastatic sites [18, 19]. In paper II we further show

increased levels of NETs in the circulation of breast cancer patients with metastatic disease compared to patients with local breast cancer, suggesting a potential role of NETs in later stages of breast cancer.

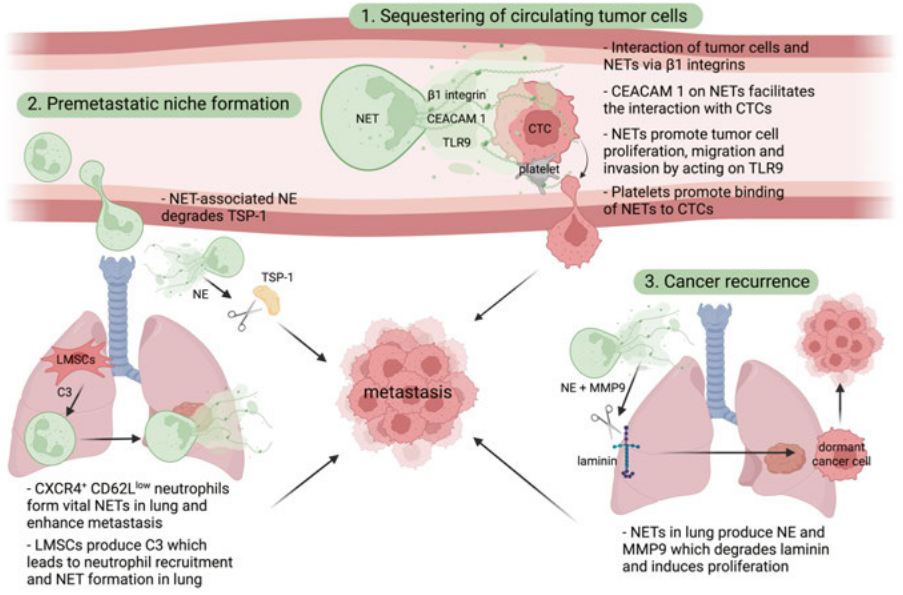

Figure 3: *Schematic illustration showing NETs in metastasis formation*. NETs contribute to metastasis formation in various ways 1) NETs sequester circulating tumor cells (CTCs). NETs and tumor cells were shown to interact e.g. via β1-integrin or CEACAM1. NETs promote tumor cell proliferation, migration and invasion by acting on Toll-like receptor 9 (TLR9). Further, platelets promote binding of NETs to CTCs. 2) NETs are involved in premetastatic niche formation. NET-associated neutrophil elastase (NE) degrades the anti-tumorigenic factor thrombospondin-1 (TSP-1). In addition, so called 'aged neutrophils' form vital NETs in the lung that contribute to premetastatic niche formation. Finally, lung-resident mesenchymal stem cells (LMSCs) produce complement factor 3 (C3) in the lung, which leads to neutrophil recruitment and NET formation in the lung. 3) NETs play a role in cancer recurrence. NETs contain neutrophil elastase (NE) and matrix metalloproteinase 9 (MMP9), which degrade laminin leading to proliferation of dormant cancer cells. Image from Herre et al. [30]

NETs and organ dysfunction

The occurrence of multiple organ dysfunction and failure in cancer patients has been known since several decades [61]. The underlying mechanisms and causes are however poorly understood. Even organs that are not sites of primary or metastatic tumor growth are affected. In the IRMA study it was shown that approximately 50% of all cancer patients were diagnosed with renal insufficiency before the initiation of cancer treatment [62]. Organ dysfunction

causes severe complications for the already ill patient and can lead to the necessity to decrease cancer treatment dose to a less efficient concentration in order to avoid further organ damage by the anticancer drug. Besides the kidney, it has been shown that also the heart of treatment-naïve cancer patients can be affected. Cardiac biomarkers like NT-proBNP or high-sensitive troponin T were elevated before the initiation of cardiotoxic anticancer therapy [22]. In paper II we observed that tumor-bearing MMTV-PyMT mice that form mammary carcinomas with metastasis to the lung have altered levels of the liver enzymes aspartate aminotransferase and alkaline phosphatase compared to their healthy littermates, indicating another organ affected with organ dysfunction caused by systemic effects of the tumor.

NETs have been shown to cause organ damage in various diseases **(Figure 4)**. In 2007 Clark et al. demonstrated that sepsis-induced NET formation was associated with endothelial damage, a decreased perfusion of the liver sinusoids and liver damage characterized by increased levels of alanine aminotransferase [63]. Mice with LPS-induced sepsis were shown to have increased NET levels, and several organ dysfunction markers for heart, lung and kidney were elevated. Histological analysis of these organs revealed significant damage. Removal of NETs by DNase I treatment decreased the level of these markers, normalized the histology and increased the survival [64]. In our previous studies we identified NETs as contributors to organ dysfunction in mice with cancer [18, 19]. Two tumor mouse models, MMTV-PyMT and RIP1-Tag2, showed impaired vascular function characterized by reduced vessel perfusion and increased leakiness. Vessel damage was caused by the presence of NET-platelet complexes partly clogging the blood vessels, and the subsequent hypo-perfusion of peripheral organs. The decreased perfusion led to endothelial activation, increased numbers of infiltrating neutrophils, upregulation of pro-inflammatory cytokines and renal dysfunction. NET formation was suggested to be induced by tumor-secreted G-CSF. Both tumor models expressed high levels of G-CSF and were found to induce NET formation while B16 melanoma-bearing mice neither secreted high levels of G-CSF nor were shown to induce the formation of NETs. Antibody-mediated removal of G-CSF in the MMTV-PyMT model restored kidney vascular function. Removal of NETs with DNase I or inhibition of NET formation with a PAD4 inhibitor lowered endothelial activation and normalized kidney function [18, 19]. In paper I in the current thesis we have shown that tumor-bearing MMTV-PyMT mice displayed increased inflammatory markers in the heart together with elevated numbers of infiltrating immune cells. Biomarkers for myocardial strain like B-type natriuretic peptide (BNP) or growth/differentiation factor-15 (GDF-15) were upregulated. Removal of NETs by peritoneal injection of DNase I for three days normalized the thrombotic phenotype, reduced the myocardial inflammation and the expression of biomarkers for myocardial strain in the tumor-bearing individuals. Further, cancer patients with various malignant disorders showed increased levels of NETs compared to a healthy control

group. NET levels correlated with cardiac disease biomarkers NT-proBNP and soluble tumor necrosis factor receptor-1 (sTNFR1) [23].

The mechanisms by which NETs can induce organ dysfunction are manyfold. It has been shown that NETs have direct cytotoxic effects on endothelial and epithelial cells. Especially histones exert deleterious effects on the endothelium and organ function [65]. Histones activate TLRs or the inflammasome pathway resulting in the release of pro-inflammatory cytokines. Histones also directly bind to endothelial cells, which leads to cell permeabilization, calcium influx and endothelial injury. Further, histones induce platelet activation, aggregation and in turn thrombin formation, which increases the risk for thrombosis, impaired perfusion and in turn endothelial activation [66]. Besides extracellular histones, neutrophil proteases have been shown to cause endothelial and epithelial cell damage. Saffarzadeh et al. showed that the preincubation of NETs with antibodies against histones or an MPO inhibitor but not a NE inhibitor reduced the cytotoxicity caused by NETs [67].

In conclusion, NETs contribute to organ inflammation and dysfunction in cancer in several ways: 1) NETs are directly cytotoxic and cause endothelial damage and in turn endothelial activation. 2) NETs impair blood flow either via platelet activation and subsequent clot formation or by physically blocking the blood flow, which leads to hypoperfusion and again endothelial activation that in turn facilitates immune cell extravasation into the inflamed organ.

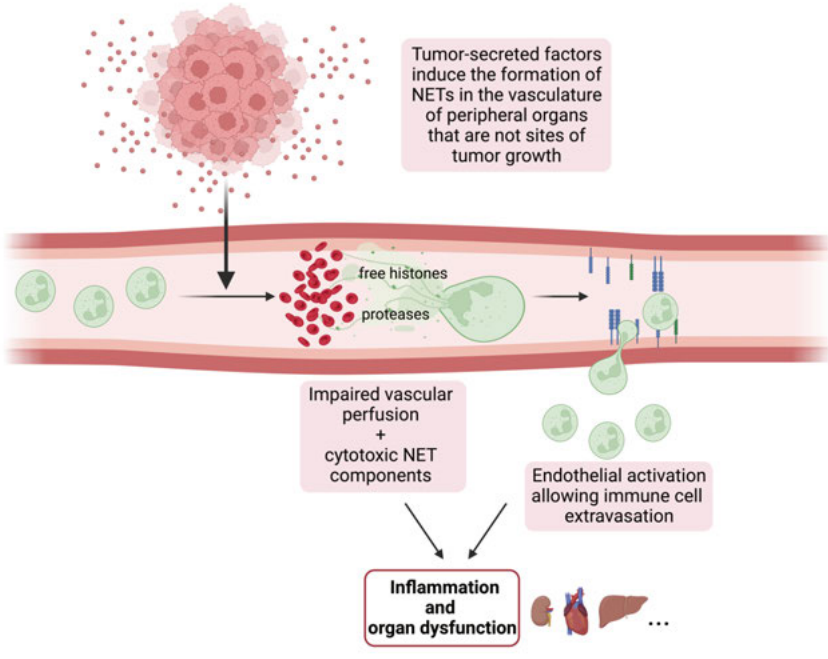

Figure 4: *Schematic illustration showing the contribution of NETs to inflammation and organ dysfunction.* Tumors secrete factors that induce the formation of NETs in the vasculature of peripheral organs. Intra-vascular NET formation leads to impaired vascular perfusion and the release of cytotoxic NET components. This in turn can lead to endothelial activation allowing immune cell extravasation and subsequent organ inflammation and organ dysfunction. Image from Herre et al. [30]

NETs and thrombosis formation

NETs are involved in thrombosis formation **(Figure 5)**. This was for the first time reported by Fuchs et al. in 2010. The authors showed that platelets suspended in plasma adhered to NETs and that adherent platelets became activated. Simultaneous DNase I treatment removed both NETs and platelets confirming their interaction [68]. Besides the use of DNase I, treatment with heparin, which has a high affinity for histones, destabilized the NETs and removed platelet aggregates. They further showed that NETs did not only form a scaffold for platelets but also for red blood cells. In addition, they demonstrated that von Willebrand factor (vWF), fibronectin and fibrinogen bound to the NETs. Treatment of clotting blood with tissue plasminogen activator (tPA) or DNase I revealed that clot formation could only be resolved by DNase I treatment and subsequent NET degradation but not by tPA-induced fibrin degradation suggesting that NETs can form a clot scaffold independent from fibrin [68].

A study in the journal Science in 2017 investigated the importance of endogenous DNase expression in the prevention of vascular occlusion in a mouse model of neutrophilia. The researchers knocked out two endogenously expressed DNases, DNase I and DNase 1L3, which are the only secreted DNases in our body. The knockout led to the formation of clots in blood vessels of lung, liver and kidneys during chronic neutrophilia. The clots were positive for DNA and other NET markers like MPO and citrullinated histones. Interestingly some of the clots were negative for fibrin and neither platelet depletion nor thrombin inhibition could resolve the blood clots. This study confirmed that NETs form a scaffold for platelets and erythrocytes in a fibrin-independent way [69].

In 2010 a Nature Medicine article described the procoagulant effects of the neutrophil serine proteases NE and cathepsin G in a model of both chemically- and mechanically-induced vessel injury and systemic E. coli infection [70]. They concluded that both neutrophil serine proteases contributed to fibrin deposition and in turn thrombus formation and stabilization. Nucleosomes released during NET formation were found to capture substrates of serine proteases like tissue factor pathway inhibitor (TFPI) that were subsequently cleaved and deactivated leading to a decreased inhibition of the coagulation cascade [70].

Several studies also showed that NETs are decorated with active TF [71-74]. In a mouse model of deep vein thrombosis (DVT) it was shown that neutrophils accumulated at the vessel wall and formed NETs during the initiation of DVT formation [72]. The NETs were decorated with TF. Removal of NETs with DNase I or heparin significantly reduced the growth of DVT [72]. In addition, NETs provided a scaffold for FXII activation and the authors hypothesized that FXII could be activated by the negatively charged DNA of the NET backbone. In 2018 Wang et al. investigated the role of NETs in thrombin generation [73]. In a model of sepsis induced by CLP they observed the activation of neutrophils and subsequent NET formation. Activated neutrophils further shed off microparticles (MPs) that bound to the NETs. NET-MP complexes were important for thrombin generation and it was suggested that thrombin generation was mainly mediated by activation of FXII of the intrinsic coagulation pathway [73]. In addition, TF expressing MPs secreted from circulating cancer cells have been shown to adhere to NETs formed at the site of thrombosis formation leading to an increased incidence of cancer-associated venous thrombosis [74].

Besides the contribution of NETs to the induction of thrombosis formation, NETs have also been found to play a role in the stabilization of already formed thrombi. Longstaff et al. showed that the presence of mostly histones but also DNA in the thrombotic clot led to an increased fibrin fiber thickness and more stable clots upon challenging them with shear forces [75]. The presence of DNA and histones also significantly prolonged plasma clot lysis by tPA, however lysis time could be reduced by DNase treatment in combination with tPA

[75, 76]. NETs also stabilize clots independent on tPA. Gould et al. showed that NETs formed in the plasma of septic individuals impaired the fibrinolysis of clots. The anti-fibrinolytic action was caused by the formation of a non-productive ternary complex between plasmin, cell-free DNA and fibrin that interfered with plasmin-mediated fibrin degradation [77]. Further, it was shown that PAD2 and PAD4 enzymes that may be released during NET formation could citrullinate fibrinogen. Citrullination of fibrinogen reduced clot stability however the resistance to clot lysis was increased [78].

Several murine models have been used to study the role of NETs in thrombosis formation in cancer-associated thrombosis (CAT). Leal et al. found increased levels of NETs in the murine 4T1 mammary carcinoma model. Tumor-bearing mice had significantly reduced occlusion times compared to control mice. DNase I treatment resulted in occlusion times that were similar in tumor-bearing and control individuals. The formed thrombi contained both neutrophils and were rich in DNA content, indicating the presence of NETs [79]. We have previously shown that the formation of NETs in two spontaneous, orthotopic mouse models of mammary carcinoma, MMTV-PyMT, and insulinoma, RIP1-Tag2, led to vessel occlusion. Both the treatment with DNase I and PAD4 inhibition resulted in improved vessel perfusion [18]. We further showed that tumor-bearing MMTV-PyMT mice have increased thrombin-antithrombin (TAT) levels, which could be reversed by DNase I treatment [23].

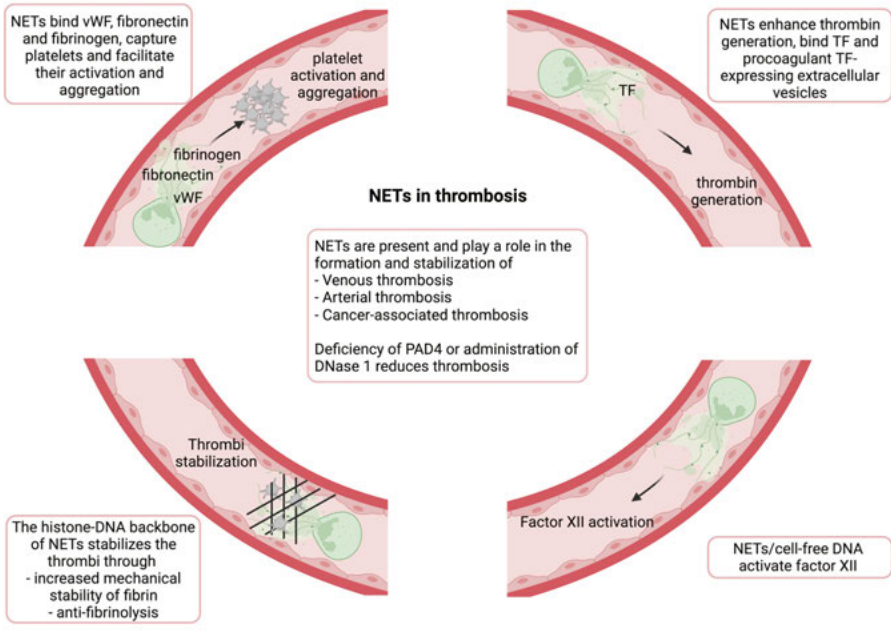

Figure 5: *Schematic illustration showing the role of NETs in thrombosis formation.* 1) NETs bind von Willebrand factor (vWF), fibronectin and fibrinogen, capture platelets and facilitate their activation and aggregation. 2) NETs enhance thrombin generation and bind tissue factor (TF). 3) NETs activate the intrinsic coagulation pathway by binding and activating factor XII. 4) NETs stabilize thrombi through increasing the mechanical stability of fibrin and through anti-fibrinolysis. Image from Herre et al. [30]

The role of NETs in metastasis, cancer-induced organ dysfunction and thrombosis formation identify them as an interesting prognostic marker and therapeutic target.

Therapeutic targeting of NETs

Neutrophil extracellular traps play a role in various diseases such as severe infections, cardiovascular diseases, autoimmune diseases and cancer. In most of the diseases NETs seem to have a negative effect on patient health. Investigating NETs as therapeutic targets could therefore be useful for the treatment of various diseases. Considering NETs as therapeutic targets, their removal has to be safe. If the inhibition of NET formation is safe in severe infections is not clear, since studies with controversial outcomes have been published. One study showed that the inhibition of NET formation using a PAD4 knockout mouse led to a higher susceptibility of the mice to develop bacterial infections in a disease model of necrotizing fasciitis [80]. However, in another study investigating the importance of NETs in acute lung inflammation during

influenza A infection, the use of a PAD4-deficient mouse strain did not result in aggravated health parameters [81]. In an additional study Martinod et al. challenged PAD4 knockout mice with mild and severe sepsis caused by cecal ligation and puncture. The PAD4 knockout mice showed similar survival as the control mice. Further, PAD4 knockout even seemed to protect the mice from septic shock [82]. Considering these data, effects of NET inhibition might be disease-dependent.

There are two options how to use NETs as therapeutic targets: the degradation of already formed NETs or the inhibition of NET formation **(Figure 6)**. One option for the degradation of NETs is the use of DNase I. DNase I is an endogenously expressed DNA-digesting enzyme that degrades single- and double-stranded DNA in the blood. Treatment with DNase I has been suggested to be beneficial in pathological conditions such as ischemia-reperfusion injury, kidney dysfunction and lupus nephritis, all having in common the presence of neutrophil extracellular traps [19, 73, 83]. Recombinant human DNase I is already in clinical use for the treatment of cystic fibrosis, a chronic airway disease in which the inhalation of DNase I alleviates disease symptoms by breaking down the DNA in the sputum and thereby reducing the sputum viscosity [84]. Intravenous injection of DNase I in patients with lupus nephritis has also been considered safe [85]. DNase I is to date commercially available only as bovine and human protein. This makes the long-term use of the enzyme in immunocompetent mouse models impossible due to the developing immune reaction against the foreign protein and the subsequent degradation of it. In paper II we expressed recombinant mouse DNase I in an adeno-associated virus (AAV) vector to be able to evaluate the long-term treatment effects in a breast cancer mouse model. Adeno-associated viruses (AAVs) are small, single-stranded DNA viruses belonging to the parvovirus family. They have been used in more than 100 clinical trials for gene therapy and are considered safe due to the small risk of genome integration, their sustained expression and low immunogenicity [86]. The fact that already five AAV vectors have been approved for clinical use shows the feasibility of this therapy approach.

Another option to destabilize NETs is the use of heparins. Heparin is a glycosaminoglycan and has been in clinical use as an anticoagulant drug for many years. Histones are components of NETs that have been shown to trigger inflammation, coagulation and cause endothelial injury. Heparin has a high affinity to histones [87] and was shown to dismantle NETs by releasing histones from the DNA backbone [68]. It was further shown that low molecular weight heparins inhibited the formation of NETs in response to IL-8, PMA and HMGB1 stimulation [88]. However, there is also a study that suggests that heparin might even induce the formation of NETs [89]. Additional investigations are needed to fully understand the effect of heparin and potential use for the destabilization of NETs.

Besides the destabilization and degradation of NETs, the inhibition of NET formation is another therapeutic strategy. Peptidyl arginine deiminases (PADs) are enzymes converting positively charged arginine residues into neutral citrulline. PAD4, which is needed for the formation of NETs, is the only PAD enzyme that translocates to the nucleus and citrullinates histones and transcription factors [90]. During NET formation PAD4 citrullinates histones and causes a weakening of the electrostatic forces between DNA and histones leading to chromatin decondensation. NET formation can be inhibited by preventing the activity of PAD4. One possibility is to use the drug Cl-amidine, which inhibits all PAD isoforms [91]. In 2015 PAD4-specific small-molecule-inhibitors GSK484 and GSK199 were introduced and found to fully inhibit mouse and partly human NET formation [92]. Another PAD4 inhibitor, BMS-P5, was recently developed by Bristol-Myers Squibb. It was shown that BMS-P5 could inhibit myeloma-induced histone citrullination and NET formation in a mouse model and that it inhibited NET formation also by human neutrophils [93].

Besides PAD4, neutrophil elastase has been shown to be required for NET formation *in vitro* and *in vivo* [35]. The use of NE inhibitors GW311616A and Sivelestat was successful in inhibiting NET formation both in a mouse model in which NET formation was induced by cecal ligation and puncture [49] and in mice with lung carcinoma [94]. NE inhibition also inhibited metastasis formation in both studies.

Gasdermin D (GSDMD) is a pore-forming protein that was shown to be active in neutrophils of septic patients and mice and involved in the formation of NETs. Inhibition of GSDMD with disulfiram or genetic deletion inhibited NET formation during sepsis [95].

Very recently, an additional way to inhibit NET formation and initiate NET uptake by macrophages by using an anti-citrullinated protein antibody (tACPA) was described [96]. The antibody alleviated disease symptoms in several NET-associated diseases i.e. inflammatory arthritis, pulmonary fibrosis, inflammatory bowel disease and sepsis.

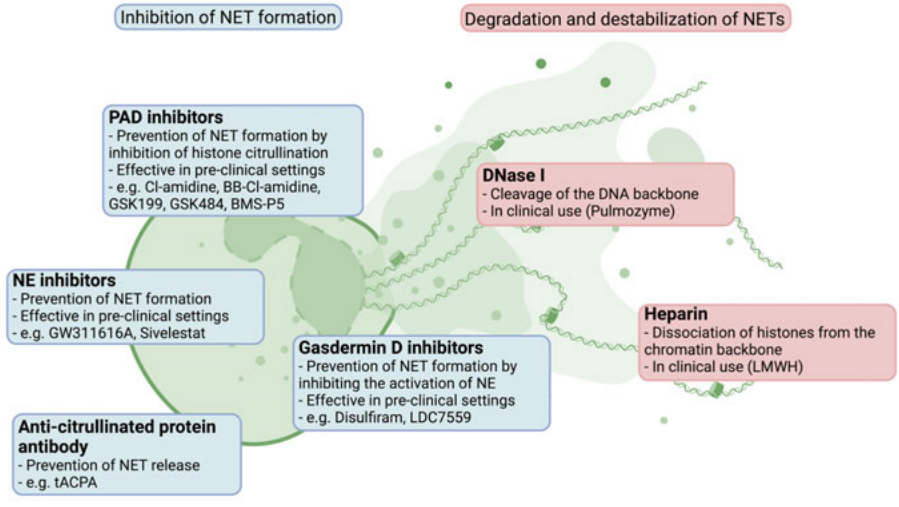

Figure 6: *Schematic illustration listing different therapeutic strategies to degrade NETs or inhibit NET formation*. 1) Inhibition of NET formation through the use of peptidyl-arginine deiminase (PAD) inhibitors, neutrophil elastase (NE) inhibitors, gasdermin D inhibitors or an anti-citrullinated protein antibody. 2) Degradation and destabilization of NETs through the use of DNase I or heparin. Image from Herre et al. [30]

Mouse models

Cultured tumor cells have been a valuable tool to study cancer cell behaviour, their interactions or reactions to drugs. These models still deliver valuable findings and newly developed 3D *in vitro* models resemble the tumor and its environment more and more closely. *In vitro* models do however hold certain limitations for example the absence of the immune system, which plays an important role during tumor development and progression. Further, these models do not allow the investigation of distant tissues or organs that can be affected by the tumor.

At this point mouse models come into play. Subcutaneous tumor models in which cancer cells are injected under the skin have the advantage of growing surrounded by other tissues, more closely resembling the tumor microenvironment and allow the study of tumor affected organs or metastatic sites. Subcutaneous models can be divided into syngeneic and xenograft models. While syngeneic models use cells originating from the same mouse strain as the one they are engrafted in, xenograft models use foreign cells, which are engrafted into immunodeficient mice to avoid immune rejections. Also here certain limitations like the use of immunodeficient animals or the speed of tumor development do exist: Subcutaneous tumors develop within days or weeks while human tumors grow for years and develop through multi-step tumorigenesis.

Further, tumor models can be either orthotopic, meaning that the tumor cells grow in the corresponding tissue, or heterotopic, which describes tumor growth at a site distant from the tumor cell origin, usually subcutaneously. The advantage of orthotopic tumor models is that the tumor growth takes place in a more relevant tumor microenvironment.

A third type of tumor model is the genetically engineered mouse, which spontaneously forms tumors from normal cells due to the insertion of oncogenes or the silencing of tumor-suppressor genes. These tumors, in contrast to the subcutaneous models, are orthotopic, develop spontaneously, are under the influence of the immune system since they grow in immunocompetent mice and mimic the steps of human tumor development more closely. Two examples of transgenic mouse models are the MMTV-PyMT and the RIP1-Tag2 models, which are used in the studies of this thesis work.

MMTV-PyMT

MMTV-PyMT mice develop mammary adenocarcinomas with metastasis to the lung. These mice have the polyoma middle T oncoprotein (PyMT) expressed under control of the mouse mammary tumor virus (MMTV) promoter [97]. Tumors develop in the mammary tissue of female mice as a result of hormone-induced activation of the promoter. Binding of PyMT to proto-oncogenes of the c-src family and proteins of the ras and PI3 kinase pathways are critical steps in tumor induction. This model shows morphological and biomarker-related similarities to human breast cancer progression such as the loss of the hormonal oestrogen and progesterone receptors and persistent expression of the epidermal growth factor receptor (Her2) leading to extensive proliferation and inhibition of apoptosis [98].

RIP1-Tag2

RIP1-Tag2 mice develop pancreatic neuroendocrine tumors with metastasis primarily to the liver but also to the lung. These mice express the simian virus 40 T-antigen (Tag) under the control of the rat insulin gene promoter (RIP), which leads to the development of insulinoma, i.e. carcinoma in the insulin-producing beta cells in the islets of Langerhans. Tag binds and inhibits the two tumor-suppressors p53 and pRB thereby blocking apoptosis and cell cycle arrest. Tag further interferes with cell growth, differentiation and the cell cycle by binding to other proteins [99]. Tumor development undergoes a multi-step tumorigenesis, beginning with islet hyperplasia after 4 weeks of age. At 7-8 weeks dysplastic islets undergo an angiogenic switch, develop into adenomas until 12 weeks of age and finally into invasive islet cell carcinoma at 14-15 weeks [100, 101].

VEcadTRAP mouse

A third transgenic tumor model used in this thesis is the VEcadTRAP mouse. TRAP stands for translating ribosome affinity purification. TRAP mice are healthy, transgenic mice that express an eGFP-tagged ribosomal protein, L10a, in any genetically defined cell type, depending on the promoter. The specific TRAP mouse used in this thesis was generated in the lab of Prof Eric Holland (Fred Hutchinsson Cancer Research Center, Seattle, US) and expresses the eGFP-tag under the VE-cadherin promoter, specific for endothelial cells **(Figure 7)**.

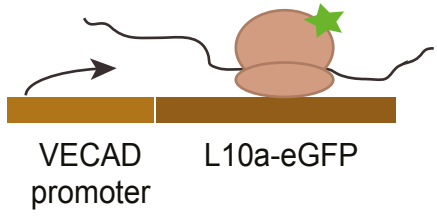

Figure 7: *Schematic illustration of the TRAP construct*. Enhanced green fluorescence protein (eGFP) fused to the ribosomal protein L10a. L10a-eGFP expression is under control of the endothelial-specific vascular endothelial (VE) cadherin promoter.

The fact that ribosomes also carry the mRNA that is about to become translated into protein makes the TRAP mouse a valuable tool to study the traslatome in the cell type of interest. Affinity purification with an anti-GFP antibody purifies the tagged ribosomes and indirectly the associated mRNA. The extracted RNA can then be used in any downstream analysis like qPCR or RNA sequencing [102].

The TRAP model has several advantages over common RNA purification techniques: RNA purification from total organ preparations does not give information about gene expression in specific cell types. Further, tissues don't have to be fixed or dissociated, the extracted mRNA reflects the protein content of the cells more closely than total RNA does, and eGFP-tagged cell types can be visualized using immunohistochemical methods [102, 103].

In paper III we have crossed healthy VEcadTRAP mice expressing eGFP-tagged ribosomes in the endothelial cells with tumor-bearing RIP1-Tag2 mice. This allows us to study tumor-induced changes in endothelial cells specifically.

Present investigations

Aims

The aim of this thesis was to investigate the mechanisms leading to the systemic effects of cancer and potential therapeutic strategies with a special focus on NETs. In **paper I** we investigated how cancer and cancer-associated NETs affect cardiac function both in a mouse breast cancer model and in human cancer patients. The aim of **paper II** was to investigate if long-term NET removal using a murine DNase I-expressing AAV vector is possible and if it reduces metastasis formation in a murine breast cancer model. **In paper III** we explored how cancer and cancer-associated NETs affect endothelial gene expression in organs that are distant from the primary tumor.

Paper I

Neutrophil extracellular traps promote cancer-associated inflammation and myocardial stress

For paper I we made use of the MMTV-PyMT mouse model ($PyMT^+$) for mammary carcinoma. These mice develop palpable mammary tumors with 8-10 weeks of age and form spontaneous metastases in the lungs. First, we measured the level of NETs with an in-house developed ELISA that detects complexes of H3Cit and DNA in plasma. $PyMT^+$ mice had elevated NET levels compared to their healthy littermates. To investigate if $PyMT^+$ mice also showed increased coagulation, TAT complexes were measured. Tumor-bearing mice had significantly higher TAT complexes. DNase I treatment for three days reduced both NET levels and TAT complexes. These results suggest a causal relationship between the formation of NETs and the pro-coagulant state in our tumor-bearing mice. NETs have in many studies been shown to contribute to thrombosis in various ways, e.g. by forming a scaffold for platelets that facilitates platelet activation and aggregation and by stabilizing the fibrin network with their DNA backbone. Further, NETs were shown to increase thrombin generation by binding tissue factor [104].

In a previous publication from our group, we show that the perfusion of the myocardium in $PyMT^+$ mice is impaired and that increased numbers of neutrophils are present in the heart [18]. To investigate if these findings go along with cardiac inflammation, we analyzed the expression of inflammatory

markers by qPCR. Both TNFα and IL-1β were increased in the PyMT$^+$ mice. TNFα levels could be reduced by treating the mice with DNase I, which indicates an involvement of NETs. Shedding of TNFRs from the heart can be induced as a response to inflammation to avoid extensive inflammatory signalling [105]. We found that sTNFR1 levels were significantly increased in the serum of PyMT$^+$ mice compared to the healthy littermates and that the sTNFR1 levels positively correlated with the TNFα expression in the heart. In addition to the elevated inflammatory cytokines in the heart, the number of CD45+ leukocytes was increased in PyMT$^+$ mice. The majority of CD45+ cells was found to be CD68+ cells. Removal of NETs by DNase I treatment reduced the number of CD45+ and CD68+ cells significantly. We further investigated the presence of NETs in the heart using 3D confocal microscopy. We found Hoechst-stained DNA fibers in the proximity of fragmented Gr1+ cells in the PyMT$^+$ mice while these structures could not be detected in PyMT$^+$ mice treated with DNase I or their healthy littermates. Electron microscopy on heart tissue of tumor-bearing mice showed blood stasis and platelet aggregation in the capillaries. These results correspond to findings from our previous publication where we found the capillaries of the heart to be poorly perfused in PyMT$^+$ mice. Further, mitochondria with swollen cristae in the endothelial cells indicated oxidative stress. In the cardiomyocytes we found focally broken cell membranes and widened and distorted desmosomal gaps of the intercalated discs that connect cardiomyocytes.

To investigate if cardiac remodelling was ongoing in PyMT$^+$ mice, we immunostained for Ki67. Significantly more cells were proliferating in the tumor-bearing mice and the majority of these cells were CD31+ endothelial cells. NET removal by DNase I treatment reduced the number of proliferating cells to numbers comparable to healthy individuals. This is an interesting finding since other studies have shown that NETs formed inside the vessels cause damage to the vessel-lining endothelial cells.

Inflammation and cardiac remodelling suggest that the heart could be damaged. We therefore investigated cardiac biomarkers for inflammation and strain in the heart and blood. BNP a marker for cardiac stress, increased pressure, heart failure and myocardial strain was increased in the tumor-bearing PyMT mice compared to the healthy individuals and could be significantly reduced by DNase I treatment. GDF-15, a marker for inflammation in the heart was upregulated in the heart tissue and elevated in the serum of PyMT$^+$ mice compared to healthy individuals. DNase I treatment reduced the GDF-15 RNA expression in the heart tissue however, it did not reduce the GDF-15 levels in the serum. It is possible that the short-term DNase I treatment is sufficient to affect mRNA levels, but that a more long-term DNase I treatment would be needed to also affect protein levels. In paper I we have treated our mice with bovine DNase I, which is the only DNase that is commercially available in sufficient amounts to treat the mice. The use of a foreign protein however,

induces an immune response and degrades the foreign protein. To avoid this, we only treated our mice with daily injections for three days. In paper II we made use of an AAV vector expressing species-specific DNase I, which made the treatment over a longer time possible. RNA expression of both troponin I and T was increased in the $PyMT^+$ mice. DNase I treatment reduced the troponin expression to levels comparable to healthy individuals.

To investigate the cardiac function, echocardiography was performed. In $PyMT^+$ mice cardiac output was increased due to the higher stroke volume. This came along with increased mitral valve flow and a shortened isovolumic relaxation time and posterior wall hypertrophy, which indicates an increased work load compared to the heart of healthy littermates. DNase I treatment could not change these parameters. It would be interesting to see if a more long-term DNase I treatment would have an effect on the measured functional parameters. The increased cardiac output can be caused by the increased metabolic activity caused by the presence of the tumor, however it can also be a sign of reduced peripheral oxygenation, which would agree with our previous findings of disturbed peripheral organ perfusion.

To investigate if our findings in the mouse model are also true for humans, we measured biomarkers for cardiac strain in treatment-naïve cancer patients with various malignancies and correlated the biomarkers to the NET levels. NET levels, measured with an ELISA detecting H3Cit-DNA and MPO-DNA complexes, were elevated in the cancer patients. Also, NE, a component of NETs was increased. Since the $PyMT^+$ mice showed increased inflammation, we measured the inflammatory status in the cancer patients. C-reactive protein (CRP) was increased in the cancer patients and correlated well with both NET ELISAs and the NE level, suggesting that the NET levels are associated with inflammation also in cancer patients. GDF-15, sTNFR1, sTNFR2 and NT-proBNP, markers for cardiac inflammation, strain and damage were elevated in the cancer patients compared to the healthy controls. There was a correlation between H3Cit-DNA and NT-proBNP and H3Cit-DNA and sTNFR1, which indicates a connection of NET formation with the cardiac strain in the cancer patients. It is interesting that despite the very heterogenous patient group with more than 15 different cancer types significant differences in both NET formation and cardiac strain related biomarkers could be found. This suggests that both NET formation and organ damage occur in a broad variety of malignancies.

The efficacy of DNase I as a treatment for cancer-related pathology has been demonstrated in several pre-clinical studies. Also, the use of DNase I in human patients has been investigated. DNase I is in frequent use as an aerosol spray in cystic fibrosis. Systemic administration of DNase I has been done in a small group of systemic lupus erythematosus patients with no obvious side-effects.

We conclude that NETs contribute to inflammation and myocardial strain in malignant disease and identify NETs as potential therapeutic targets in cancer patients to prevent cardiac inflammation and dysfunction.

Paper II

AAV-mouse DNase I sustains long-term DNase I expression in vivo and suppresses breast cancer metastasis

In paper II we measured the levels of NETs using the H3R8cit ELISA in the plasma of treatment-naïve breast cancer patients with and without metastases and a control group consisting of previous breast cancer patients considered to be cured at 5-year follow up. There was no difference in NET levels between the control group and the patients with local breast cancer, however, the level of NETs was significantly higher in breast cancer patients with metastatic disease. This suggests a role of NETs in the progression of breast cancer at a more advanced stage of the disease.

In our previous studies we have used bovine DNase I to remove NETs from the circulation. The use of a foreign protein in immunocompetent mice however most likely induces an immune reaction that would remove the protein from the circulation making the long-term treatment impossible. To confirm this hypothesis, we injected mice with bovine DNase I daily for two weeks and collected serum one week after initiation of the injections and at the end of the study. Using an ELISA detecting anti-bovine DNase I antibodies we found that two out of three mice developed antibodies already after one week. The third mouse developed a lower antibody response after one week, however the response doubled in the second week. This finding shows that for the long-term DNase I administration the foreign bovine DNase I is not suitable.

Since long-term DNase I treatment requires a species-specific DNase I protein and murine DNase I is not commercially available in sufficient amounts, we decided to express murine DNase I with an AAV vector. We chose the AAV-2 serotype with tropism to the liver, heart and muscle. Due to the liver-specific promoter, mDNase I expression was however only induced in the liver. To study the effect of AAV-mediated expression of DNase I on the systemic effects of breast cancer, we made use of the MMTV-PyMT mouse model ($PyMT^+$) for mammary carcinoma. These mice develop palpable mammary tumors with 8-10 weeks of age and form spontaneous metastases in the lungs. The mice were injected with an AAV vector expressing murine DNase I (AAV-mDNase I) or a control vector missing the transgene (AAV-null) when the mice turned 6 weeks. Before vector injection, serum samples and urine samples were collected. After vector injection, mice were serum sampled every other week until they reached approximately 14 weeks of age. At the termination of the study organs were harvested and urine and plasma samples were collected.

To examine if the AAV-mDNase I vector could induce the expression of DNase I in the liver we injected two different doses of AAV-mDNase I, AAV-null or PBS into healthy FVB mice. Liver DNase I expression was clearly increased after injection of the higher dose (10^11 GC/mouse) of AAV-mDNase I. Also, serum DNase activity measured eight weeks after vector injection, at the termination of the study, revealed an increase in mice treated with the high vector concentration. The increase in serum DNase activity could already be detected two weeks after vector injection and stayed stably high until study termination eight weeks after vector injection. To investigate how long DNase activity would be measurable in the circulation, we injected healthy mice with the AAV-mDNase I vector and collected serum samples up to 34 weeks after vector injection. DNase I activity was increased at least until 25 weeks after the injection. Two of the four injected mice died toward the end of the study but the DNase activity of the other two mice stayed high. If the death of the two mice has a connection to vector injection is currently not clear. Spontaneous deaths however can happen on a low frequency in the colony.

We further examined if the injection of the AAV vector would cause liver toxicity. For this we measured alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in the serum of injected, healthy FVB mice. We could not find changes in the levels of these enzymes in the first eight weeks after vector injection. To investigate if long-term AAV-mDNase I injection could induce changes in liver enzymes we injected healthy FVB mice, and collected serum samples 20, 25, 30 and 45 weeks after vector injection. We could not detect clear changes in the liver enzymes. Only the AST levels at 20 weeks post injection were increased, which was normalized in the following measurements. Interestingly, we found that $PyMT^+$ mice that were not injected with the AAV vectors showed significant changes in AST and ALP liver enzymes suggesting that the cancer itself induces a certain level of liver toxicity. Also yet another example of systemic effects of cancer. It will be interesting to confirm this finding in future analyses of liver enzymes both in tumor-bearing mice and cancer patients.

To investigate if the treatment with the AAV-mDNase I vector could alleviate breast cancer-induced pathology, we injected $PyMT^+$ mice. As in the healthy FVB mice, injection of the high dose AAV-mDNase I led to an increased serum DNase activity and to increased expression of mDNase I in the liver. The amount of nucleosomes was measured and revealed a trend to decreased levels in the AAV-mDNase I treated group, however the difference was not significant and would probably need a higher number of individuals.

We have previously shown that kidney function is impaired in $PyMT^+$ mice compared to their healthy littermates. To examine if the use of the AAV-mDNase I vector had an effect on organ function, we measured the NGAL level, a marker for reduced vascular perfusion and hypoxia in the kidney. NGAL in the urine was significantly higher in $PyMT^+$ mice that had received

the AAV-null vector compared to mice that received the DNase I expressing vector. This suggests that DNase I treatment alleviated cancer-induced renal pathology.

To investigate if long-term DNase I expression attenuated spontaneous lung metastasis, we collected lungs and primary tumors eight weeks after vector injection. There was no difference in primary tumor weight comparing AAV-null and AAV-mDNase I injected mice. Three of the AAV-null injected mice had to be terminated two weeks before the experimental endpoint due to the development of a big primary tumor. Still, the proportion of mice that had developed metastases in the lungs was significantly higher in the AAV-null group.

We conclude that long-term, species-specific expression of DNase I is possible and does not lead to liver pathology in form of elevated liver enzymes. Moreover, elevated mDNase I expression improved kidney function and reduced the proportion of mice with breast cancer lung metastasis. AAV-mediated expression of mDNase I can be seen as a promising approach to suppress systemic effects of breast cancer.

Paper III

Global gene expression analysis of distant organ vasculature in mice with cancer

In previous studies we observed that RIP1-Tag2 (RT2) mice, which develop pancreatic neuroendocrine tumors in the insulin-producing beta cells and form spontaneous metastasis in primarily the liver but also the lung showed an impaired vascular function in the heart and the kidney, organs distant from tumor growth. To investigate how the tumor affects vascular function in more detail we made use of the VEcadTRAP mouse model. VEcadTRAP mice express an eGFP-tagged ribosomal protein under control of the endothelial specific promoter VE-cadherin. This eGFP tag enables us to isolate endothelial ribosomes using magnetic beads coated with anti-GFP antibodies. The endothelial ribosomes also carry the RNA that is about to be translated into protein, the endothelial translatome. In order to investigate the endothelial gene expression in our tumor mouse model we crossed the VEcadTRAP mice with RT2 mice. We generated tumor-bearing RT^+ mice and healthy littermates (RT^-) that were used as healthy controls. To investigate if the mice indeed expressed eGFP-tagged ribosomes, we stained for eGFP and co-stained for the vascular marker CD31. We confirmed the presence of eGFP in the endothelial cells.

To get an overall picture about the endothelial gene expression in various organs we extracted RNA from kidney and heart, two organs that are not sites of tumor growth, and from liver and lung, which are sites of metastatic growth. To investigate if primary tumor size plays a role in endothelial gene expression, we harvested organs at two different time points (13 weeks and 14.5

weeks), which generated two groups of mice; one with mice with a tumor volume < 100 mm^3 (RT^+) and one with mice with a tumor volume > 100 mm^3 (RT^{++}). In a previous study we found that tumor-bearing RT2 mice had impaired vascular function with decreased perfusion when their primary tumor was bigger than 100 mm^3 [18]. Further, we wanted to investigate a potential role of NETs in the tumor-induced effects on the vasculature. We therefore also added groups of mice RT^+, RT^{++} and RT^- that were DNase I treated. The extracted RNA was sequenced with the AmpliSeq technique.

We performed a principal component analysis (PCA) including all six experimental groups and found that samples belonging to the same organ rather than samples belonging to one experimental group clustered together. This is not surprising as endothelial cells in different organs perform different functions. In the PCA result, lung and heart showed a more similar gene expression while both kidney and especially liver seemed to be less related to the other organs.

We further investigated the number of differentially expressed genes (DEG) comparing RT^+ to RT^- mice and RT^{++} to RT^- mice and found that the number pf DEG was manyfold higher in liver and lung than in kidney and heart. A possible explanation for this finding is that liver and lung are sites of metastatic tumor growth while kidney and heart are not. Interestingly, the number of DEG increased in mice with tumors > 100 mm^3 compared to mice with tumors < 100 mm^3 in all four organs, which suggests that the cancer induces these systemic effects also in organs that are not sites of tumor growth.

We performed gene set enrichment analysis (GSEA) to identify over-or underrepresentation of proteins involved in certain molecular pathways. In general, pathways associated with metabolism and the complement and coagulation cascade were positively and negatively enriched. More in detail, the amino acid metabolism was enriched in liver and lung of RT^+ mice and in liver, kidney and heart of RT^{++} mice compared to the RT^- mice. In response to DNase I treatment it was negatively enriched in the lung of RT^+ mice and in the liver, lung and heart of RT^{++} mice. This suggests that NETs might play a role in the regulation of this pathway in a tumor situation.

Another pathway that was frequently enriched was the complement and coagulation pathway as well as the blood clotting cascade. Positive and negative enrichments of these pathways were however not consistent between organs and treatment groups: In the lung we found a positive enrichment in RT^+ mice compared to RT^- mice. Interestingly this finding was reversed after DNase I treatment. In the kidney of RT^{++} mice the pathway was enriched however this was not affected by DNase I treatment. In contrast, a negative enrichment was found in the heart vasculature of RT^{++} mice compared to DNase I-treated RT^{++} mice. In liver and heart of RT^+ compared to RT^- mice we found a negative enrichment. Moreover, there was a positive enrichment in DNase I-treated kidneys from RT^+ mice and in the liver vasculature of RT^{++} mice. It is interesting to see that all organs seem to be affected by the complement and

coagulation cascade, considering that NETs have been shown to be pro-thrombotic in different ways, e.g. by the activation of platelets or by activating the intrinsic coagulation cascade with their DNA backbone [104]. In addition, NETs are cytotoxic towards the vessel-lining endothelial cells. Endothelial damage can also induce the upregulation of pro-coagulant factors. The reason why different organs seem to behave differently is not known, but it is well established that ECs display large heterogeneity between organs due to their specialized functions. Additional analyses investigating the specific genes responsible for the positive and negative enrichment of the pathway is needed to understand the experienced discrepancies. One possibility is that e.g. the lung shows a positive enrichment in the coagulation pathway in tumor-bearing mice contributing to the pro-coagulant state, while the kidney tries to compensate and downregulate the coagulation pathway.

As previously shown, the number of DEG in organs that are sites of metastatic growth i.e. liver and lung comparing tumor-bearing mice with healthy littermates, was much higher than in the kidney and the heart. An increased primary tumor size increased the number of DEG in all organs. Interestingly, DNase I treatment of mice with tumors > 100 mm^3 resulted in a large number of DEG, while DNase I treatment of mice with tumors < 100 mm^3 did not. These data match our previous findings where we showed that the NET-impaired vessel function was only present in mice with tumors > 100 mm^3 [18].

In the liver 42 genes were upregulated and 123 were downregulated comparing RT^+ with RT^- mice. In RT^{++} compared to RT^- mice we found 421 genes upregulated and 589 to be downregulated. IL-1β is one of the genes that is significantly upregulated in RT^+ mice. This is consistent with our previous findings that IL-1β is upregulated in the kidney from mice with breast cancer (MMTV-PyMT). An additional gene that was upregulated in the endothelial cells of both RT^+ and RT^{++} mice is Nr4a1. Nr4a1 was found to be involved in endothelial cell survival and proliferation and acts downstream of VEGF-A, where it mediates proliferation and survival of endothelial cells. Snord15a, a non-coding RNA predicted to guide the methylation of 28S ribosomal RNA, is a third gene that was found to be significantly upregulated in the endothelial cells of RT^{++} mice.

In the lung one gene was upregulated and 41 genes were downregulated comparing RT^+ with RT^- mice. In RT^{++} compared to RT^- mice we found 662 genes upregulated and 654 genes downregulated. Tmem27, encoding collectrin, a homologue of angiotensin converting enzyme 2, was downregulated. Collectrin has been suggested to act as a chaperon for L-arginine transporters. There are publications that show that collectrin knockout in mice decreases endothelial L-arginine and leads to the uncoupling of endothelial nitric oxide synthase, production of superoxides, reduced nitric oxide and endothelial dysfunction and hypertension. Also in the lung, the non-coding RNA, Snord15a, was upregulated in RT^{++} mice.

In the kidney no genes were differentially expressed comparing RT^+ with RT^- mice. In RT^{++} compared to RT^- mice we found 17 upregulated genes and no downregulated genes. As in the liver and the lung, Snord15a was upregulated in RT^{++} mice and DNase I treatment led to a downregulation. Interestingly we found that nine out of the 17 upregulated genes are usually expressed by the liver. This phenomenon has been detected earlier in kidney tissue as a response to kidney injury and was termed "renal hepatization" [106-108]. To confirm that the upregulation of liver specific genes can also be seen on protein level, we immunostained RT^{++} kidneys for hemopexin and co-stained for the vascular marker CD31. We found a significantly increased expression of hemopexin in the vasculature of tumor-bearing mice. To investigate if this was also the case in another tumor model, we stained the kidneys of MMTV-PyMT mice and also found a significant upregulation of hemopexin in the kidney vasculature in this model. Hemopexin is a heme binding protein. Its expression has been shown to be triggered by inflammation [107]. These findings suggest that renal hepatization also happens as a response to cancer.

In the heart two genes were upregulated and three genes were downregulated comparing RT^+ with RT^- mice. Also in RT^{++} compared to RT^- mice we found a small number of DEG, 11 genes upregulated and four genes downregulated. As in the other organs we found Snord15a to be upregulated in heart endothelial cells of RT^{++} mice. DNase I treatment significantly lowered the Snord15a expression. As in the liver we found Nr4a1 to be upregulated in RT^+ mice. Nr4a1 has a vascular protective role and its knockdown has been associated with worsened heart dysfunction in myocardial infarction [109]. Foxf1 was one of the genes upregulated in the cardiac vasculature of RT^{++} mice. DNase I treatment reversed the expression.

In addition to the organ-specific findings described before, we also made some more general observations affecting all organs. Specifically affected by the DNase I treatment we found several genes differentially expressed that belong to the peripheral circadian clock. Disruption of the peripheral circadian clock has been associated with chronic inflammation and cardiovascular events like myocardial infarction, stroke and thrombosis.

In conclusion, this study gives a global overview of the gene expression changes that take place in the vasculature of distant organs in individuals with cancer and the potential role of NETs. More focused studies are required to determine the mechanisms behind our findings.

Popular science summary

The first thought that most people get when they hear the word cancer is the growth of a tumor in the body. This primary tumor marks the initiation of the cancer and usually gives the cancer its 'name', e.g. breast cancer, lung cancer, pancreatic cancer,…What is not so well-known is the fact that cancer is not limited to the primary tumor, but that cancer is a systemic disease meaning that it affects many parts of the body, even parts and organs that are not sites of tumor growth. The cancer is doing this by sending tumor cells and by releasing molecules from the primary tumor that travel to distant sites in the body via the blood stream. The relevance of these systemic effects becomes especially clear when looking at the causes of the majority of cancer-related deaths, which are all systemic effects of the cancer: The primary cause of cancer-related death is metastasis formation, the spread of cancer cells to distant sites and the formation of additional tumors there. The second cause of death is thrombosis formation, pathologic blood coagulation and formation of blood clots that can occlude small blood vessels causing e.g. lung embolisms, stroke or myocardial infarction. And the third cause of cancer related death, organ dysfunction, not necessarily in the organ where the primary tumor grows, but in any organ of the body. For example, there are studies that show that approximately 50% of cancer patients have a decreased kidney function already at the time of cancer diagnosis. This is a problem since most chemotherapies are secreted via the kidney and are by themselves harmful for the kidney. Starting chemotherapy with already impaired kidney function means that chemotherapy dose has to be reduced to avoid harming the kidney further, but it also means that the patient will be treated with a non-optimal dose for the cancer. The kidney is only one example, also heart function can be affected by the cancer and we have studied this in paper I of this PhD thesis.

The mechanisms behind the systemic effects of cancer are various. It is well known that tumors secrete factors into the blood stream and also affect the body's own cells e.g. immune cells that instead of working for the health and well-being of the host start to work in favour of the developing cancer. However, the exact mechanisms are up to now relatively poorly understood.

In my PhD work I have investigated the role of neutrophil extracellular traps (NETs) in tumor-induced organ dysfunction, metastasis formation and thrombosis. NETs originate from neutrophils, one of the immune cells of our body. When neutrophils receive stimuli e.g. during a bacterial infection, they

can release their DNA together with other molecules that are usually safely stored inside the cell into the blood stream. The NETs then form a web similar to a spider web that can trap the bacteria in the blood stream. The molecules released from the neutrophil are antibacterial and also present in this web. These molecules can then kill the trapped bacteria. NETs do however not only form during bacterial infections but they can also form during cancer through molecules secreted by the tumor. While NETs can be beneficial for the patient during severe bacterial infections they also come with a cost, especially in cancer patients. In several studies it was shown that NETs contribute to metastasis formation, thrombosis and also organ dysfunction in individuals with cancer. In my PhD thesis I have investigated the systemic effects of cancer with a special focus on NETs.

In paper I I have studied the effect of tumor-induced NET formation on cardiac function. In a mouse model that develops breast cancer we found that mice with cancer (PyMT+) had increased levels of NETs compared to the healthy mice. In addition, the level of proteins i.e. molecules produced by the tissue, that are related to organ inflammation were higher than in healthy mice. We also found increased numbers of immune cells in the hearts of PyMT+ mice indicating an inflammation in the heart. In addition to the inflammation, we wanted to explore if the heart function is already negatively affected. We found that proteins related to cardiac strain were higher in the PyMT+ mice than in healthy individuals. To find out if NETs are involved in these inflammatory processes, we treated the mice with DNase I, a protein that cleaves DNA and thereby destroys the NETs. Several of the proteins that were increased in the PyMT+ mice were lower after DNase I treatment, which indicates that NETs are partly responsible for cardiac inflammation and cardiac strain. We further wanted to explore if the situation we found in mice could be similar for human cancer patients. We therefore collected blood from cancer patients with different types of cancer and again determined the levels of proteins related to inflammation and heart function. Also, in the human patients we found that several of the proteins were higher than in healthy individuals. Interestingly, for some of the proteins we found a positive correlation to the levels of NETs, meaning that high protein levels come along with high NET levels and low protein levels come along with low NET levels. This indicates that NETs contribute to cardiac disease also in human cancer patients. NETs should therefore be studied further since their removal might help to prevent cardiac complications in cancer patients.

In paper II we explored if the destruction of NETs would lead to the formation of less metastases. In this study we again made use of the PyMT mouse breast cancer model. To destroy the NETs, we again wanted to use the protein DNase I. However, the only DNase I that is commercially available is extracted from cows. Since we treat mice, this presents us with a problem. The immune system of mice will recognize the foreign cow protein and will destroy it. The mechanisms are similar when we get infected by a virus or

bacteria. Our immune system recognizes foreign parts and removes them from our body to protect us. In our previous studies the use of the cow DNase I was possible since we treated the mice for only three days, which is short enough to avoid that the immune system completely destroys the foreign protein. However, in this study the action of DNase I was needed for a longer time since we investigated the formation of metastases, which is a process that takes much longer than only three days. We solved this problem by using a virus vector, more specifically an adeno-associated virus (AAV) vector, that we equipped with the DNA of the murine DNase I protein. Injection of the AAV vector into the mice will lead to the production of the murine DNase I protein, that will not be recognized by the immune system. After injection of the AAV vector into the mice we could measure the presence and activity of DNase I in the blood for at least 25 weeks. We did not find adverse effects of virus injection in the liver, which is the place where the DNase I is produced. Further, we found that NGAL, a protein that indicates kidney impairment was lower than in PyMT mice that were not treated with the DNase I-producing AAV vector. Most interestingly, we found that fewer of the mice that received the DNase I-producing AAV vector developed metastases compared to mice that did not receive DNase I. This study indicates that the production of a species-specific DNase I protein with the help of an AAV vector is possible and that NET removal reduces metastasis formation in the breast cancer developing mice.

In paper III we studied how cancer and NETs produced by the cancer affect the endothelial cells, which are the cells that line the inside of our blood vessels. This is interesting to know since the endothelial cells are the cells that come in contact with tumor cells that travel through our body and that can form metastases if they pass through the endothelial cells. In addition, endothelial cells are constantly in contact with blood and the immune cells that travel through our body. Activated endothelial cells allow immune cells to pass into the tissue of organs, which is an indication of organ inflammation. In this study we used the RIP1-Tag2 mouse model, which develops tumors in the insulin producing cells in the pancreas. We found that the organs in which metastases grow, liver and lung, show more changes in the endothelial cells than organs that are not sites of metastatic growth. Further, we found that mice with bigger tumors had more changes in the endothelial cells. This shows us that it is the tumor cells and molecules released from the tumor that affect the endothelial cells. Interestingly, we found proteins that in healthy mice are mostly present in the liver, now also present in the kidney of the mice with cancer. This is a phenomenon termed ‘renal hepatization’ and indicates diminished kidney function. In conclusion, this study gives a global overview on the endothelial cells in individuals with cancer. More focused studies are required to determine the mechanisms behind our findings.

Populärwissenschaftliche Zusammenfassung

Der erste Gedanke, den die meisten Menschen haben, wenn sie das Wort Krebs hören, ist das Wachstum eines Tumors im Körper. Dieser Primärtumor markiert den Beginn der Krebserkrankung und gibt dem Krebs in der Regel seinen "Namen", z. B. Brustkrebs, Lungenkrebs, Bauchspeicheldrüsenkrebs... Weniger bekannt ist die Tatsache, dass Krebs nicht auf den Primärtumor beschränkt ist, sondern dass Krebs eine systemische Erkrankung ist, was bedeutet, dass er viele Bereiche des Körpers betrifft, auch Bereiche und Organe, die keine Orte des Tumorwachstums sind. Der Krebs tut dies, indem er Tumorzellen aussendet und Moleküle aus dem Primärtumor freisetzt, die über den Blutkreislauf an entfernte Stellen im Körper gelangen. Die Relevanz dieser systemischen Auswirkungen wird besonders deutlich, wenn man sich die Ursachen der meisten krebsbedingten Todesfälle ansieht, die allesamt auf systemische Effekte des Krebses zurückzuführen sind: Die Hauptursache für krebsbedingte Todesfälle ist die Bildung von Metastasen, also die Absiedelung des Primärtumors in andere Körperregionen und die Bildung weiterer Tumore dort. Die zweite Todesursache ist die Thrombosebildung, die pathologische Blutgerinnung und die Bildung von Blutgerinnseln, die kleine Blutgefäße verschließen können und z. B. Lungenembolien, Schlaganfälle oder Herzinfarkte verursachen. Und die dritte Ursache für krebsbedingte Todesfälle sind Funktionsstörungen von Organen, nicht unbedingt in dem Organ, in dem der Primärtumor wächst, sondern in jedem Organ des Körpers. Es gibt zum Beispiel Studien, die zeigen, dass etwa 50 % der Krebspatienten bereits zum Zeitpunkt der Krebsdiagnose eine verminderte Nierenfunktion haben. Dies ist problematisch, da die meisten Chemotherapien über die Nieren ausgeschieden werden und selbst schädlich für die Nieren sind. Wenn eine Chemotherapie bei bereits eingeschränkter Nierenfunktion begonnen wird, bedeutet dies, dass die Chemotherapie Dosis reduziert werden muss, um eine weitere Schädigung der Niere zu vermeiden, aber auch, dass der Patient mit einer für die Krebserkrankung nicht optimalen Dosis behandelt wird. Die Niere ist nur ein Beispiel, auch die Herzfunktion kann durch den Krebs beeinträchtigt werden, was wir in "paper I“ dieser Dissertation untersucht haben.

Die Mechanismen hinter den systemischen Auswirkungen von Krebs sind vielfältig. Es ist bekannt, dass Tumore Faktoren in den Blutkreislauf absondern und auch körpereigene Zellen beeinflussen, z. B. Immunzellen, die statt für die Gesundheit und das Wohlergehen des Wirts zu arbeiten, beginnen,

zugunsten des sich entwickelnden Krebses zu arbeiten. Die genauen Mechanismen sind jedoch bisher relativ schlecht verstanden.

In meiner Doktorarbeit habe ich die Rolle von "neutrophil extracellular traps“ (NETs) bei tumorbedingten Organdysfunktionen, Metastasenbildung und Thrombose untersucht. NETs stammen von Neutrophilen, einer der Immunzellen unseres Körpers. Wenn Neutrophile einen Reiz erhalten, z. B. bei einer bakteriellen Infektion, können sie ihre DNA zusammen mit anderen Molekülen, die normalerweise sicher in der Zelle gespeichert sind, in den Blutstrom abgeben. Die von den Neutrophilen freigesetzten Moleküle sind antibakteriell und ebenfalls in diesem Netz vorhanden. Diese Moleküle können dann die eingeschlossenen Bakterien abtöten. NETs bilden sich jedoch nicht nur bei bakteriellen Infektionen, sondern auch bei Krebs durch vom Tumor selbst gebildete Moleküle. Während NETs bei schweren bakteriellen Infektionen für den Patienten von Vorteil sein können, haben sie auch ihren Preis, insbesondere bei Krebspatienten. In mehreren Studien wurde gezeigt, dass NETs bei Krebspatienten zur Metastasenbildung, Thrombose und auch zu Organdysfunktionen beitragen. In meiner Doktorarbeit habe ich die systemischen Auswirkungen von Krebs mit besonderem Fokus auf NETs untersucht.

In "paper I“ habe ich die Auswirkungen der tumorinduzierten NET-Bildung auf die Herzfunktion untersucht. In einem Mausmodell, das Brustkrebs entwickelt, fanden wir heraus, dass Mäuse mit Krebs (PyMT+) im Vergleich zu gesunden Mäusen erhöhte Mengen an NETs aufwiesen. Darüber hinaus war die Menge der vom Gewebe produzierten Proteine bzw. der Moleküle, die mit Organentzündungen in Zusammenhang stehen, höher als bei gesunden Mäusen. Wir fanden auch eine erhöhte Anzahl von Immunzellen in den Herzen von PyMT+-Mäusen, was auf eine Entzündung im Herzen hindeutet. Zusätzlich zur Entzündung wollten wir untersuchen, ob die Herzfunktion bereits negativ beeinträchtigt ist. Wir fanden heraus, dass die Proteine, die charakteristisch für eine Belastung des Herzens sind, bei den PyMT+-Mäusen höher waren als bei gesunden Individuen. Um herauszufinden, ob NETs an diesen Entzündungsprozessen beteiligt sind, behandelten wir die Mäuse mit DNase I, einem Protein, das die DNA spaltet und dadurch die NETs zerstört. Mehrere der Proteine, die in den PyMT+-Mäusen erhöht waren, waren nach der DNase I-Behandlung niedriger, was darauf hindeutet, dass die NETs teilweise für die Herzentzündung und die Belastung des Herzens verantwortlich sind. Wir wollten außerdem untersuchen, ob die Situation, die wir bei Mäusen gefunden haben, bei menschlichen Krebspatienten ähnlich sein könnte. Wir entnahmen daher Blut von Krebspatienten mit verschiedenen Krebsarten und bestimmten erneut die Werte von Proteinen, die mit Entzündungen und der Herzfunktion zusammenhängen. Auch bei den menschlichen Patienten stellten wir fest, dass mehrere der Proteine höher waren als bei gesunden Personen. Interessanterweise fanden wir für einige der Proteine eine positive Korrelation mit den Werten der NETs, was bedeutet, dass hohe Proteinwerte mit hohen NET-Werten und niedrige Proteinwerte mit niedrigen NET-Werten einhergehen. Dies

deutet darauf hin, dass NETs auch bei menschlichen Krebspatienten zur Herzerkrankung beitragen. NETs sollten daher weiter untersucht werden, da ihre Entfernung dazu beitragen könnte, kardiale Komplikationen bei Krebspatienten zu verhindern.

In "paper II“ haben wir untersucht, ob die Zerstörung von NETs zur Bildung von weniger Metastasen führen würde. In dieser Studie verwendeten wir erneut das PyMT-Maus-Brustkrebsmodell. Zur Zerstörung der NETs wollten wir wieder das Protein DNase I verwenden. Die einzige DNase I, die im Handel erhältlich ist, wird jedoch aus Kühen extrahiert. Da wir Mäuse behandeln, stellt uns dies vor ein Problem. Das Immunsystem der Mäuse erkennt das fremde Kuheiweiß und vernichtet es. Die Mechanismen sind ähnlich, wenn wir uns mit einem Virus oder einer Bakterie infizieren. Unser Immunsystem erkennt die Fremdkörper und entfernt sie aus unserem Körper, um uns zu schützen. In unseren früheren Studien war die Verwendung von Kuh-DNase I möglich, da wir die Mäuse nur drei Tage lang behandelten, was kurz genug ist, um zu verhindern, dass das Immunsystem das fremde Protein vollständig zerstört. In dieser Studie war die Wirkung von DNase I jedoch über einen längeren Zeitraum erforderlich, da wir die Bildung von Metastasen untersuchten, ein Prozess, der viel länger als drei Tage dauert. Wir lösten dieses Problem durch die Verwendung eines Virusvektors, genauer gesagt eines AAV (Adeno-assoziiertes Virus) Vektors, den wir mit der DNA des murinen DNase I-Proteins ausgestattet haben. Die Injektion des AAV-Vektors in die Mäuse führt zur Produktion des murinen DNase I-Proteins, das vom Immunsystem nicht erkannt wird. Nach der Injektion des AAV-Vektors in die Mäuse konnten wir das Vorhandensein und die Aktivität von DNase I im Blut für mindestens 25 Wochen messen. Wir konnten keine nachteiligen Auswirkungen der Virusinjektion in der Leber feststellen, dem Ort, an dem die DNase I produziert wird. Außerdem stellten wir fest, dass NGAL, ein Protein, das auf Nierenschäden hinweist, niedriger war als bei PyMT-Mäusen, die nicht mit dem DNase I produzierenden AAV-Vektor behandelt wurden. Interessanterweise entwickelten weniger Mäuse, die den DNase I produzierenden AAV-Vektor erhielten, Metastasen als Mäuse, die keine DNase I erhielten. Diese Studie zeigt, dass die Herstellung eines speziesspezifischen DNase I-Proteins mit Hilfe eines AAV-Vektors möglich ist und dass die Entfernung von NETs die Metastasenbildung in Mäusen, die Brustkrebs entwickeln, reduziert.

In "paper III“ untersuchten wir, wie sich Krebs und die vom Krebs ausgelöste NETs Bildung auf die Endothelzellen auswirken, also auf die Zellen, die das Innere unserer Blutgefäße auskleiden. Dies ist interessant zu wissen, da die Endothelzellen die Zellen sind, die mit den Tumorzellen in Kontakt kommen, die durch unseren Körper wandern und Metastasen bilden können, wenn sie die Endothelzellen durchdringen. Außerdem stehen Endothelzellen ständig in Kontakt mit dem Blut und den Immunzellen, die durch unseren Körper wandern. Aktivierte Endothelzellen lassen Immunzellen in das Gewebe von Organen eindringen, was ein Hinweis auf eine Organentzündung ist. In dieser

Studie verwendeten wir das RIP1-Tag2-Mausmodell, bei dem sich in den Insulin produzierenden Zellen der Bauchspeicheldrüse Tumore entwickeln. Wir fanden heraus, dass die Organe, in denen Metastasen gebildete werden, Leber und Lunge, mehr Veränderungen in den Endothelzellen aufweisen als Organe, in denen keine Metastasen wachsen. Außerdem stellten wir fest, dass Mäuse mit größeren Tumoren mehr Veränderungen in den Endothelzellen aufwiesen. Dies zeigt uns, dass es die Tumorzellen und die vom Tumor freigesetzten Moleküle sind, die die Endothelzellen beeinflussen. Interessanterweise fanden wir Proteine, die bei gesunden Mäusen hauptsächlich in der Leber vorkommen, nun auch in der Niere der Mäuse mit Krebs. Dieses Phänomen wird als "Nierenhepatisierung" bezeichnet und deutet auf eine eingeschränkte Nierenfunktion hin. Zusammenfassend lässt sich sagen, dass diese Studie einen globalen Überblick über die Endothelzellen bei Menschen mit Krebs gibt. Gezieltere Studien sind erforderlich, um die Mechanismen hinter unseren Ergebnissen zu ermitteln.

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