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Characterization of high density and low density neutrophils isolated from patients with newly diagnosed lung cancer compared to healthy individuals.

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Abstract

Neutrophil granulocytes are the first responders to invading pathogens, counteracting them through various cytotoxic mechanisms. It has been shown that neutrophils are not a homogenous population but are comprised of heterogenous subpopulations that can be precisely differentiated, and which also undergo changes in context of cancer. Data providing detailed insights into these changes however are still scarce. Neutrophils can also release their own DNA as so-called neutrophil extracellular traps (NETs), characterized by the presence of citrullinated histone H3 (H3Cit).

The aim of this study was to investigate cancer-associated changes of high density neutrophils (HDNs) and low density neutrophils (LDNs) isolated from patients with newly diagnosed lung cancer regarding their cell size and granularity, potential of activation, subpopulation distribution, DNA release and H3Cit positivity, compared to cells from sex- and age-matched healthy individuals.

HND and LDN subpopulations were separated using density gradient-based centrifugation and stimulated with ionomycin (IO) and phorbol 12-myristate 13-acetate (PMA). Results were obtained via photometric and flow cytometric analyses.

It was shown that LDNs from patients with lung cancer are significantly smaller in size measured by their forward scatter (FSC). Also, both HDNs and LDNs from patients displayed lower granularity according to their side scatter (SSC). A decrease in SSC upon IO and PMA stimulation could also be observed.

Furthermore, both, patient derived HDNs and LDNs showed significantly increased CD11b expression upon IO and PMA stimulation, which was measured as a marker for cellular activation.

An increase in activated ($CD62^{low}$/$CD16^{high}$) neutrophils was observed within LDNs from patients with lung cancer.

DNA release was found to be significantly reduced in cells isolated from patients under various stimulation conditions. Interestingly, HDNs from patients showed a significant increase in H3Cit positivity only upon IO stimulation, while patient LDNs displayed a significant H3Cit positivity reduction only at PMA stimulation.

In summary, in this study we demonstrated that patient derived HDNs and LDNs showed significant differences in cell size and granularity, subpopulation distribution as well as in expression levels of activation marker CD11b *in vitro*, leading to the conclusion that neutrophils derived from patients with lung cancer might be activated more easily. However, the true nature of DNA release and NET formation remains to be further investigated to gain clearer insights into the true characteristics of neutrophil subpopulations in patients with cancer.

Kurzfassung

Neutrophile Granulozyten reagieren unmittelbar auf eindringende Krankheitserreger und bekämpfen diese durch verschiedenste zytotoxische Mechanismen. Es wurde gezeigt, dass Neutrophile keine homogene Population sind, sondern aus präzise voneinander differenzierbaren heterogenen Subpopulationen bestehen, welche sich auch im Zusammenhang mit Krebserkrankungen verändern. Daten über diese Veränderungen sind jedoch rar. Ebenso können Neutrophile ihre DNA als sogenannte Neutrophil Extracellular Traps (NETs) freisetzen, welche durch das Vorhandensein von citrulliniertem Histon H3 (H3Cit) charakterisiert sind.

Der Fokus dieser Studie lag auf der Erforschung krebsassoziierter Veränderungen von High Density Neutrophils (HDNs) sowie Low Density Neutrophils (LDNs) isoliert aus Patienten mit neu diagnostiziertem Lungenkrebs bezüglich Zellgröße und Granularität, Aktivierungspotential, Subpopulationsverteilung, DNA-Freisetzung und Positivität von H3Cit im Vergleich zu Zellen von Geschlechts- und Alters-angepassten gesunden Personen.

HND- und LDN-Subpopulationen wurden mittels Dichtegradienten-basierter Zentrifugation aufgetrennt und mittels Ionomycin (IO) und Phorbol-12-Myristat-13-Acetat (PMA) stimuliert. Die Ergebnisse wurden über photometrische sowie durchflusszytometrische Analyseverfahren erhalten.

Es wurde gezeigt, dass LDNs aus Lungenkrebspatienten signifikant an Größe, gemessen durch ihre Vorwärtsstreuung (FSC), abnahmen. Außerdem zeigten HDNs und auch LDN aus Patienten signifikant reduzierte Granularität gemäß ihrer seitlichen Streuung (SSC). Stimulation mit IO und PMA führte ebenso zu einer Reduktion in SSC.

Zusätzlich zeigten von Lungenkrebspatienten isolierte HDNs sowie LDNs nach Stimulation eine signifikant erhöhte CD11b-Expression, ein Marker für Zellaktivierung.

Innerhalb der LDN-Subpopulationen von Lungenkrebspatienten zeigte sich eine signifikante Zunahme aktivierter Neutrophilen ($CD62^{niedrig}/CD16^{hoch}$).

Weiters wurde festgestellt, dass DNA-Freisetzung unter gewissen Stimulationsbedingungen in Zellen aus Patienten signifikant verringert war. Patienten-HDNs zeigten bei IO-Stimulation einen signifikanten Anstieg der H3Cit-Positivität, während LDN bei PMA-Stimulation eine signifikante H3Cit-Verringerung aufwiesen.

Zusammenfassend konnte durch diese Studie gezeigt werden, dass sich isolierte HDNs und LDNs in ihrer Größe und Granularität, der Subpopulationsverteilung sowie in ihrem Expressionsmuster von CD11b *in vitro* signifikant unterscheiden. Dies legt die Annahme nahe, dass Neutrophile im Zuge einer Lungenkrebserkrankung leichter aktivierbar zu sein scheinen. Um konkretere Einblicke zur Freisetzung von DNA und zur Ausbildung von NETs bei Neutrophilen Subpopulationen in Krebserkrankungen zu gewinnen, bedarf es jedoch weiterer Untersuchungen.

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List of Abbreviations

°C	degrees celsius
µL	microliter
µm	micrometer
µM	micromolar
ADC	adenocarcinoma
ADP	adenosine diphosphate
AF	Alexa Fluor®
Akt2	RAC-beta serine/threonine-protein kinase
APC	activated protein C
APC	allophycocyanin
APCs	professional antigen presenting cells
APS	antiphospholipid syndrome
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CaCl2	calcium chloride dihydrate
CATS	Vienna Cancer and Thrombosis Study
CD	cluster of differentiation
cfDNA	cell-free DNA
CFU	colony forming unit
CFU-Baso	basophil colony forming unit
CFU-E	erythrocyte colony forming unit
CFU-Eo	eosinophil colony forming unit
CFU-GM	granulocyte, monocyte colony forming unit
CFU-MC	mast cell colony forming unit
CFU-Meg	megacaryocyte colony forming unit
DC	dendritic cell
ddH_2O	double-distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOAC	direct oral anticoagulants
DVT	deep-vein thrombosis
EC-No	ethics committee number
EOC	epithelial ovarian cancer
ETs	extracellular traps

ex./em.	excitation/emission
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FSC	forward scatter
G-CSF	granulocyte colony stimulating factor
GDPase	guanosine triphosphatase
GPIb	glycoprotein Ib
h	hour
H3Cit	citrullinated histone H3
HBSS	Hanks' Balanced Salt Solution
HD	healthy donors
HDNs	high density neutrophils
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency viruses
HNSCC	head and neck squamous cell carcinoma
IL-8	interleukin 8
IO	ionomycin
IQR	interquartile range
LDGs	low density granulocytes
LDNs	low density neutrophils
LLC	Lewis lung carcinoma
LMWH	low-molecular-weight heparin
LPS	lipopolysaccaride
Mac-1	macrophage-1 antigen
METs	macrophage extracellular traps
MFI	median fluorescent intensity
mg	milligram
MHC	major histocompatibility complex
min	minutes
min-max	minimum-maximum
mL	milliliter
MPO	myeloperoxidase
MPV	mean platelet volume
n	number
n.s.	not significant
NADPH	nicotinamide adenine dinucleotide phosphate

NDNs	normal density neutrophils
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NK	natural killer cells
NOX2	NADPH oxidase 2
NSCLC	non-small cell lung carcinoma
PAD4	peptidylarginine deaminase 4
PAMPs	pathogen-associated molecular patterns
PB	Pacific Blue™
PBMCs	peripheral blod mononuclear cells
PBS	Dulbecco's Phosphate Buffered Saline
PE	phycoerythrin
PenStrep	Penicillin-Streptomycin
PFA	paraformaldehyde
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PMNs	polymorphonuclear leukocytes
Rac1/2	Ras-related C3 botulinum toxin substrate 1/2
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SLE	systemic lupus erythematosus
SSC	side scatter
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor beta
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TxA2	thromboxane A2
unstim	unstimulated
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
VIBS	Vienna Bleeding Study
VKA	vitamin K antagonists
VTE	venous thromboembolism
VWF	von Willebrand factor

1. Introduction

1.1. The immune system

The vast majority of earth´s population is comprised of microorganisms. Hence, humans are in constant contact with these organisms, which are persistently threatening the individuals' normal homeostasis. To sustain host survival, an intricate system has evolved to counter this massive array of pathogenic, allergenic as well as toxic substances: the immune system. The central role of the immune system is to protect the host and to defend it from harmful intruders, which is mediated through the activation and control of an organized response to invading pathogens (Gilbert et al. 2018). This involves an interplay of many different players, with responsibilities ranging from recognition to elimination, without dealing excessive damage to host tissue. The mechanism of distinguishing between exogenous and host matter is termed self-nonself discrimination. But not every microorganism is harmful, some microbes are even essential for the host to support or sustain normal organ and tissue function, such as the microbiomes found on the skin or within the gastrointestinal tract (Gilbert et al. 2018). This makes it an additional task for the immune system to tolerate them and hold them in check (Chaplin 2010).

The immune system uses a complex assortment of protective mechanisms which rely on the detection of distinct structural features on intruders. These mechanisms can be broken down into two general groups, the innate and the adaptive immune system (Chaplin 2010).

1.1.1. Innate and adaptive immune system

The initial and non-specific host response to pathogen invasion is conducted by the innate immune system, which enfolds physical and chemical barriers as well as a variety of cellular defence responses (Alberts et al. 2015). It is a fast-acting system which is encoded in the host´s germ line and relies on the recognition of molecular patterns (so-called pathogen-associated molecular patterns – PAMPs) that are shared by many intruders (Chaplin 2010). Effector cells of the innate immune system are neutrophils, monocytes and macrophages, dendritic cells (DC), natural killer cells (NK) and components of the complement system (Alam 1998).

Compared to the innate response, the effective response of the adaptive immune system is slower. Lymphocytes, namely B and T cells, are the effector cells of the adaptive immune response. To develop their effectiveness, T cells have to encounter the antigen first, establish the antigen-binding structure and then proliferate (Chaplin 2010).

This encounter is mediated via professional antigen-presenting cells (APCs), which phagocytose microbes, cleave their proteins and carry the resulting peptide fragments to their surface via major histocompatibility complex (MHC) proteins. The peptide-MHC complexes are then transported to a lymph organ, where they are presented to T cells to activate them. Activated T cells are capable of either killing infected cells themselves or stimulating other immune cells (such as B cells and phagocytes) to eliminate pathogens. This interaction directly links the innate and adaptive immune system (Alberts et al. 2015).

When B cells come in contact with an antigen, either via direct pathogen interaction or though other immune cells, they produce highly specific structures, so-called antibodies. In this response of the adaptive immune system, antibodies are secreted into the bloodstream, where they circulate and finally bind pathogens. This renders them unable to interact with host cell receptors and marks them for destruction by components of the innate immune system (Alberts et al. 2015).

During lymphocyte development, cells are capable of producing a vast variety of antibodies (more than 10^{12}) and receptors, guaranteeing a highly specific targeting and binding of foreign structures (Alberts et al. 2015).

The most crucial feature of the adaptive response is the establishment of the so-called immune memory, characterised by the production of long-lived B cells that memorize a specific antigen structure of unique intruders. These cells can endure in a dormant state over a long period of time until the host is confronted with the same pathogen again, thereby contributing to a much faster and more effective response upon a second time encounter (Chaplin 2010).

1.1.2. Peripheral blood mononuclear cells (PBMCs)

PBMCs are crucial components of both the innate and the adaptive immune system. They are comprised of monocytes, lymphocytes including T-, B- and NK cells and dendritic cells in different frequencies. With a frequency between 70 and 90%, lymphocytes by far constitute the majority of PBMCs, with the most of them being T cells (45 to 70% of lymphocyte) and only a small percentage of B cells (5 to 15% of lymphocytes). Monocytes cover 10 to 30% of total PBMC count, whereas NK cells provide 5 to 10% and dendritic cells only between 1 to 2% of the total peripheral cells (Muir 2019).

1.1.3. Polymorphonuclear leukocytes (PMNs)

PMNs are part of the innate immune system and are often referred to as granulocytes. They can be subdivided in three groups; neutrophils, eosinophils and basophils. Basophils (<1% of leukocytes) and eosinophils (<4% of leukocytes) are the least abundant granulocytes but are very important for allergic reactions, parasite and virus defence (Abbas, Lichtman, and Pillai 2014; Karasuyama et al. 2011). Neutrophils on the other hand are the most abundant leukocyte with 50 to 75% of circulating leukocytes (KIMCL 2019).

1.2. Neutrophils

Neutrophils, the first responders to invaders, can be recruited to the side of infection and show a huge repertoire of functions dedicated to find, interact with and finally exterminate intruders. Pathogens ranging from bacteria to fungi, protozoa and even viruses are being killed by various cytotoxic mechanisms (Mayadas, Cullere, and Lowell 2014).

1.2.1. Neutrophil production, mobilization and life span

Haematopoiesis, the process of blood cell forming, takes place in the bone marrow. Neutrophils derive from hematopoietic stem cells, which are located in niches with low oxygen tension and little blood flow provided by osteoblasts (Winkler et al. 2010). In a healthy adult human, between 1 and 2 x 10^{11} neutrophils are generated daily. The quantity of produced neutrophils is principally regulated by their apoptotic rate and tuned by granulocyte colony stimulating factor (G-CSF) (Borregaard 2010).

Large numbers of mature neutrophils are stored in the bone marrow, which can be quickly mobilized as a response to infection or during inflammation, leading to a rapid increase in numbers of circulating neutrophils (Furze and Rankin 2008). Neutrophils retention in the bone marrow and their subsequent release is mediated via various chemokine receptors and their corresponding ligands which are expressed by vascular endothelial cells and osteoblasts (Lapidot and Kollet 2002). Neutrophil release is controlled by G-CSF, as it facilitates the regulation of chemokine receptor and ligand expression by bone marrow endothelial cells. When signalling is tilted in the favour of certain chemokine receptors, neutrophils are released from the bone marrow. (Eash et al. 2010; Theilgaard-Mönch et al. 2004).

Once released, the life span of neutrophils is rather short. In circulation, they survive for only about 7 hours before they are degraded in the spleen (Mayadas and Cullere 2005). In tissue, neutrophils undergo apoptosis after 1-2 days (Mayadas et al. 2014).

1.2.2. Neutrophil defence mechanisms

Neutrophils use a variety of mechanisms to counteract pathogenic microorganisms. These mechanisms are often combined and include processes termed degranulation and phagocytosis as well as the formation of so-called neutrophil extracellular traps (NETs). A schematic representation of these mechanisms can be seen in Figure 1.

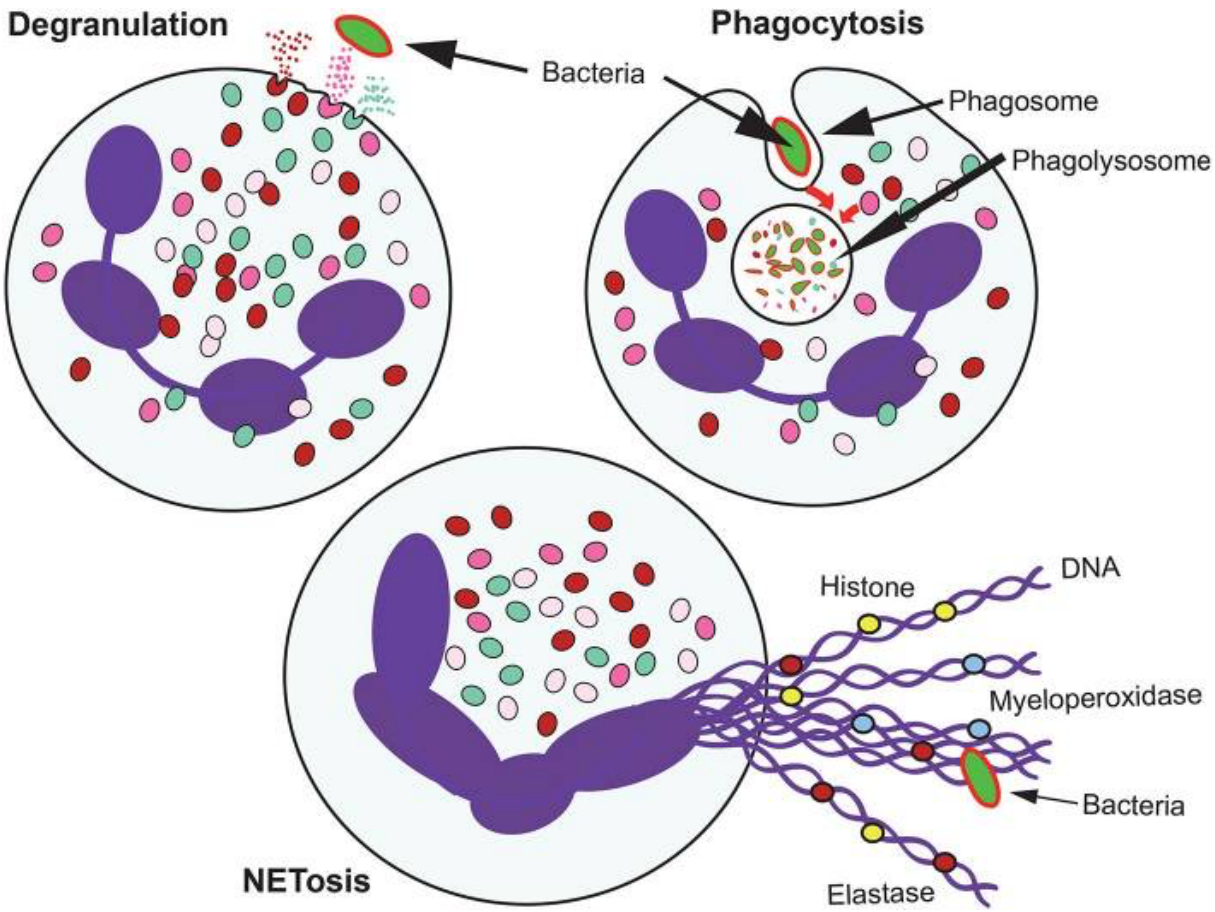

Figure 1: Schematics of Neutrophil Defence Mechanisms. When neutrophils come into contact with invading pathogens, three distinct mechanisms of host defence can occur. Graphic taken form Rosales et al. (2018).

1.2.2.1. Degranulation

Antimicrobial peptides and proteinases are stored in neutrophil granules. These granules can either fuse with the phagosome during phagocytosis, thereby contributing to the microbicidal properties of the phagosome (Lee, Harrison, and Grinstein 2003) or can be released into the extracellular space. This is termed degranulation, a process where granules fuse with the plasma membrane upon neutrophil activation and release their content (Mayadas et al. 2014).

Neutrophil granules can be categorized in four distinct groups:

1. Azurophilic (or primary) granules, characterized by the presence of myeloperoxidase (MPO)
2. Specific (or secondary) granules, containing lactoferrin
3. Tertiary granules, which contain gelatinase
4. Secretory vesicles, containing express alkaline phosphatase as well as albumin (Lee et al. 2003)

Neutrophils mainly contain cationic peptides. These can interact with negatively charged membrane components of invading pathogens, which results in nonspecific membrane permeabilization and the formation of pores. Intracellular targets are bound as well, leading to the inhibition of RNA and DNA biosynthesis as well as the disruption of bacterial biofilms (Choi, Chow, and Mookherjee 2012).

Accompanying degranulation is the generation of reactive oxygen species (ROS) by activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is associated with a rapid increase of oxygen consumption by neutrophils. This is known as the respiratory burst (Mayadas et al. 2014). To assemble NAPDH oxidase and thereby make it functional, a translocation of its cytosolic components ($p67^{phox}$, $p47^{phox}$ and $p40^{phox}$) to the phagosome membrane has to occur. There, guanosine triphosphatase (GTPase) Rac2 (or Rac1) and cytochrome b558 (comprised of $gp22^{phox}$ and $gp91^{phox}$ (NOX2)) are located (Kuijpers and Lutter 2012).

ROS play a crucial role in the destruction of pathogenic fungi and bacteria, but it is not yet fully understood if killing of these microorganisms is mediated directly via protein and lipid peroxidation, decarboxylation and deamination or indirectly through phagocyte proteinase activity modulation (Klebanoff and Williams 2006).

1.2.2.2. Phagocytosis

Together with macrophages, neutrophils are termed phagocytes, as with the help of specific receptors, they can recognize, internalize and in the end kill both opsonized (immunoglobulin-coated) as well as non-opsonized pathogens. The initially formed phagosome is not yet microbicidal, as it lacks antimicrobial components at its primary state. A series of events has to occur, altering both the vacuoles' inner composition as well as the vacuole membrane to gain the ability of killing the engulfed microorganism and dispose the remainders. This process is called maturation, where fusion with endosomes and lysosomes results in the formation of a phagolysosome. These interactions lead to the acquisition of vacuolar adenosine triphosphatase (ATPases), the NADPH oxidase complex as well as several antimicrobial enzymes, resulting in a hydrolase-rich, acidic environment capable of degrading its content (Lee et al. 2003).

1.2.2.3. Neutrophil extracellular traps

The controlled release of DNA by activated neutrophils is known as NET formation. NETs act as a defence mechanism capable of deactivating and killing intruding pathogens, which is possible due to their specific composition of nuclear and granular components (Brinkmann et al. 2004).

With DNA as the main structural element, NET fibres are not membrane-surrounded and contain proteins from granules such as neutrophil elastase (NE), MPO and cathepsin G, lactoferrin, gelatinase as well as histones (Brinkmann et al. 2004).

The formation of NETs is a highly regulated process. It occurs significantly faster than apoptosis, with the release occurring as early as 10 minutes post activation. Activation happens *in vivo* through stimulants like interleukin-8 (IL-8) or bacterial lipopolysaccharide (LPS) (Brinkmann et al. 2004) and can be triggered *in vitro* using phorbol 12-myristate 13-acetate (PMA) or calcium ionophores such as A23187 and ionomycin (IO) (Hamam, Khan, and Palaniyar 2019). Several pathways lead to nuclear NET formation.

One NET formation pathway, known as the NADPH oxidase (NOX)-independent pathway, is controlled by peptidylarginine deaminase 4 (PAD4) as the leading enzyme. It is responsible for the conversion of arginine residues on histones to citrulline, which leads to a loss of the histones positive charge. This subsequently leads to rapid chromatin decondensation, making citrullinated histones (especially H3 = H3Cit) a hallmark of this pathway (Hamam et al. 2019). *In vitro* stimuli of this pathway are calcium ionophores such as IO and A23187 (Douda et al. 2015).

The NOX-dependent NET formation pathway relies on the *in vivo* induction of NOX2 via bacterial LPS or *in vitro* using PMA. This induction leads to elevated ROS production and an increase in MPO-related activity, followed by the translocation of NE and MPO into the nucleus. This promotes DNA decondensation, rupture of the nuclear envelope and neutrophil granulae membranes, decorating the DNA strands with granular proteins prior to extracellular release (Papayannopoulos et al. 2010). Both pathways are schematically represented in Figure 2.

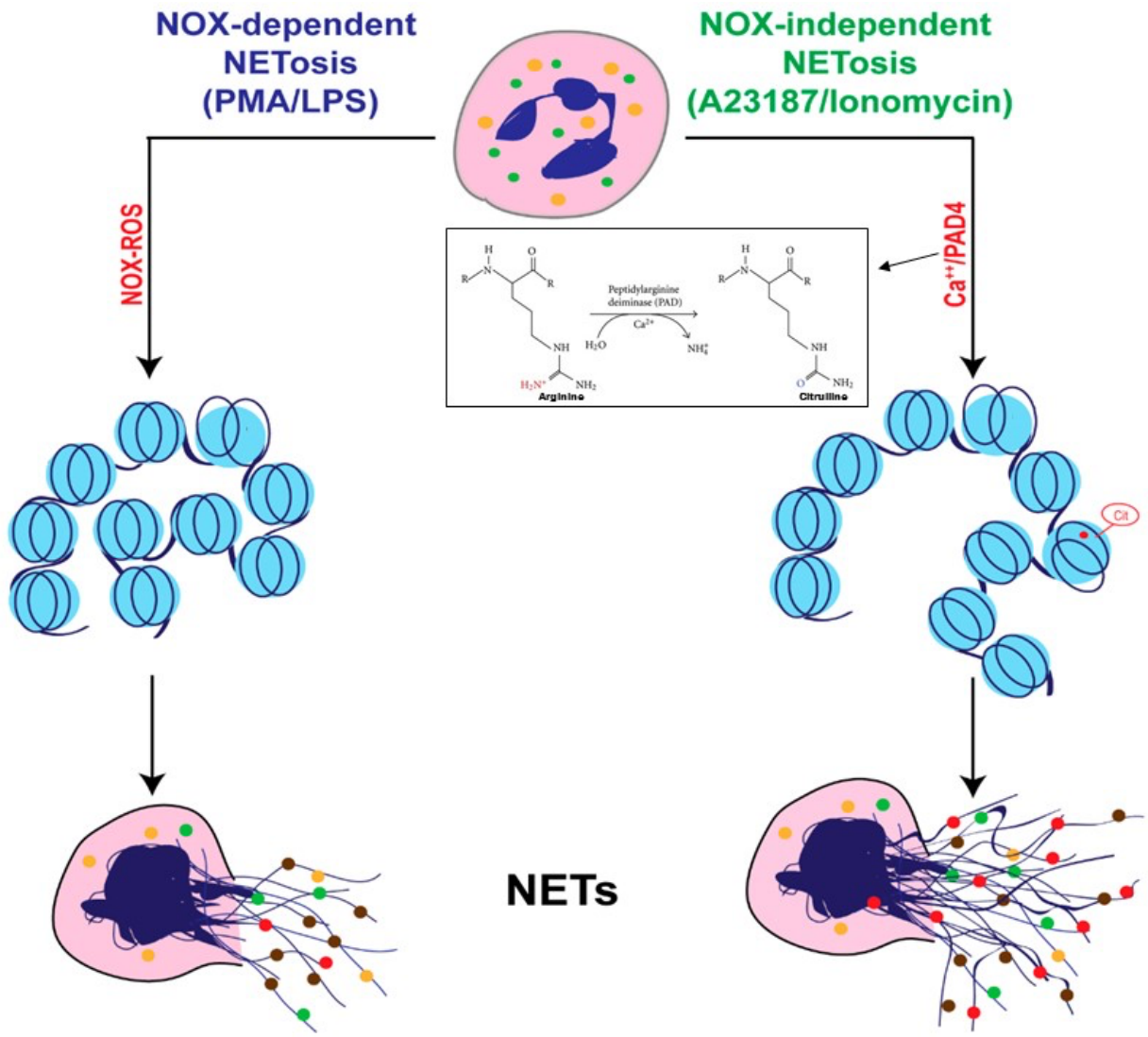

Figure 2: NET formation pathways. Two major pathways lead to the formation of NETs, dependent and independent on the involvement of NADPH oxidase (NOX). During the NOX-dependent pathway, ROS and NOX promote DNA decondensation, rupture of the nuclear and neutrophil granulae envelope, leading to subsequent NET release. The NOX-independent pathway is controlled by peptidylarginine deaminase 4 (PAD4), which is responsible for the conversion of arginine residues on histones to citrulline, leading to a loss of the histones positive charge, subsequently resulting in chromatin decondensation and NET release. Graphics taken from Mohanan et al. (2012) and Hamam et al. (2019).

1.2.3. Neutrophil subpopulations

Originally neutrophils were thought to be homogeneous, but it has been shown that they are a rather heterogeneous population. There are currently two ways to discriminate neutrophils: subpopulations according to surface marker expression (Pillay et al. 2012) and subpopulations according to their density (Sagiv et al. 2015).

As reported by Pillay et al. (2012), neutrophil subdivision is usually performed according to different expression patterns of the surface markers L-selectin (CD62L) and FcγRIII (CD16). This was established in an experimental setup, where Pillay and colleagues administered LPS to healthy donors and morphologically analysed circulating neutrophils after 3 hours post administration. In total, three subpopulations of circulating neutrophils could be observed. Banded neutrophils, which were most likely newly released from the bone marrow, showed a $CD16^{dim}/CD62L^{bright}$ expression pattern whereas neutrophils with a morphologically hypersegmented nucleus expressed $CD16^{bright}/CD62L^{dim}$. In addition, the $CD16^{bright}/CD62L^{dim}$ neutrophils showed an upregulated expression of CD11c, CD54 and CD11b, leading to the assumption that these cells are activated. But variances in surface marker expression were not the only differences found between newly released and activated neutrophils. $CD16^{dim}$-banded neutrophils displayed a higher survival rate in culture after 24 hours compared to $CD62L^{dim}$ cells. Also, activated neutrophils showed a 3-fold higher activation potential of the NADPH oxidase upon *ex vivo* stimulation compared to newly released neutrophils. These two subpopulations could not be seen in healthy control individuals before LPS administration. The third subpopulation was observed in the same gate as cells from control individuals and expressed $CD16^{bright}/CD62L^{bright}$ and were thereby characterized as mature neutrophils (Figure 3) (Pillay et al. 2012).

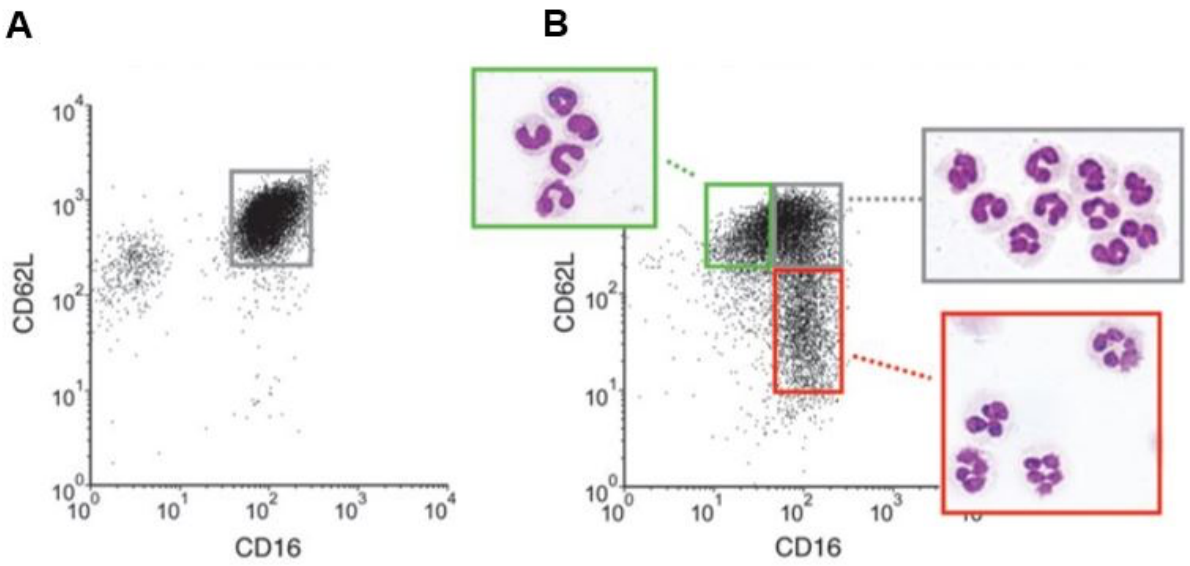

Figure 3: Neutrophil subpopulations according to CD16/CD62L surface marker expression. (A) $CD16^{bright}/CD62L^{bright}$ mature circulating neutrophils (grey) from healthy donors were analysed before LPS administration **(B)** 180min post administration of LPS, three distinct neutrophil subpopulations could be observed: $CD16^{dim}/CD62L^{bright}$ (newly released; green), $CD16^{bright}/CD62L^{dim}$ (activated; red) and $CD16^{bright}/DC62L^{bright}$ (mature; grey). Graphic taken from Pillay et al. (2012).

Additionally, neutrophils can be distinguished by density differences. When blood is layered over a density gradient and centrifuged, two different layers are visible: The PBMC layer, which usually contains monocytes and lymphocytes, and the PMN layer, containing granulocytes (Figure 8). Interestingly, neutrophils were also found in the PBMC layer, which were named low density neutrophils (LDN) (Hacbarth and Kajdacsy-Balla 1986), also referred to as low density granulocytes (Carmona-Rivera and Kaplan 2013). To continue this nomenclature, neutrophils within the PMN layer are called high density neutrophils (HDNs) (Sagiv et al. 2015) or normal density neutrophils (NDNs) (Hong 2017).

Low density neutrophils (LDNs) are described to be a mixed population comprised of immature, myeloid-derived suppressor cells and mature neutrophils. Upon flow cytometer analysis, LDNs show an increase in forward scatter (FSC) but a similar side scatter (SSC) to HDNs, indicating that LDNs are increased in size whilst a similar granularity to HDNs is maintained (Sagiv et al. 2015). Expression levels of the neutrophil activation surface markers CD11b and CD66b were found to be elevated in LDNs from healthy individuals, from patients with antiphospholipid syndrome (APS) (Mauracher et al. 2020) and from patients with cancer (Sagiv et al. 2015). Additionally, H3Cit levels in LDNs were shown to be highly elevated in both healthy individuals as well as in APS

patients, indicating that NET formation is increased in LDNs compared to HDNs (Mauracher et al. 2020). LDNs also display impaired neutrophil function, act immunosuppressive and pro-tumorigenic, promote angiogenesis in cancer as well as metastatic tumour cell seeding to distant organs (Sagiv et al. 2015). Furthermore, their ability to transmigrate through tissue is impaired and phagocytotic activity is decreased. It was also observed in a murine model, that upon treatment with TGF-β, mature HDNs were capable to become LDNs. This transition however was also observed to occur spontaneously, but only in blood taken from mice bearing late-stage tumours. Compared to blood from mice bearing early-stage tumours, the HDN/LDN ration changed to the favour of LDNs in late tumour bearing mice, with 90% of all neutrophils being located within the LDN fraction after 3 hours of *ex vivo* incubation. This transition might be mediated via advanced tumour-associated changes of the chemokine/cytokine milieu (Sagiv et al. 2015).

Elevated levels of blood LDNs was not only shown in cancer, but were also implicated in other severe pathologies such as HIV (Cloke et al. 2012), severe infection (Morisaki et al. 1992) and autoimmune disease (Denny et al. 2010).

1.3. Haemostasis

Under normal conditions, an anticoagulant surface is maintained within the blood vessels' endothelium to keep blood in its fluid state. If damage occurs to the blood vessel, bleeding has to be stopped at the site of injury without hindering blood flow somewhere else in circulation. This process is called haemostasis, a tightly regulated procedure forming a haemostatic plug, mainly containing platelets and fibrin, to counteract blood loss. Haemostasis is a combination of two main processes that occur simultaneously, which are activated by components of the subendothelial matrix when exposed to blood. These processes become active within seconds after injury and under normal conditions are localized to only the site of injury. Haemostasis is tightly controlled by the fibrinolysis pathway and various anticoagulant mechanisms to uphold blood fluidity when no injury is present as well as regulating the size of the forming clot, holding it proportional to the sustained wound (Gale 2011).

1.3.1. Primary haemostasis

The major components involved in primary haemostasis are platelets, small anuclear cells that derive from megakaryocytes (Schulze and Shidasani 2005). In healthy blood vessels, they do not aggregate with one another nor adhere to the surface. However, when injury occurs, components of the subendothelial matrix such as von Willebrand

factor (VWF) and collagen become exposed and subsequently bind platelets, starting their activation (Gale 2011; Ruggeri 2007).

The decisive factor for linking primary to secondary haemostasis is the serine protease thrombin. It activates platelets at the final step of the coagulation cascade, which results in platelet cytoskeleton remodelling, granule secretion and integrin activation (Kahn et al. 1998). Stabilization of the platelet plug is mediated by insoluble fibrin, generated during secondary haemostasis (Gale 2011).

1.3.2. Secondary haemostasis

Fibrin, the key player of secondary haemostasis, is generated during the proteolytic coagulation cascade via the cleavage of fibrinogen by thrombin (Kahn et al. 1998). It forms an insoluble mesh, which is integrated into the platelet plug and its surroundings, leading to a stable and strong blood clot. The coagulation cascade and hence the formation of fibrin occurs concurrently with platelet aggregation during primary haemostasis (Falati et al. 2002).

Upon vascular injury, blood comes into contact with extravascular tissues. These tissues express large amounts of tissue factor (TF), a transmembrane protein localized on fibroblasts and other extravascular cells. TF acts as a cofactor for factor VIIa, the first serine protease of the coagulation cascade (Kirchhofer and Nemerson 1996). TF and VIIa form a complex capable of activating factor X and IX, transforming them to factor Xa and IXa. Factor X can also be activated by factor IXa, when VIIIa is present as a cofactor. The cofactor Va together with factor Xa then activates prothrombin, leading to the generation of thrombin (Dahlbäck 2000).

Within the coagulation cascade, thrombin is responsible for several major reactions, such as the activation of cofactors V and VIII as well as factor XI (which in turn facilitates factor IX activation), and is also crucial for clot propagation as it ensures coagulations positive feedback activation (Lane, Philippou, and Huntington 2005).

The mode of action of the proteolytic coagulation cascade is schematically represented in Figure 4.

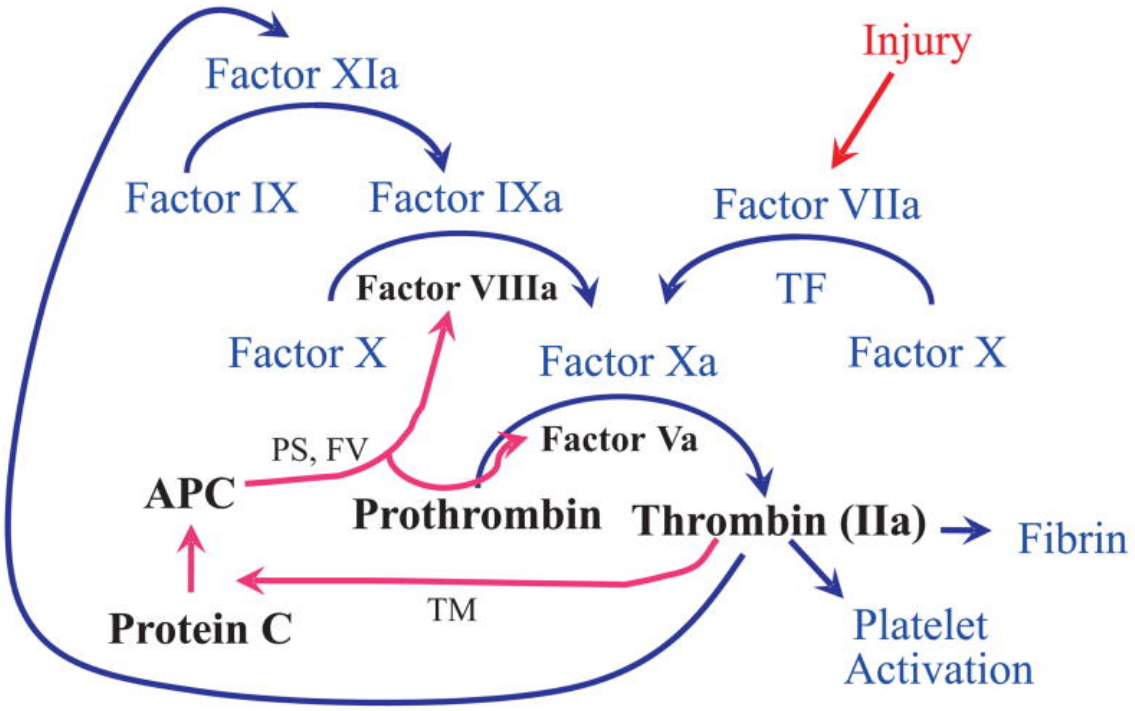

Figure 4: The coagulation cascade. As a response to injury, tissue factor (TF) initiates the coagulation cascade, which ultimately results in the generation of thrombin and fibrin. Graphic taken form Graham et al. (2011).

When blood vessels are intact, the coagulation cascade is inactive. Activation is prevented by numerous anticoagulant mechanisms, one of which is controlled via thrombomodulin, a cofactor for thrombin. Together they stimulate the anticoagulant serine protease protein C (APC), which is capable of converting thrombin from procoagulant to anticoagulant. This makes it of major importance when the already active coagulation cascade has to be downregulated (Esmon and Owen 1981). Also located on vessel endothelial cells are heparan sulfate proteoglycans. They activate a serine protease inhibitor called antithrombin, which suppresses the function of several factors within the coagulation cascade (Quinsey et al. 2004; Shimada et al. 1991)

1.3.3. Fibrinolysis

When wounds are healing, blood clots have to be dissolved again. This happens in a process called fibrinolysis. But dissolving clots is not the only task of the fibrinolytic system, it is also responsible for preventing blood clot formation in healthy vessels. This system is comprised of several serine proteases. They are produced either directly on the fibrin clot surface or bind to it, thereby leading to plasminogen activation and subsequent fibrin degradation. (Hoffman 2005).

It is crucial for procoagulant and anticoagulant systems to be in adequate balance to avoid pathologies. If one or multiple of these systems are defect, the result might be bleeding (not enough clotting) or pathological thrombus formation (too much clotting), known as thrombosis (Gale 2011).

1.4. Thrombosis

Thrombosis is defined as the formation of a blood clot that either partially or completely blocks an arterial or venous blood vessel, leading to limitations in the natural blood flow. Multiple factors contribute to the development of thrombosis, where, as mentioned before, the adequate balance of haemostasis and endogenous anticoagulation plays an important role. In 1856, the German physician Rudolf Virchow proposed three leading factors predisposing to thrombosis, including endothelial vessel wall damage, a hypercoagulable state and venous or arterial blood stasis (Virchow 1856). These factors became commonly known as the “Virchow’s triad” (Figure 5) (Damilola and Fernandez 2019).

Hypercoagulability, a haematological concept generally defined as an increased thrombogenic risk, can arise from various changes within the haemostatic system. The changes can result from increased levels of prothrombotic proteins and cytokines, deficiencies of anticoagulant factors (both natural and endogenous), variations in blood components and viscosity as well as inflammatory factors. These can either be inherited or acquired. Protein C and S deficiencies, antithrombin III deficiency, factor V Leiden and prothrombin gene mutations are the most common inherited risk factors for thrombosis. Far more common than the inherited are acquired factors, which can be a result of inflammatory pathologies, morbid obesity, heavy smoking or medication (especially hormonal drugs such as contraceptives), amongst many others. Blood stasis can also occur due to many different factors such as pregnancy, immobility or impaired blood flow due to blood vessel remodelling, fibrosis or atherosclerosis (Damilola and Fernandez 2019).

The pathogenesis of thromboembolic disorders differs vastly in regards to whether a thrombotic event occurs within the venous or the arterial system (Turpie and Esmon 2011).

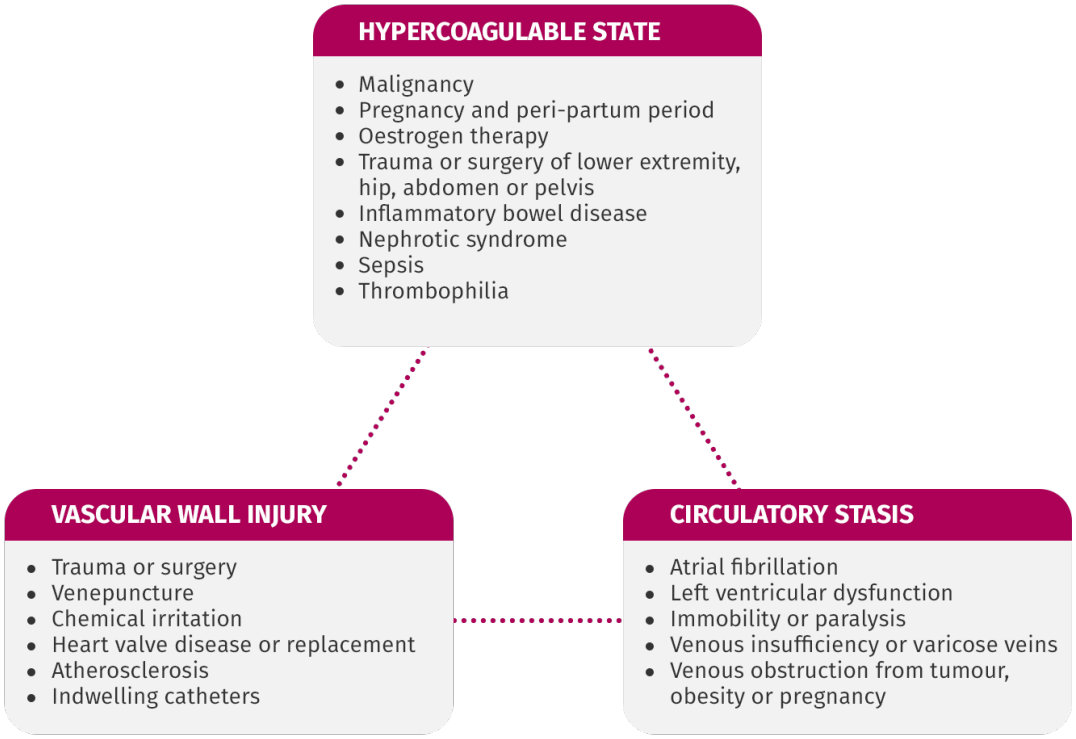

Figure 5: Virchow´s triad. A graphical representation of the three leading factors predisposing to thrombosis and their underlaying causes. Graphic taken from: https://www.thrombosisadviser.com/thrombus-formation/ (accessed 05.05.2020)

1.4.1. Arterial thrombosis

Arterial thrombosis is mainly triggered through rupture of an atherosclerotic plaque (Engelmann and Massberg 2013), leading to the release of liquid from the plaques core. This liquid contains high levels of strong thrombogenic factors like TF and collagen, that are capable of initiating the coagulation cascade and platelet activation immediately after plaque rupture (Figure 6) (Ardissino et al. 1997). Thrombin formation further activates platelets, thereby promoting additional platelet recruitment, adhesion, aggregation and thus rapid thrombus growth. A thrombus developed through rupture of a coronary atherosclerotic plaque is the major cause for acute myocardial infarctions and ischaemic heart disease, resulting in sudden death at roughly 50 to 70% when newly occurring (Davies 2000).

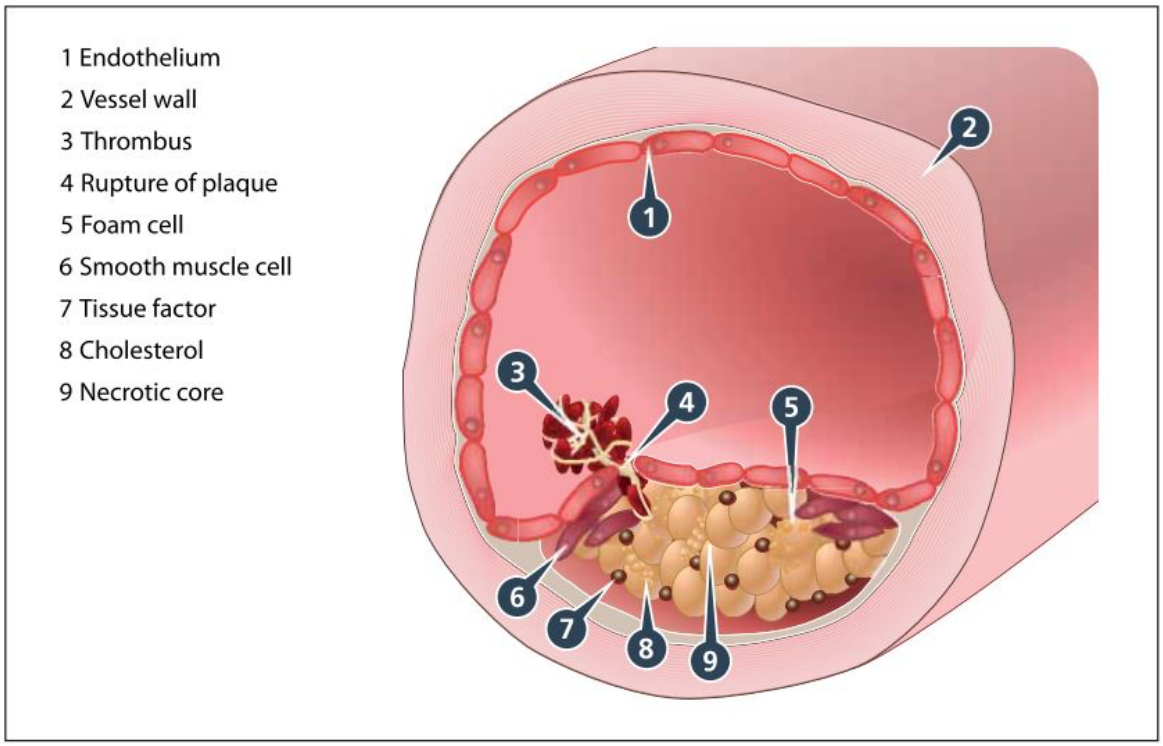

Figure 6: Arterial thrombus formation – a schematic overview. After plaque rupture, thrombogenic factors are released into the lumen of the vessel, where a thrombus starts to form. Graphic taken from Turpie & Esmon (2011).

1.4.2. Venous thrombosis

Venous thromboembolism (VTE) is a widespread pathology with potentially fatal outcome. It is comprised of two forms of thrombotic events: deep-vein thrombosis (DVT), occurring mainly in the large leg veins, and pulmonary embolism, which is the result of thrombus parts breaking away from DVT and travelling through circulation until blocking a pulmonary artery. Reduced blood flow in veins, termed venous stasis, is the main reason for thrombus formation in affected regions (Figure 7). Several medical conditions can cause venous stasis, amongst the most common inducers are reduced mobility, increased venous pressure or surgical orthopaedic interventions (Kroegel and Reissig 2003). Stasis promotes thrombus formation because coagulation factors are failed to be cleared rapidly enough after vascular injury. This leads to endothelial activation and the subsequent expression of adhesion molecules on the endothelial cell surface. Venous thrombosis is primarily caused by the activation of the coagulation cascade. Hence, venous thrombi mainly consist of red blood cells intertwined in great amounts of fibrin (López, Kearon, and Lee 2004).

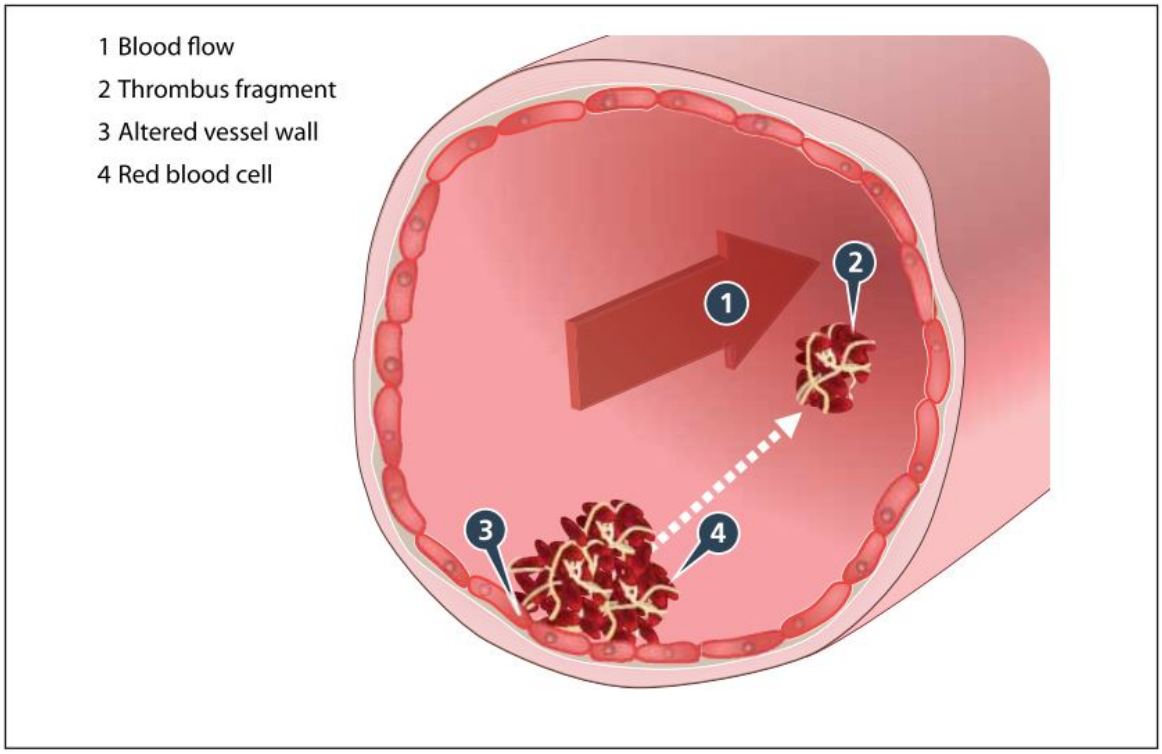

Figure 7: Deep-vein thrombus formation – a schematic overview. Venous stasis as well as vessel wall damage can be causes for DVT. When the thrombus breaks loose, it can occlude a pulmonary artery, leading to pulmonary embolism. Graphic taken from Turpie & Esmon (2011).

1.4.3. Neutrophils and NETs in thrombosis

Over a century ago, the link between immune cells and thrombotic events has been described by Bizzozero for the first time, as he stated that not only platelets but also leukocytes are involved in thrombus formation (Bizzozero 1881, 1882). In the last decade however, the attention has focused specifically on neutrophils, as it was shown that they bind activated endothelium and adhere to the site of vessel injury even before platelets (Darbousset et al. 2012).

Neutrophils play an important role in the context of thrombosis in various ways. Their main effectors in pathogen defence, neutrophil elastase and cathepsin G, were shown to promote coagulation and thereby play a crucial role in thrombus growth (Massberg et al. 2010). Additionally, neutrophil-associated TF activity has a significant impact on thrombus formation as well. However, if TF is produced by neutrophils directly or rather passively acquired from other cells has been up for debate for a long time. Initially it was shown in several studies conducted in monkeys as well as in humans, that neutrophils are capable of producing large amounts of TF (Nakamura, Imamura, and Okamoto 2004). This was countered by studies claiming that neutrophils produced no TF at all (Østerud 2004) or only in very limited amounts (Østerud 2010). Today it is widely

accepted that no synthesis of TF occurs in normal human neutrophils, but it may rather be passively acquired from monocytes (Noubouossie et al. 2019). Nevertheless, neutrophils play a crucial part in thrombus initiation and formation, as it has also been shown that depletion of neutrophils in mouse models lead to a decrease of thrombus formation (von Brühl et al. 2012).

Due to recent findings, more studies started to focus on the thrombosis associated role of NETs (Kapoor, Opneja, and Nayak 2018). This initially started as experimental DVT-thrombi in baboons showed the presence NETs (Fuchs et al. 2010) and thrombi with high contents of deoxyribonucleic acid (DNA) were found in mice (von Brühl et al. 2012). In humans, the first reported observations of extracellular DNA traps in thrombi were made in intraluminal thrombi from patients with abdominal aortic aneurysm (Delbosc et al. 2011), in a patient with microscopic polyangiitis that was complicated by DVT and pneumonia (Nakazawa et al. 2012) and in thrombi taken from acute myocardial infarction patients (de Boer et al. 2013). Another study focusing on thrombus maturation found that in histological samples of human venous thrombi, H3Cit positive neutrophils were present mostly in organizing thrombi. H3Cit-positivity was observed both intracellular as well as diffuse extracellular. This suggests that NET formation occurs predominantly during thrombus maturation, specifically during the inflammatory thrombus development stage (Savchenko et al. 2014).

The pro-coagulant role of NETs has recently emerged, providing clear evidence on how different NET mechanisms are involved in VTE initiation and progression. It is assumed that the pro-thrombotic nature of NETs originates from their capability of interacting with the endothelium, with platelets and several coagulation factors. As described before, two major components of NETs are histones and DNA. The DNA matrix extruded during NET formation can provide a scaffold for thrombus initiation, as it is capable of binding not only pathogens but also platelets and erythrocytes (Fuchs et al. 2007, 2010), which in turn supports further platelet aggregation and activation. The exact mechanism of NET-platelet interaction is not yet fully understood, but findings from studies in a deep vein thrombosis murine model showed that H3Cit, a hallmark of citrullination mediated NET formation, is capable of interacting with von Willebrand factor (vWF) (Brill et al. 2012). It is also believed that platelets are directly activated by histones via platelet receptor TLR2 and TLR4 stimulation (Semeraro et al. 2011). These activated platelets can in turn bind neutrophils via glycoprotein Ib (GPIb), leading to further NET formation and thereby propagating thrombus formation (von Brühl et al. 2012). Histones were also observed to have a high affinity to membrane phospholipids and were shown to facilitate pore formation and thereby a subsequent release of endothelial coagulation activation factors (Fuchs, Brill, and Wagner 2012). Additionally, NETs activate the

coagulation cascade, as protease activity of clotting factors is enhanced by extracellular nucleic acids, which induces the generation of thrombin (Kannemeier et al. 2007). As an example, the intrinsic pathway can be initiated via factor XII activation through NETs (von Brühl et al. 2012). Factor XII itself was also shown to initiate histone citrullination via urokinase plasminogen activator receptor (uPAR)-mediated phosphorylation of Akt2, resulting in NET formation. Blocking of factor XII-uPAR or specific inhibition of Akt2 was demonstrated to result in reduced NET formation *in vitro* (Stavrou et al. 2018). Again, TF plays an additional role in NET-induced VTE. It is present on extracellular DNA together with protein disulfide isomerase, which is capable of activating TF derived from other blood cells and thereby supporting the activation of the extrinsic pathway (von Brühl et al. 2012; Manukyan et al. 2008; Reinhardt et al. 2008). Serine proteases such as neutrophil elastase and cathepsin G are also present in NETs. They proteolytically inactivate tissue factor pathway inhibitor (TFPI) (thereby enhancing the activity of factor Xa) and thus promoting the production of thrombin and fibrin (Massberg et al. 2010). NETs were also shown to co-localize with fibrin and VWF in thrombi and thereby providing a thrombolysis-resistant scaffold (Fuchs et al. 2012). All these facets give strong evidence that multiple aspects of the coagulation cascade are affected by NETs, but the definite underlying mechanisms to all aforementioned interactions still need to be investigated.

1.4.4. Neutrophils and NETs in cancer and cancer-associated thrombosis

In human cancer biology, neutrophils play an important role. They constitute for a major portion of inflammatory cell infiltrates and can be a predictive factor for disease progression and outcome, as a high ratio of neutrophils to lymphocytes as well as an increased number of peripheral or intratumoural neutrophils was linked to a poor prognosis (Donskov 2013). They were also shown to have both pro- and antitumoral properties (Brandau, Dumitru, and Lang 2013). These opposing properties can be accredited to the two different subpopulations of neutrophils. Several in vitro and in vivo studies conducted by Sagiv and colleagues in 2015 reported that LDNs showed insignificant cytotoxicity as well as impaired phagocytosis, reduced chemotactic activity and weakened oxidative burst. Gene expression patterns of LDNs were also observed to have a reduced expression of a variety of chemokine receptors, chemokines and proinflammatory molecules, all associated with a reduction in inflammatory capacity. Taken together, LDNs were reported to have defective antitumor activity. In HDNs however, these basic neutrophil functions were all observed to be directed towards antitumor protection, with a capability of displaying antitumor functions in early- as well as late-stage tumours (Sagiv et al. 2015).

The different tumour-associated characteristics may also be connected to the neutrophil activation stage (Gregory and Houghton 2011) and are also assumed to be linked to NET formation. NETs might influence the tumour microenvironment as well as crucial steps in tumour development and progression such as immune suppression, angiogenesis, tumour growth and metastasis (Demers and Wagner 2013). These assumptions arise from findings of NET-like structures and neutrophils within tumour samples from Lewis lung carcinoma (LLC) patients. The observed structures were mainly hypercitrullinated histones on extracellular chromatin. Present neutrophils were observed to be a mix of dead, fully intact and even primed, hypercitrullinated neutrophils on the brink to form NETs (Ho-Tin-Noé et al. 2009). Neutrophil stimulation for NET formation is believed to occur due to hypoxia at the tumour site, where hypoxia-induced factor 1α might activate neutrophils (McInturff et al. 2012). Tumour-induced neutrophil priming on the other hand might be induced by G-CSF (Demers and Wagner 2014).
Interestingly, the tumour stage is also thought to have a significant impact on neutrophil properties, as a recent study was able to demonstrate. With increasing tumour age, neutrophil presence shifts from antitumoural neutrophils surrounding the tumour at early stage to protumoural neutrophils infiltrating the tumour at later stages (Mishalian et al. 2013). This substantiates the claim that NETs might have advantageous effects on primary tumour growth, since NETs were observed inside tumour samples in the previously mentioned study (Ho-Tin-Noé et al. 2009).

NETs were also observed to play a crucial role in metastasis. A study conducted in a model of systemic infection showed that NETs deposited on the micro-vasculature were capable of immobilizing and trapping circulating tumour cells in a similar manner as how it occurs with intruding pathogens. These cancerous cells were observed to then proliferate and form nodules, thereby again supporting the tumour-enhancing properties of NETs (Cools-Lartigue et al. 2013). However, it is still unclear if NETs just physically anchor tumour cells or if additional mechanisms of cancer cell protection are involved as well (Demers and Wagner 2014).

A condition that has gained more and more significance amongst physicians is cancer-associated thrombosis. Current estimates reckon that cancer is the most prevalent cause for VTE, with 20% of all thrombotic incidences being caused by malignancies (Heit, Spencer, and White 2016). Patients with cancer have a 0.5% annual risk to develop VTE compared to 0.1% within the general population (Elyamany, Alzahrani, and Bukhary 2014). Patients with cancer-associated thrombosis were shown to have the worst survival among all VTE patients (Puurunen et al. 2016). A cohort study which included 10315 VTE patients reported that 54.3% of all deaths were related to cancer (Ageno et al. 2019). Amongst all possible causes for death in patients with

cancer, VTE is the second most prevalent one, only after disease progression itself (Khorana et al. 2007).

In general, a multitude of factors predispose patients with cancer to an increased risk of thrombus formation. They often have to undergo chemotherapy, multiple surgical interventions or long-term catheter placements, all of which are known factors that lead to endothelial injury. Oncological pain is often experienced as a side effect of treatment or caused by the disease itself and patients are therefore rendered immobile for long time periods (Fernandes et al. 2019). The risk to experience an event of VTE also depends on the type of cancer. For example, lung, brain, pancreas and haematological malignancies (amongst others) are associated with a high thrombosis risk (Fernandes et al. 2019; Stein et al. 2006), whereas prostate and breast cancers pose a lower risk of clot formation (Gade et al. 2017). Regardless of cancer type and genetic background, further risks factors that contribute to cancer-associated thrombosis are higher histological tumour grade (Ahlbrecht et al. 2012) and metastasis. Specific chemotherapeutic treatments such as taxane-based drugs and platinum derivates as well as immunomodulatory agents are associated with elevated VTE risk as well (Fernandes et al. 2019).

Over the years, several laboratory parameters have also been identified to either have a direct association with venous thromboembolism in patients with cancer and which could be used as predictive parameters on thrombosis development. High platelet count (Simanek et al. 2010) and high plasma levels of soluble P-selectin (Riedl et al. 2016) were shown to be significant risk factors for VTE development in patients with solid tumours, with soluble P-selectin levels even being a predictive marker for VTE development (Ay et al. 2008). Further predictive biomarkers were found to be increased levels of prothrombin fragment 1+2 and D-dimer (Ay et al. 2009), elevated levels of clotting factor VIII (Vormittag et al. 2009) as well as an increased potential of thrombin generation (Ay et al. 2011). In patients without cancer, increased mean platelet volume (MPV) is associated with both venous and arterial thrombosis. Interestingly, contrary observations were described in patients with cancer, as high MPV was found to be associated with a decrease in VTE development risk compared to patients with low MPV (Riedl et al. 2013). Decreased platelet reactivity however was shown to also be associated with VTE and poor disease prognosis in patients with cancer (Riedl et al. 2017). Certain growth factors were observed to be associated with VTE development as well. Vascular endothelial growth factor (VEGF) is necessary to stimulate angiogenesis, which is crucial for oxygen supply to the tumour site. However, the formed vessels are not only leaky and highly thrombogenic (Brat and Van Meir 2004), but VEGF itself is capable of inducing the expression of TF (Mechtcheriakova et al. 1999). An association

between high levels of its soluble form (sVEGF) and VTE risk in patients with cancer was observed by Posch et al. (2016).

In 2018, a new model to predict cancer-associated VTE has been made public. Involving only two factors (levels of D-dimer and cancer risk group assignment), it can be used fairly easily, making it possible to individually predict VTE risk in the first six months after the patient has been diagnosed (Pabinger et al. 2018).

Cancer-associated VTE distinguishes from other VTE cases by risk factors and pathophysiological mechanisms. One of the most critical protein involved in the progression of cancer-associated thrombosis might be TF produced by cancer cells (Mukai and Oka 2018). As discussed before, TF activates the extrinsic coagulation pathway, which consequently leads to fibrin generation and activation of platelets. This, however, may not be the case for all types of cancer. A previous study conducted in patients with brain tumours found no statistically significant association between TF-expression levels and future incidences of VTE (Thaler et al. 2013). The pro-thrombotic nature of brain tumours might derive from the overexpression of podoplanin, a protein capable of activating platelets (Mir Seyed Nazari et al. 2018). But cancer cells also sequester other distinct pro-coagulant factors, which are capable to directly stimulate factor X. Moreover, the generation of inflammatory cytokines, which leads to endothelial damage, as well as the production of so-called carcinoma mucins, which obstruct the coagulation cascade, furthers the pro-coagulative nature of cancerous tumours. Furthermore, cancer cells are capable to synthesize plasminogen activator inhibitor-1, thereby inhibiting the fibrinolytic system. All those factors result in a pro-anticoagulation imbalance and the consequent generation of cancer-associated thrombosis (Mukai and Oka 2018).

Neutrophils have been linked to cancer-associated venous thrombosis, as they become activated in inflammatory disorders such as cancer and were found to be dominantly present within the local tumour-surrounding infiltrate (Gregory and Houghton 2011). In a study using a murine mammary carcinoma model, isolated neutrophils were seen to be highly hypercitrullinated, prepared to undergo NET formation. At late disease stage, thrombotic events inside the lungs of mice occurred. Upon further blood analyzation it could be seen that levels of hypercitrullinated neutrophils were low, whereas plasma DNA levels were significantly elevated and H3Cit was present. This suggests the spontaneous occurrence of NET formation (Demers et al. 2012). Several factors secreted by tumour cells are capable of priming neutrophils towards NET formation, where granulocyte G-CSF constitutes as a very important priming agent. Tumours producing G-CSF have also been associated with poor disease prognosis (Demers and Wagner 2014).

But not only the disease itself is believed to have an impact on neutrophils and subsequent thrombus formation, the treatment, specifically chemotherapy, might lead to elevated occurrences of thrombotic events in patients with cancer as well. Chemotherapy leads to cell death and subsequent DNA release, where large fragments can be found in the plasma even after the first cycle (Deligezer et al. 2008; Kwee et al. 2012). It was originally believed that these DNA fragments originated from necrotic tumour cells. More recent insights however suggest that certain chemotherapies are capable of inducing NET formation. Increased levels of cell-free DNA (cfDNA) were observed in plasma from early-stage breast cancer patients 24 hours after chemotherapy. It was shown that cfDNA is capable of inducing thrombin generation in whole blood, hence forming the link between chemotherapeutic treatment and the occurrence of thrombotic events (Demers and Wagner 2014).

In a recent study, NET biomarkers H3Cit, nucleosomes and cfDNA were investigated as possible predictive biomarkers for VTE in patients with cancer. It was observed that patients with H3Cit levels over the 75th percentile of the study population showed 14.5% of VTE incidences over a period of 2 years compared to 8.5% of incidences in patients with H3Cit levels below the 75th percentile. It could also be seen that patients with increased levels of nucleosomes and cfDNA during the first 3 to 6 months had a higher risk to experience a thrombotic event (Mauracher et al. 2018). An association with a higher mortality rate in patients with cancer could be observed for both elevated cfDNA and H3Cit levels (Grilz et al. 2019).

1.5. Hypothesis and Aim

The aim of this study was to investigate potential differences in neutrophils between healthy volunteers and patients with newly diagnosed lung cancer, specifically changes in neutrophil activation potential, subset distribution, DNA release and H3Cit positivity.

We hypothesise that in patients with lung cancer, LDNs occur in larger quantities compared to healthy individuals. Furthermore, we hypothesise that isolated HDNs and LDNs from patients with cancer have a higher potential of activation, release more DNA and show an increased predisposition to undergo NET formation upon stimulation with certain stimuli.

2. Materials and Methods

2.1. Study cohort

Between October 2018 and June 2019, 20 healthy adult individuals (median age [min-max]: 62 [50-72] years, female n=11) and 20 newly diagnosed, pre-treatment patients with lung cancer (median age [min-max]: 67 [46-82] years, female n=11) were included into the Vienna Cancer and Thrombosis Study (CATS) (Ay et al. 2008). CATS is an ongoing, single centre prospective cohort study involving patients with active malignancies to investigate parameters that could be predictive for VTE occurrence in patients with cancer. For inclusion, all of the following criteria have to be met: 1) patients must be older than 18 years; 2) willing to participate; 3) newly diagnosed with lung, brain, breast, pancreas, prostate or kidney cancer, cancer of the gynaecologic system or the lower/upper gastrointestinal tract; haematological malignancies; sarcoma; or disease progression after partial or complete remission; 4) the malignancy has to be histologically confirmed and 5) patients have to give written informed consent. Patients were not included if 1) they had a bacterial or viral infection two weeks before; 2) a thrombotic event (either arterial or venous) within three months prior to inclusion; 3) are anticoagulated continuously with low molecular weight heparin or vitamin K antagonists; 4) underwent surgical intervention or radiotherapy two weeks before; 5) or chemotherapy within the last three months. Patients were excluded if just one of the exclusion criteria was met. Endpoints of CATS were either the occurrence of (symptomatic or fatal) VTE, withdrawal of consent, loss of follow-up or death. Ethical approval for this study was given by the Ethics Committee of the Medical University of Vienna (EC-No 126/2003).

Healthy donors were included into the Vienna Bleeding Study (VIBS). In VIBS, patients with a bleeding tendency of unknown origin, as well as healthy individuals (control cohort) are included. In the present study only the group of healthy controls were included. Those had the following exclusion criteria: if they had 1) cases of bleeding related diseases within their family; 2) experienced any thrombotic event before in their life; 3) have a general bleeding tendency; 4) took any form of platelet aggregation inhibitor (e.g. Aspirin) ten days before the blood draw or 5) experienced any form of acute disease six weeks prior to inclusion. This study was ethically approved by the Ethic Committee of the Medical University of Vienna (EC-No 039/2006).

For our study, written informed consent was given by each participant before inclusion. Each donor, both healthy as well as patients with lung cancer, underwent a structured interview on their medical history, where they were also informed of the study details, and a blood draw for routine parameters and experimental setup. All methods were conducted in conformity with the Declaration of Helsinki.

2.2. Blood draw

For experimental setups, 27 mL blood was taken from each participant using a tourniquet, VACUETTE® Blood Collection + Luer Adapter Sets (Greiner Bio-One, Kremsmünster, AUT) and VACUETTE® K3EDTA blood vials 9 mL (Greiner Bio-One). Blood draw was performed by a nurse or medical doctor and transported to the laboratory within a timeframe of maximum 20 minutes (min) for further handling.

2.3. Buffer preparation

Hanks' Balanced Salt Solution with 1.26 mM calcium chloride dihydrate ($CaCl_2$) ($HBSS^+$) was used as the buffer of choice for the DNA release assay as well as to dilute stimulants for neutrophil activation and H3Cit measurement. To prepare 500 mL of $HBSS^+$, 50 mL of 10X HBSS (Gibco by Thermo Fisher Scientific, Waltham MA, US) was diluted in 450 mL double-distilled water (ddH_2O) and 92.61 mg $CaCl_2$ (Merck, Darmstadt, GER) were added. The solution was mixed by gently inverting and sterile filtered using a FILTER TOP 500 "rapid"-FILTERMAX (pore size: 0.22 µm; TPP Techno Plastic Products AG, Trasadingen, CH) on a sterile glass bottle. $HBSS^+$ was stored at room temperature in the dark.

PBS + 2% bovine serum albumin (BSA) was used to dilute antibodies for the H3Cit measurement. To 200 mL of 1X PBS, 4 g of BSA (Sigma-Aldrich, St. Louis MO, US) were added and left on room temperature until all BSA was dissolved. It was stored at 4°C for a maximum of one week.

Complete Roswell Park Memorial Institute-medium ($RPMI^+$) was used during stimulation time in neutrophil activation and H3Cit measurement, which was prepared by mixing 450 mL RPMI 1640 (Gibco) with 50 mL fetal calve serum (FCS, Gibco), 10 mL Penicillin-Streptomycin (100 U/mL and 100 µg/mL respectively; PenStrep; Gibco) and 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco). $RPMI^+$ was stored at 4°C.

2.4. Isolation of PMN and PBMC

Two density gradients, namely HISTOPAQUE®-1119 (Sigma-Aldrich) and Ficoll-Paque™ PREMIUM 1077 (GE Healthcare, Chicago Ill, US), were used for density-based cell isolation. HISTOPAQUE®-1119 was stored at 4°C but had to be brought up to room temperature before use. From HISTOPAQUE®-1119, 3 mL were added to a 15 mL polystyrene centrifuge conical tube (Falcon by Corning Inc., Corning NY, US) and 3 mL of Ficoll-Paque™ PREMIUM 1077 were carefully layered on top using a Costar® serological pipette (Corning Inc.) and an automated pipette controller (PIPETBOY 2; Integra LifeSciences, Plainsboro Township NJ, US). On top of the two gradients 6 mL of freshly drawn whole blood were layered carefully and the tube was centrifuged using an Allegra® X-12R centrifuge (Beckman Coulter, Brea CA, US) at 700 x g for 30 min at 20°C without brakes. A schematic representation of the centrifugation tube before and after centrifugation can be seen in Figure 8. After centrifugation, the plasma layer was carefully aspirated within 0.5 cm of the PBMC cell layer. Using a plastic transfer pipette (SARSTED AG & Co. KG, Nümbrecht, GER), PBMCs were transferred into a sterile 50 mL polystyrene centrifugation tube (VWR, Radnor PA, US). The gradient layer was also aspirated within 0.5 cm of the PMN layer. PMNs were transferred to a sterile 50 mL tube using a new plastic transfer pipette. To each tube containing cells, 25 mL of $HBSS^+$ was added and centrifuged for 5 min at 800 x g at room temperature to wash away gradient residuals. The supernatant was aspirated and the cell pellet was resuspended in 1 mL of $HBSS^+$. Cell counting was performed using a Sysmex XN-350 (Sysmex Austria GmbH, Vienna, AUT).

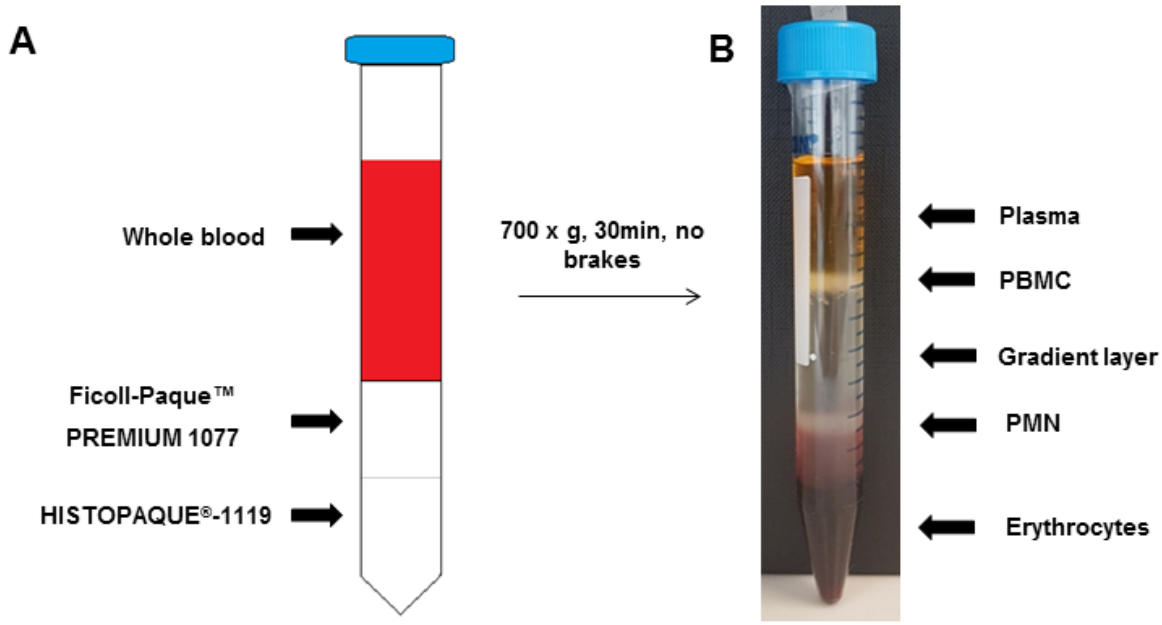

Figure 8: Schematic representation of blood isolation before and after centrifugation. (A) Whole blood layered over HISTOPAQUE®-1119 and Ficoll-Paque™ PREMIUM 1077 density gradients before centrifugation. **(B)** Separated cell layers after centrifugation. Picture taken and graphic designed by the author of this thesis.
PBMCs: peripheral blood mononuclear cells; PMNs: polymorphonuclear cells.

2.5. Neutrophil studies

2.5.1. DNA release assay

In a Nunc FluoroNunc MaxiSorp 96 well plate (Thermo Fisher Scientific), three different concentrations of both IO (2 mM stock concentration; Invitrogen, Carlsbad CA, US) and PMA (1 mM stock concentration; Sigma Aldrich) were prepared (Table 1). Table 1 shows this initial preparation. Both stimuli had then to be pre-diluted before adding the respective amounts to each well.

For measurement, SYTOX™ Green Nucleic Acid Stain (Invitrogen) was pre-diluted in a 1:100 ratio in $HBSS^+$ to a concentration of 50 µM.
Table 2 shows the exact amounts of reagents and buffer used for all pre-dilutions.

Table 1: Preparation of stock solutions

Reagent	Molecular Weight [g/mol]	DMSO	Stock Concentration
IO [1 µg]	747.07	670 µL	2 mM
PMA [10 µg]	616.83	16.21 mL	1 mM

Table 2: Pre-dilutions for all used reagents.

Reagent	From Stock [µL]	$HBSS^+$ [µL]	Dilutions	Final Concentration
IO [2 mM]	18	162	1 : 10	200 µM
PMA [1 mM]	11.7	1158.3	1 : 100	10 µM
Triton™ X-100	10	90	1 : 10	-
SYTOX™ Green [5 mM]	21.1	2090.1	1 : 100	50 µM

In the corresponding wells, calculated amounts of buffer were combined with the respective amounts of pre-diluted PMA and IO. As a positive control, Triton™ X-100 (Sigma-Aldrich), in a 1:10 dilution in $HBSS^+$, was added to the respective wells without stimuli. Triton leads to cell lysis (Borner et al. 1994), thereby it corresponds to a 100% release of DNA. Both PMNs and PBMCs were diluted in $HBSS^+$ to a concentration of 1 x 10^6 cells per mL and then added to the respective wells already containing buffer and stimuli. Thereby, final stimuli working concentrations of 4 µM, 6 µM and 8 µM for IO and 600 nM, 1 µM and 5 µM for PMA as well as a cell concentration of 1 x 10^5 cells per well was achieved. The plate was then incubated at 37°C for 2 h 45 min. After the initial incubation time, SYTOX™ Green was added to each well (final concentration: 5 µM), thereby reaching the final working volume of 300 µL per well. The plate was incubated for additional 15 min at 37°C and then immediately measured using a Multimodereader Varioskan LUX (ex. 485/em. 520; Thermo Fisher Scientific). Every condition was prepared and measured in triplicates. The respective amounts of used reagents, buffer and cell suspension are shown in Figure 9.

Condition	HBSS$^+$ [µL]	Cells (10^6 / mL) [µL]	Triton [µL from pre-dilution]	Stimulus [µL from working solution]	SYTOX™ Green [µL from pre dilution]	Total amount per well [µL]
Cells only	170	100	-	-	30	300
Positive Control	160	100	10	-	30	300
4 µM IO	164.6	100	-	5.4	30	300
6 µM IO	161.9	100	-	8.1	30	300
8 µM IO	159.2	100	-	10.8	30	300
600 nM PMA	153.8	100	-	16.2	30	300
1 µM PMA	143	100	-	27	30	300
5 µM PMA	35	100	-	135	30	300

Figure 9: Pipetting scheme for DNA release assay. The respective amounts of pre-diluted stimuli and HBSS$^+$ were added to the corresponding wells prior to cells and SYTOX™ Green. PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear cells; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HBSS: Hanks' Balanced Salt Solution; µL: microliter

2.5.2. Baseline neutrophils

Right after isolation, from both PMN and PBMC cell suspension (10^6 cells/mL in RPMI$^+$), 540 µL were fixed using 180 µL of a 4% paraformaldehyde solution (PFA, final concentration: 1%; Thermo Fisher Scientific, Waltham MA, US). These immediately fixed cells will be further referred to as baseline. For the analysis of neutrophil subsets, only baseline cells were used. In both the neutrophil activation study and in H3Cit measurement, a portion of these baseline cells were also used as control samples. In these two setups, baseline PMNs and PBMCs are stained with CD66b and propidium iodide (PI) (amongst other antibodies), which upon analysis using a CytoFLEX S (Beckman Coulter) gives information about respective neutrophil cell counts.

2.5.3. Neutrophil subset analysis

From baseline PMN and PBMC cell suspensions, 60 µL were mixed with 40 µL 1x PBS. Samples were stained with 12.5 µL of an antibody master mix consisting of CD66b, CD62L, CD16, CD41 and PI (2.5 µL of each antibody) for 15 min at room temperature in the dark. Additional information about the used antibodies can be seen in Table 4. Staining of PMN and PBMC samples was stopped with 300 µL of PBS. Unstained control samples served as negative controls. Samples were measured using a CytoFLEX S (Beckman Coulter), a detailed list of used lasers, filters and gains can be seen in Table 5B. Raw data was analysed with FlowJo V10 (FlowJo, LLC).

2.5.4. Neutrophil activation study

For this study, PMNs and PBMCs were diluted in room temperature RPMI$^+$ to a concentration of 10^6 cells/mL. Five different conditions for cell stimulation were prepared in 500 µL Eppendorf® tubes (Eppendorf AG, Hamburg, GER): unstimulated, 1 µM IO, 4 µM IO, 100 nM PMA and 600 nM PMA. To reach these concentrations, both stimuli had

to be pre-diluted. The exact amounts of stimuli and buffer used for all pre-dilutions as well as the pre-dilution concentrations can be seen in Table 3.

To stimulate cells, 45 µL of PMN or PBMC cell suspension was added to the corresponding tubes, where 5 µL of the respective pre-diluted stimuli have already been added. For unstimulated conditions, the corresponding amount of only $HBSS^+$ was added to the tube.

Cells were stimulated for 30 min at 37°C in a water bath, then fixed with 16 µL PFA (final concentration: 1%) and stored at room temperature for at least 30 min prior to further processing. During this time, an antibody master mix was prepared using 50 µL from each of the following antibodies: CD66b, CD11b, CD41 and PI. An exact overview of the used antibodies and the final concentrations used can be seen in Table 4. From each condition and respective immediately fixed baseline controls, 66 µL were transferred into 5 mL FACS tubes (Falcon by Corning Inc.) 10 µL of the antibody master mix was added to each sample and incubated for 15 min in the dark at room temperature. Staining for PMN and PBMC samples was stopped using 300 µL 1x PBS. Samples were measured using a CytoFLEX S (Beckman Coulter), a detailed list of used lasers, filters and gains can be seen in Table 5A. Raw data was analysed with FlowJo V10 (FlowJo, LLC).

2.5.5. H3Cit measurement

For H3Cit measurement, cells were diluted in pre-warmed $RPMI^+$ (37°C) to a concentration of 10^6 cells/mL. In this experimental setup the same conditions and hence the same stimuli pre-dilutions as for the neutrophil activation study were used (Table 3), which were also prepared in 500 µL Eppendorf® tubes (Eppendorf AG) prior to the addition of PMN and PBMC cell suspensions. The H3Cit measurement setup only differed in the amount of pre-dilutions and cell suspensions used, as both were doubled in volume (10 µL of stimuli, 90 µL of cell suspension). Again, final working concentrations for IO and PMA were achieved by adding the cell suspensions, thereby further diluting the already pre-diluted stimuli in a 1:10 ratio. The corresponding amount of only $HBSS^+$ was added to the tube for unstimulated conditions.

Cells were incubated at 37°C in a water bath for 3 h, with intermediate vortexing every 30 min, and then fixed with 32 µL of PFA (final concentration: 1%) for a minimum of 30 min. From each sample, 130 µL were transferred into separate 5 mL FACS tubes. Three additional tubes per cell fraction with 130 µL of immediately fixed baseline cells were prepared as well. These served as control conditions: no antibody staining at all (unstained), stained with only the secondary antibody (secondary-only) and stained the same way as stimulated samples (stained control). As a first step, all samples were washed using 500 µl PBS + 2% BSA and centrifuged at 3200 x g, 4°C for 10 min.

Afterwards the supernatant was discarded and 100 µL primary anti-H3Cit antibody (ab5103; Abcam, Cambridge, UK; diluted in PBS + 2% BSA in a 1:180 ratio) was added to all samples. Only unstained and secondary-only samples were resuspended in 100 µL PBS + 2% BSA only. After incubation for 20 min in the dark at room temperature, 500 µL of PBS + 2% BSA were added and cells were centrifuged again. The secondary antibody (goat anti-rabbit IgG) was diluted 1:10 000 in PBS + 2% BSA and one part of this dilution was used to mix with anti-CD66b antibody in a ratio of 1:40. After discarding the supernatant, the secondary antibody (100 µL) alone was used to stain secondary-only samples, whereas the secondary antibody mixed with anti-CD66b (100 µL) was used to stain the remaining samples. Again, unstained samples were resuspended in 100 µL PBS + 2% BSA. After further incubation and centrifugation as previously explained, 100 µL PI, diluted 1:50 in PBS + 2% BSA, was applied to all tubes, while unstained samples were again resuspended in only PBS + 2% BSA. Additional information to used antibodies can be found in Table 4. Again, samples were measured using a CytoFLEX S (Beckman Coulter), a detailed list of used lasers, filters and gains can be seen in Table 5C. Raw data was analyzed with FlowJo V10 (FlowJo, LLC).

Table 3: Stimuli pre-dilutions for neutrophil activation study and H3Cit measurement. (A) Pre-dilutions for Ionomycin (IO); both final concentrations were used as working solutions to further be diluted inside the test tubes. **(B)** Pre-dilutions for phorbol 12-myristate 13-acetate (PMA); the 100 µM final concentration was only used as a pre-dilution for the two working solutions (6 µM and 1 µM), which were then further diluted inside the test tubes.

Stimulus	Concentration	From Stimulus [µL]	HBSS$^+$ [µL]	Dilution	Final Concentration
A					
IO	2 mM	3	147	1 : 50	40 µM
	40 µM	25	75	1 : 4	10 µM
B					
PMA	1 mM	2	18	1 : 10	100 µM
	10 µM	6	94	1 : 16.66	6 µM
	6 µM	16.7	83.3	1 : 6	1 µM

Table 4: List of used antibodies.

Antibody	Conjugate	Company	Catalogue Nr.	Dilutions
CD66b	FITC	BioLegend	305104	1:4 in activation study; 1:5 in subset analysis
CD66b	PB	BioLegend	305112	1:40
CD62L	AF-647	BioLegend	304818	1:5
CD16	PE-Cy7	BioLegend	302016	1:5
CD41	PB	BioLegend	303714	1:4 in activation study; 1:5 in subset analysis
CD11b	APC	BioLegend	301310	1:4
H3Cit		Abcam	ab5103	1:180
Goat-anti rabbit IgG 2nd antibody	AF-647	Invitrogen	A21246	1:10000
Propidium iodide (PI)		BD Bioscience	556547	1:4 in activation study; 1:5 in subset analysis; 1:50 in H3Cit measurement

Table 5: CytoFLEX S laser and software settings. (A) Settings for neutrophil activation study. **(B)** Settings for neutrophil subset analysis. **(C)** Settings for H3Cit measurement; nm: nanometer

Channel	Antigen	Laser	Excitation (nm)	Filter	Gain
A					
FSC	-	-	-	-	83
SSC	-	-	-	-	14
FITC	CD66b	blue	488	525/40	171
APC	CD11b	red	638	660/20	422
PB	CD41	violet	405	450/45	30
PI	life-dead	yellow-green	561	610/20	70
B					
FSC	-	-	-	-	83
SSC	-	-	-	-	14
FITC	CD66b	blue	488	525/40	171
AF-647	CD62L	red	638	660/20	422
PE-Cy7	CD16	yellow	561	780/60	72
PB	CD41	violet	405	450/45	30
PI	life-dead	yellow-green	561	610/20	70
C					
FSC	-	-	-	-	83
SSC	-	-	-	-	14
AF-647	2° AB for H3Cit	red	638	660/20	422
PB	CD66b	violet	405	450/45	30
PI	life-dead	yellow-green	561	610/20	70

2.6. Flow cytometry gating strategy

2.6.1. Gating for neutrophil subsets

To correctly identify neutrophils in their different subset states amongst all recorded events within the full FACS blot, an initial gate only including single cells was defined (Figure 10A). From these cells, only CD66b positive cells (neutrophils) were gated (Figure 10B), following an additional gate separating living from dead (PI positive) neutrophils (Figure 10C). Those living, CD66b positive neutrophils were then blotted to represent their positivity for CD16 and CD62L (Figure 10D), thereby enabling a separation of the three subsets: activated (Q1 and Q4), mature (Q2) and newly released (Q3) (Figure 10E). A graphical representation of this gating strategy can be seen in Figure 10.

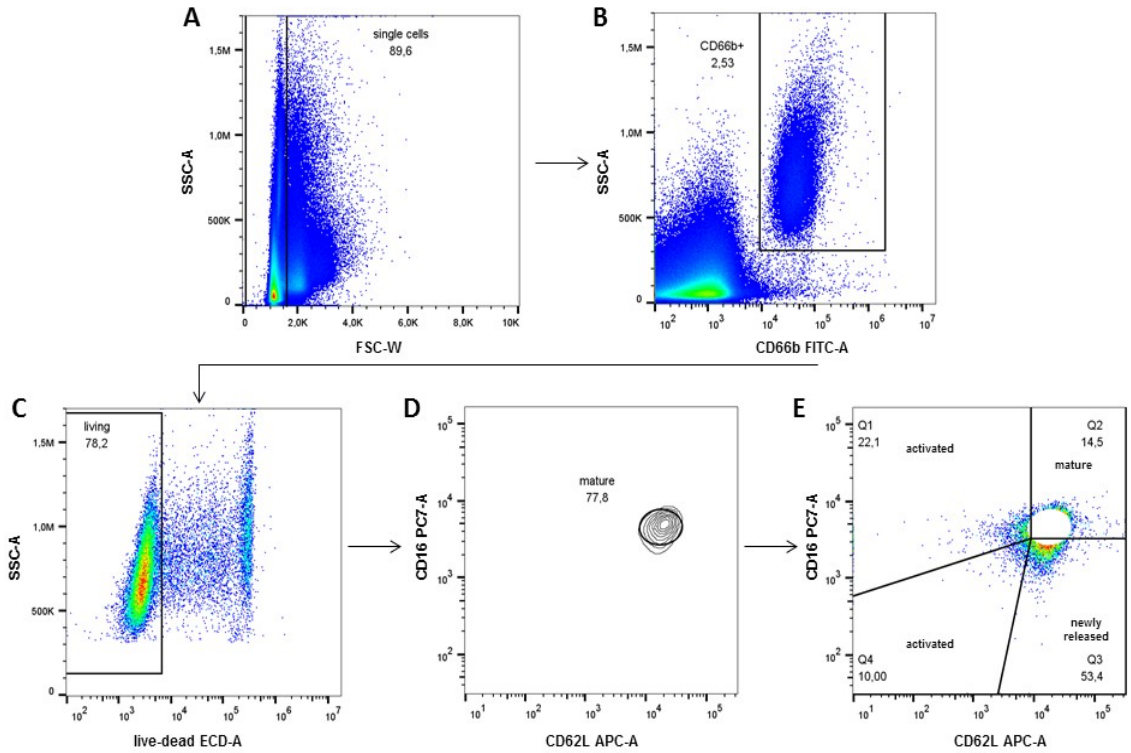

Figure 10: Gating strategy for neutrophil subset analysis. Subsets were defined by gating single, CD66b positive, living cells according to their expression of markers CD16 and CD62L.

2.6.2. Gating for neutrophil activation

In the same manner as for neutrophil subset analysis, cells for neutrophil activation studies were also gated as living, CD66b positive, single cells (Figure 11A). As a reference for activation, measured according to the increase in CD66b and CD11b positivity, the gate for both CD66b and CD11b positivity was set to 1% in immediately fixed (baseline) neutrophils (Figure 11B) and then applied to all other samples, where a significant increase in positivity for both activation markers is visible upon stimulation with 4 µM IO (Figure 11C).

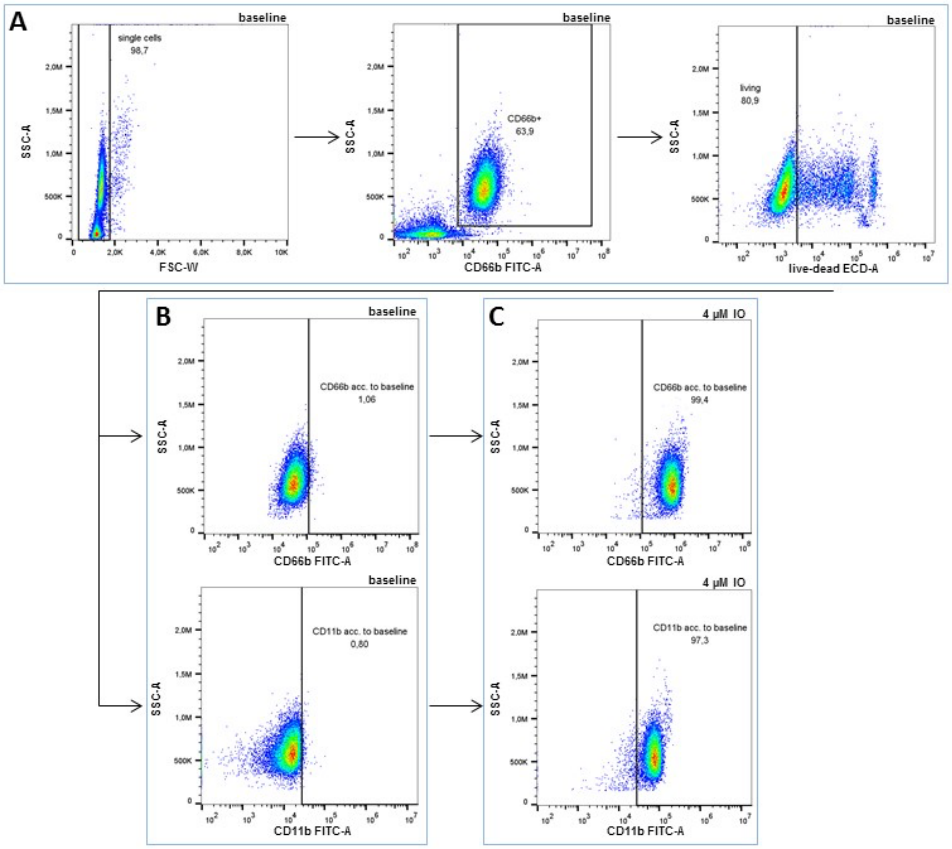

Figure 11: Gating strategy for neutrophil activation studies. An increase in neutrophil activation was measured according to the increasing positivity of activation markers CD66b and CD11b in living, CD66b positive, single cells.

2.6.3. Gating for H3Cit measurement

Gating for increased H3Cit positivity was also performed in immediately fixed (baseline) cells. Since an exact gating for H3Cit positivity is not possible under these conditions, the changes in median fluorescent intensity (MFI) of dead (Figure 12A), CD66b positive (Figure 12B) cells was measured in baseline cells and taken as a reference for changes in H3Cit MFI upon activation. In this graphical representation of the H3Cit gating strategy, baseline H3Cit MFI (Figure 12C) is compared to H3Cit MFI upon stimulation with 4 µM IO (Figure 12D).

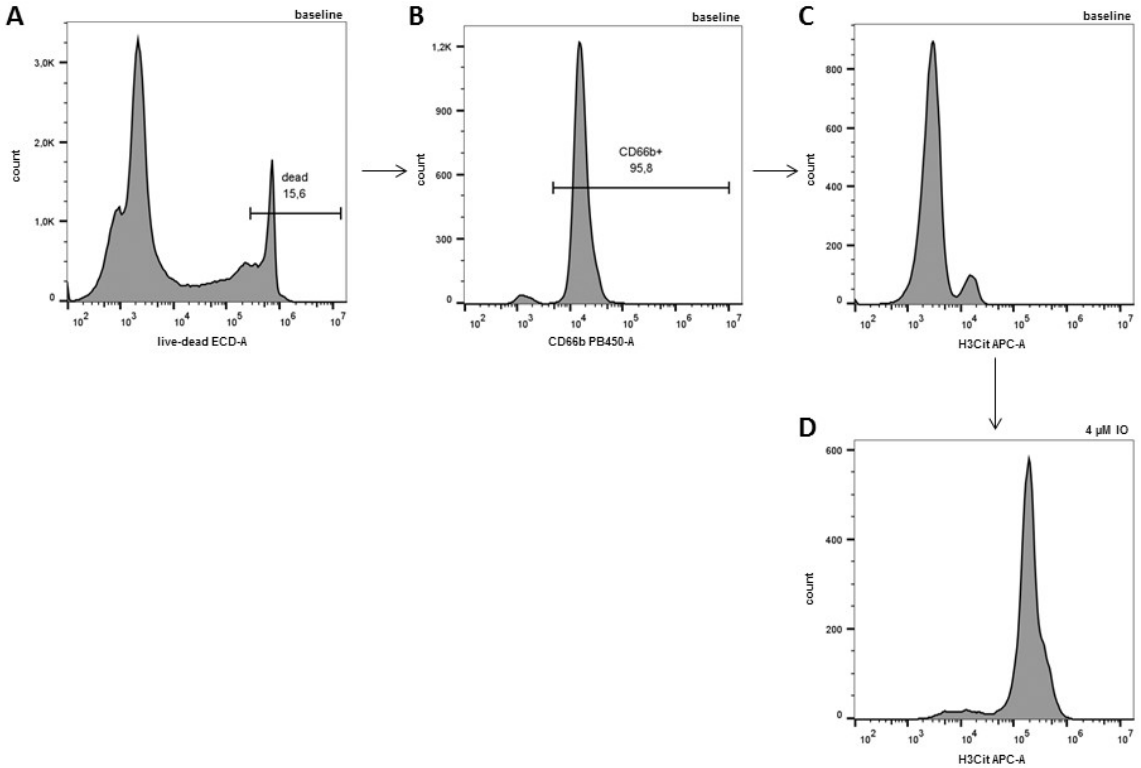

Figure 12: Gating strategy for H3Cit measurements. An increase in H3Cit MFI was measured according to baseline CD66b positive, dead cells.

3. Results

3.1. Study cohort and descriptive parameters

This study included 20 patients with newly diagnosed lung cancer from the CATS study cohort as well as 20 healthy individuals as a control cohort. Study participants were sex-matched (11 females in each cohort), but patients with lung cancer where slightly older (median age [min-max]: 67 [46-82] than healthy donors (median [min-max]: 62 [50-72]). Cancer patients had higher platelet counts, leucocyte counts and absolute as well as relative neutrophil counts. Factor VIII activity, prothrombin fragment 1 and 2 as well as fibrinogen were also elevated in patients with lung cancer. None of the patients had a history of VTE prior to study inclusion. Values can be seen in Table 6. Recruiting occurred between October 2018 and June 2019. All given information and values were determined via a structured interview as well as hematologic and routine laboratory testing of all participants.

Table 6: Descriptive parameters: healthy donors vs. patients with lung cancer (A) General information gathered via the structured interview. **(B)** Selected laboratory values. min: minimum; max: maximum; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study

		HD (n=20)	CATS (n=20)
A	**General information**		
	Age, median (min-max)	62 (50-72)	67 (46-82)
	Sex, female n (%)	11 (55)	11 (55)
B	**Laboratory values median (IQR)**		
	Platelet count	224 (214-321)	284 (211.75-377)
	Mean platelet volume	10.5 (9.85-11.17)	10.2 (9.325-10.55)
	Leucocyte count	5.29 (4.765-6.1375)	8.195 (5.9375-10.165)
	Absolute neutrophil count	3 (2.5-3.3)	5.25 (3.925-5.875)

Relative neutrophil count	54.8 (48.5-58.8)	67.5 (59.375-79.5)
Haemoglobin	14.2 (13.05-15.225)	13.6 (12.4-14.9)
D-dimer	0.3 (0.27-0.36)	0.62 (0.43-1.01)
Factor VIII activity	143.5 (135-167)	206 (175-260)
Prothrombin fragment 1+2	209.5 (148.5-233.5)	239 (182-267)
Fibrinogen	321.5 (281.75-332.75)	428.5 (385-518.5)

3.2. Neutrophil characteristics

Cell counts of HDNs and LDNs differed significantly from healthy donors to patients with lung cancer. The comparison of neutrophil subpopulation counts (measured as HDN or LDN per mL blood) revealed that cancer patients had 1.9-fold (*p=0.0009*; Figure 13A) increased HDN counts and 8.7-fold (*p=0.00065*; Figure 13B) increased LDN counts compared to healthy donors. The amount of PMN/mL is significantly increased in patients with lung cancer compared to healthy individuals (1.75-fold, *p=0.00015*; Figure 13C), whereas no significant changes in PBMC cell count could be observed between the two groups (Figure 13D).

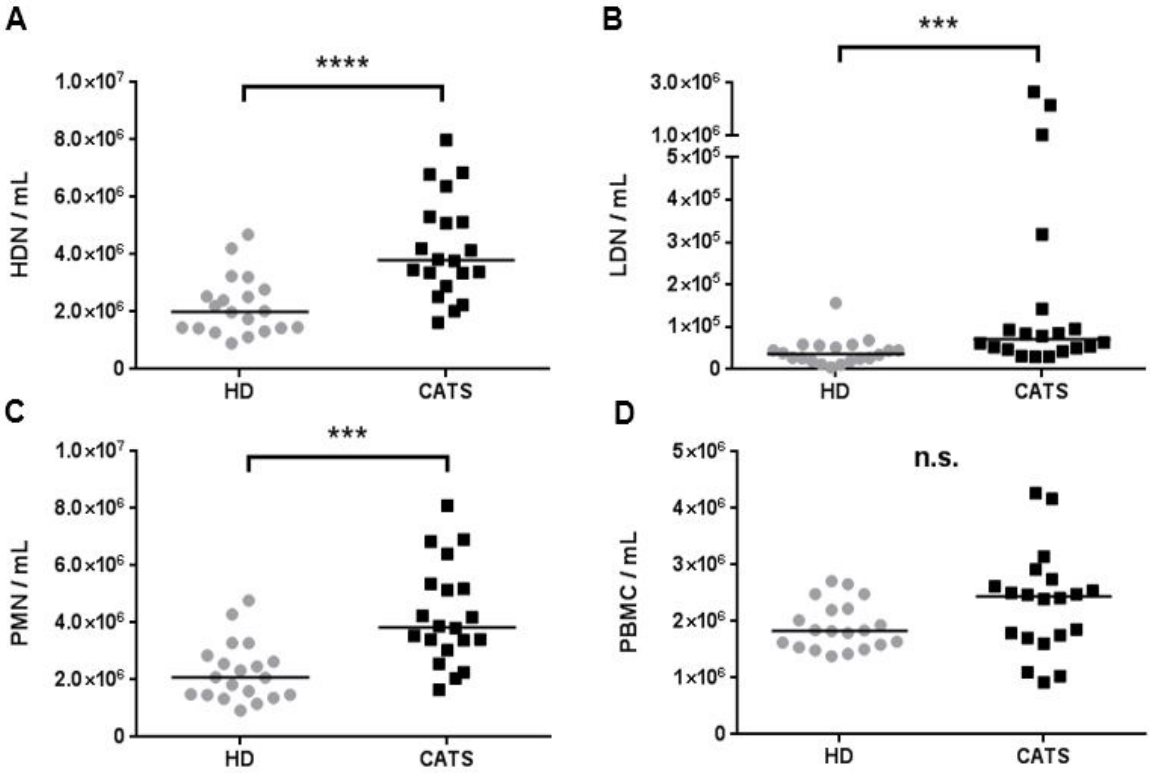

Figure 13: Differences in cell counts between healthy donors and patients with lung cancer. Differences in **(A)** HDN / mL **(B)** LDN / mL **(C)** PMN / mL and **(D)** PBMC / mL. CATS samples shown in black, HD samples shown in grey. HDN: high density neutrophils; LDN: low density neutrophils; PBMC: peripheral blood mononuclear cells; PMN: polymorphonuclear cells; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; n.s.: not significant; ***: $p < 0.001$; ****: $p < 0.0001$

3.3. Size and granularity of neutrophil subpopulations in patients and healthy controls

Differences in size and granularity according to their forward scatter (FSC) and side scatter (SSC) was observed when HDNs and LDNs from our two cohorts were compared. As these cells have been fixed immediately after isolation, they represent the most accurate picture of how cells appear inside circulation. No significant difference in FSC, representing a cells size, was observed in HDNs of patients and controls, but a trend towards increased FCS in healthy individuals could be observed. Cell size was significantly decreased in LDNs from patients with lung cancer (0.9-fold decrease, *p=0.009*). Neutrophil subpopulations from patients with lung cancer showed lower granularity according to their SSC (HDN *p=0.002*, LDN *p=0.0019*, Figure 14).

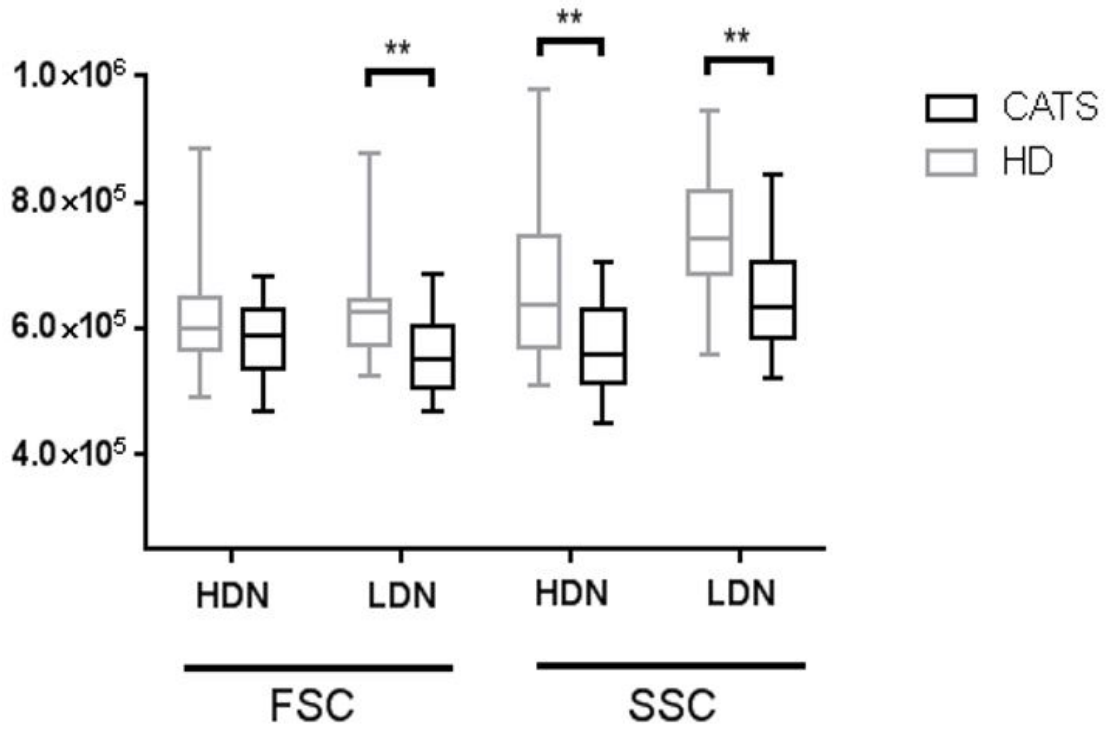

Figure 14: Changes in forward and side scatter between healthy donors and patients with lung cancer. HDNs undergo changes only in granularity, while LDNs change in both size and granularity upon disease. CATS samples shown in black, HD samples shown in grey. FSC: forward scatter; SSC: side scatter; HDN: high density neutrophils; LDN: low density neutrophils; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; **: $p < 0.01$

3.4. Effects of IO and PMA stimulation on size and granularity of neutrophil subpopulations

No differences in cell size were observed in HDNs isolated from patients with lung cancer and healthy controls, neither at baseline nor in unstimulated or stimulated conditions (Figure 15A). However, significant differences in HDN SSC between healthy individuals and patients with lung cancer were observed in all conditions. HDNs from patients with lung cancer decreased in granularity at baseline (*p=0.0012*), unstimulated (*p=0.00011*), upon activation with both concentrations of IO (1 µM: *p=0.005*, 4 µM: *p=0.01*) as well as both concentrations of PMA (100 nM: *p=0.006*, 600 nM: *p=0.004*; Figure 15B). Only stimulation with low dose IO resulted in significant decrease in size for LDNs from patients with lung cancer (1 µM: *p=0.0093*; Figure 15C). Significantly decreased granularity in LDNs from patient with cancer could be observed within the unstimulated condition (*p=0.0004*). Only cells treated with IO changed significantly in granularity (1 µM: *p=0.004*, 4 µM: *p=0.03*), whereas PMA treatment did not affect LDN SSC (Figure 15D).

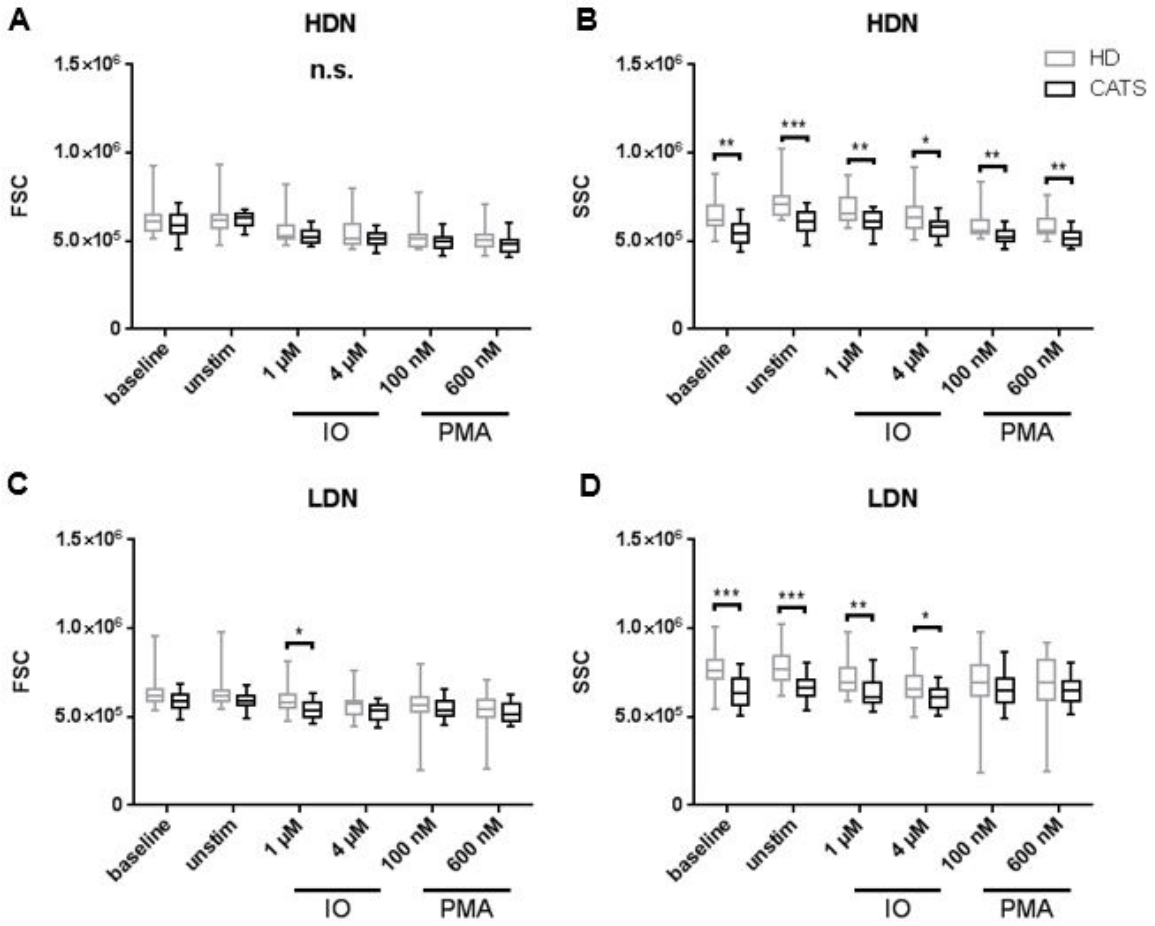

Figure 15: The effects of activation on forward and side scatter. Depiction of the effects of activation on HDN **(A)** forward scatter and **(B)** side scatter. **(C)** Effects of activation on LDN forward scatter and **(D)** side scatter. CATS samples shown in black, HD samples shown in grey. FSC: forward scatter; SSC: side scatter; HDN: high density neutrophils; LDN: low density neutrophils; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

3.5. Changes in neutrophil activation potential between HD and CATS

3.5.1. Changes in HDN activation potential

HDN stimulation for 30 min revealed a comparable increase in CD66b MFI upon stimulation with IO and PMA without significant differences between healthy individuals and patients with lung cancer (Figure 16A). Similar CD11b MFI was observed at baseline and within unstimulated samples when comparing neutrophil subsets of both cohorts, whereas stimulation with both IO (1 µM: *p<0.0001*, 4 µM: *p<0.0001*) and PMA (100 nM: *p=0.0002*, 600 nM: *p=0.0008*) resulted in significantly elevated CD11b fluorescence

intensity of CATS HDNs (Figure 16B). In a similar manner to CD66b MFI, a percentual upregulation of this activation marker was observed in both cohorts upon stimulation (Figure 16C). The results of increased CD11b MFI upon stimulation are also reflected on the corresponding percentual distributions of CD11b expressing HDNs. The measurements lead to the observation that samples from patients with lung cancer stimulated with IO (1 µM: *p<0.0001*, 4 µM: *p<0.0001*) and PMA (100 nM: *p<0.0001*, 600 nM: *p<0.0001*) were significantly higher in their percentual CD11b expression (Figure 16D).

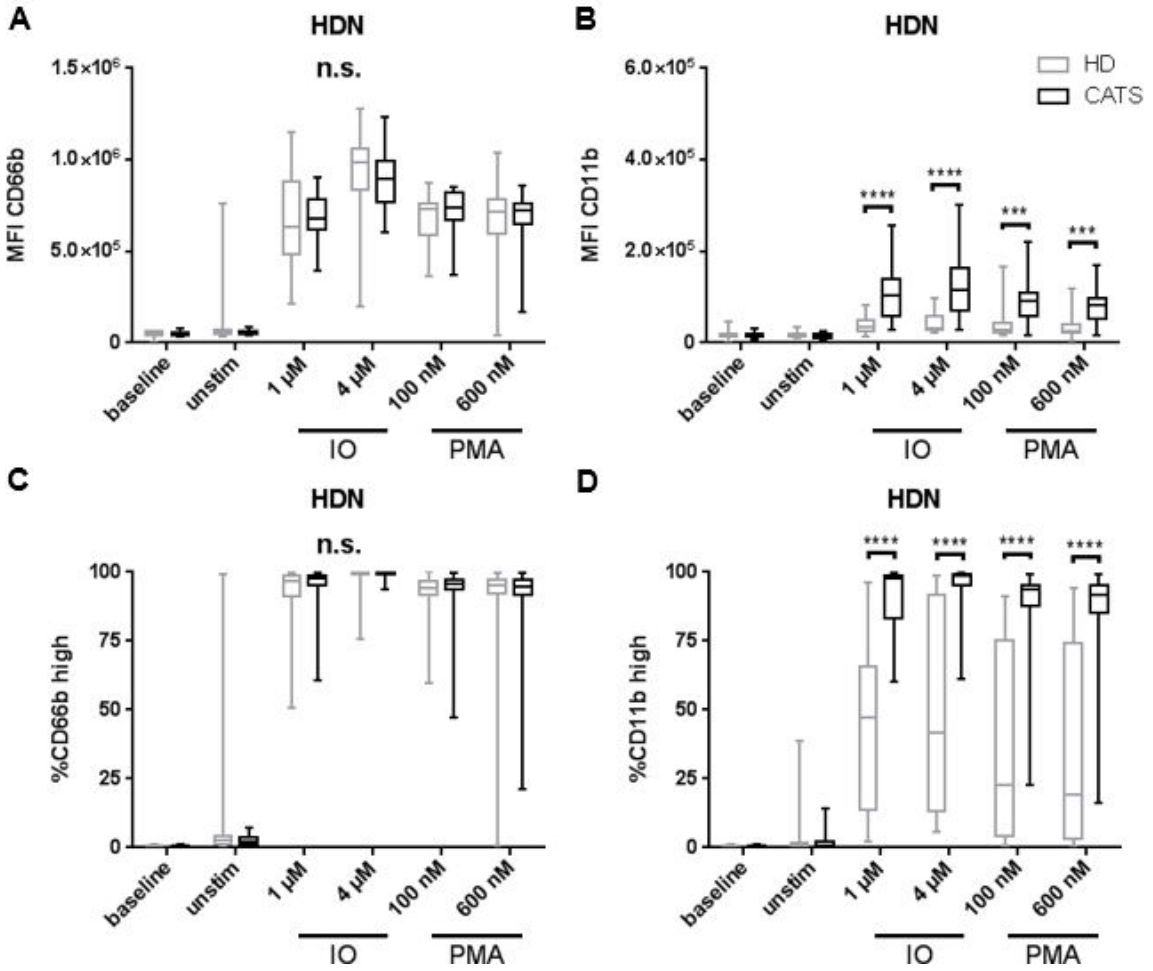

Figure 16: Differences in HDN activation potential between healthy donors and patients with lung cancer. Effect of activation on HDN **(A)** CD66b MFI, **(B)** CD11b MFI, **(C)** percentual CD66b expression and **(D)** percentual CD11b expression. CATS samples shown in black, HD samples shown in grey. MFI: median fluorescence intensity; HDN: high density neutrophils; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; n.s.: not significant; ***: p < 0.001; ****: p < 0.0001

3.5.2. Changes in LDN activation potential

Similar to results obtained from HDN measurements (Figure 16A and C), LDNs showed no significant differences in CD66b expression, neither when measuring the MFI (Figure 17A) nor the respective percentual distribution (Figure 17C). A significant increase in CD11b MFI in CATS LDNs could be observed upon stimulation with both doses of IO (1 µM: *p=0.0008*, 4 µM: *p=0.001*) and high dose PMA (600 nM: *p=0.0197*, Figure 17B). Low dose PMA (100 nM) did not result in a significant elevation in fluorescence intensity, but a trend can be assumed (*p=0.056*, Figure 17B). The percentual positivity of CATS LDNs was observed to be significantly increased in all stimulated samples, with IO stimulation leading to a significantly more intense increase in LDN activation (1 µM: *p=0.0004*, 4 µM: *p=0.0004*) than PMA stimulation (100 nM: *p=0.012*, 600 nM: *p=0.002*; Figure 17D).

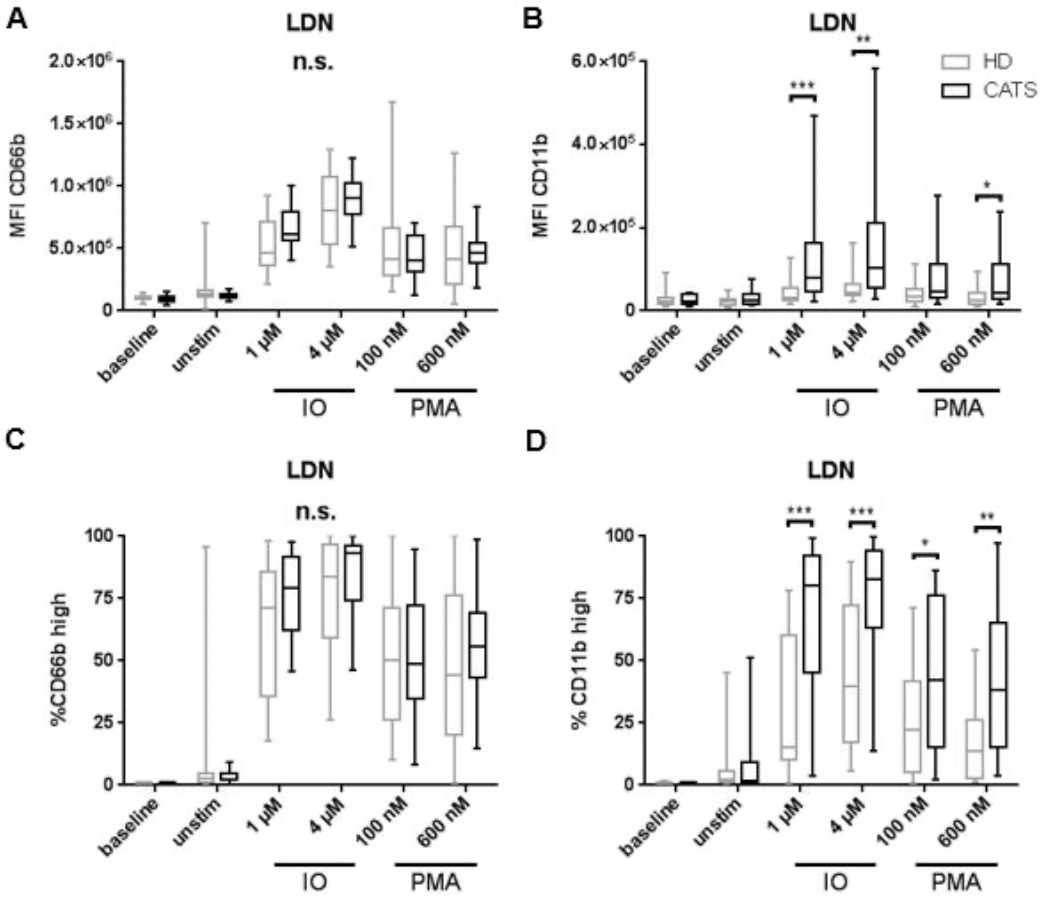

Figure 17: Differences in LDN activation potential between healthy donors and patients with lung cancer. Effects of activation on LDN, **(A)** CD66b MFI, **(B)** CD11b MFI, **(C)** percentual CD66b expression and **(D)** percentual CD11b expression. CATS samples shown in black, HD samples shown in grey. MFI: median fluorescence intensity; LDN: low density neutrophils; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

3.6. Changes in neutrophil subpopulation distribution

Neutrophil subdivision according to CD62L and CD16 expression showed no difference between HDNs from healthy controls and patients with lung cancer (Figure 18A). In LDNs however, levels of activated neutrophils ($CD62^{low}/CD16^{high}$) were significantly increased in patients with lung cancer (*p=0.035*; Figure 18B).

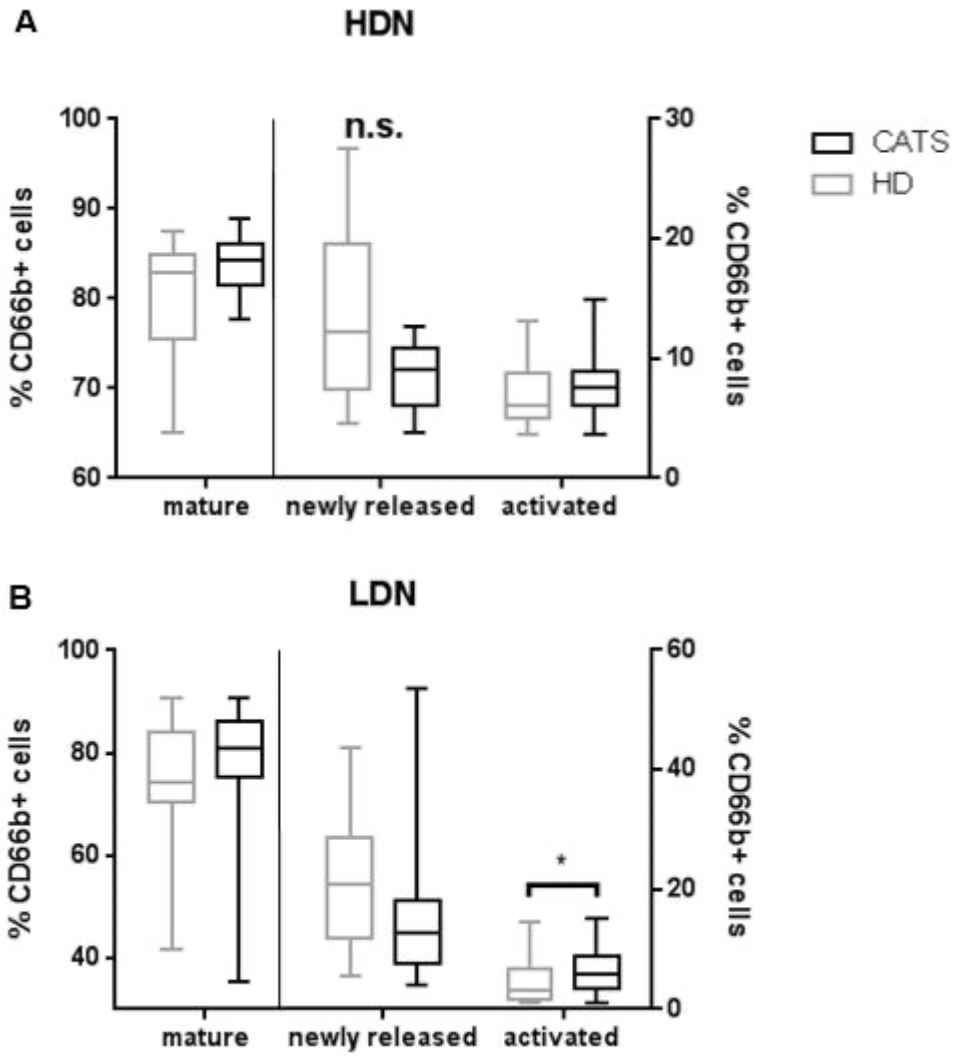

Figure 18: Maturation and activation state of HDNs and LDNs in healthy donors and patients with lung cancer. Percentage of mature ($CD62^{high}/CD16^{high}$), activated ($CD62^{low}/CD16^{high}$) and newly released ($CD62^{high}/CD16^{low}$) neutrophils in **(A)** HDN and **(B)** LDN. CATS samples shown in black, HD samples shown in grey. HDN: high density neutrophils; LDN: low density neutrophils HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; n.s.: not significant; *: $p < 0.05$

3.7. Differences in PMN and PBMC DNA release between HD and CATS

PMNs from healthy individuals showed higher spontaneous (unstim) DNA release (*p=0.0058*), which could also be seen upon high dose IO stimulation (8 µM: *p=0.043*; Figure 19A). Isolated PBMCs from healthy donors as well showed increased levels of spontaneously released DNA (*p=0.0014*). Significant differences in DNA release could be observed upon low dose IO treatment (4 µM: *p=0.025*) as well as low and middle dose PMA stimulation (600 nM: *p=0.0027*, 1 µM: *p=0.0008*; Figure 19B) of HD PBMCs.

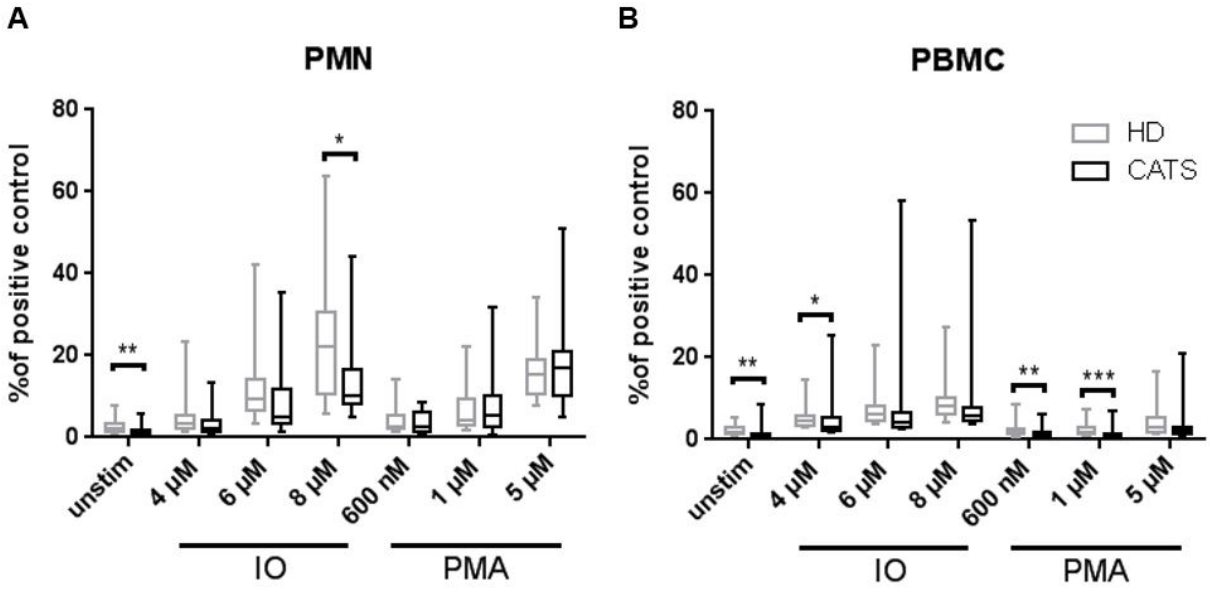

Figure 19: PMN and PBMC DNA release in healthy donors and patients with lung cancer. Effects of IO and PMA stimulation on DNA release of **(A)** isolated PMNs and **(B)** isolated PBMCs. CATS samples shown in black, HD samples shown in grey. PMN: polymorphonuclear cells; PBMC: peripheral blood mononuclear cells; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

3.8. Differences in HDN and LDN H3Cit positivity between HD and CATS

HDNs from patients with lung cancer showed elevated positivity in H3Cit, measured according to their MFI, upon stimulation with IO (1 µM: *p=0.017*, 4 mM: *p=0.017*). Stimulation with PMA however did not result in any significant differences between the two groups (Figure 20A). Interestingly, the opposite effect could be seen in isolated LDNs, as IO did not result in significant changes in H3Cit positivity, whereas both doses of PMA lead to decreased levels of H3Cit in LDNs from patients with lung cancer (100 nM: *p=0.035*, 600 nM: *p=0.045*; Figure 20B).

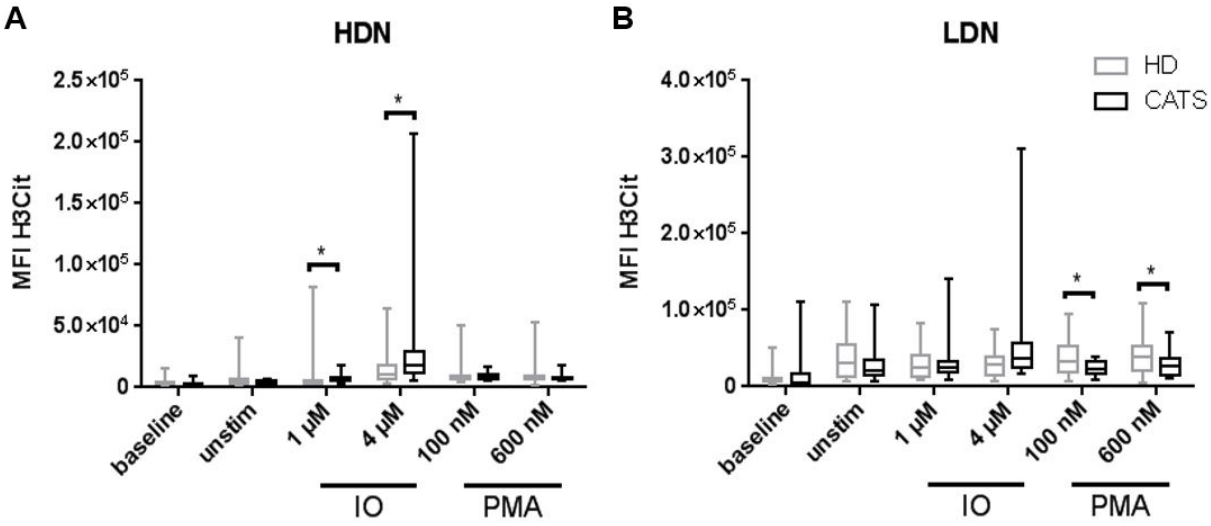

Figure 20: Median fluorescent intensity of H3Cit positive HDNs and LDNs in healthy donors and patients with lung cancer. Effects of IO and PMA stimulation on H3Cit positivity of **(A)** isolated HDNs and **(B)** isolated LDNs. CATS samples shown in black, HD samples shown in grey. HDN: high density neutrophils; LDN: low density neutrophils; IO: ionomycin; PMA: phorbol 12-myristate 13-acetate; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; *: $p < 0.05$

3.9. A technical comparison of two different cell measurement systems

To validate results of percentual occurrences of LDNs within isolated PBMC obtained from two different cell measurement techniques and systems, we directly compared the Sysmex XN-350 cell counter and the CytoFLEX S flow cytometer in regard to the respectively measured percentual occurrences of LDNs in healthy control as well as lung cancer patient samples. It could clearly be seen that, for both samples from healthy donors (Figure 21A) as well as from patients with lung cancer (Figure 21B), no significant differences between the two measurement techniques were observed.

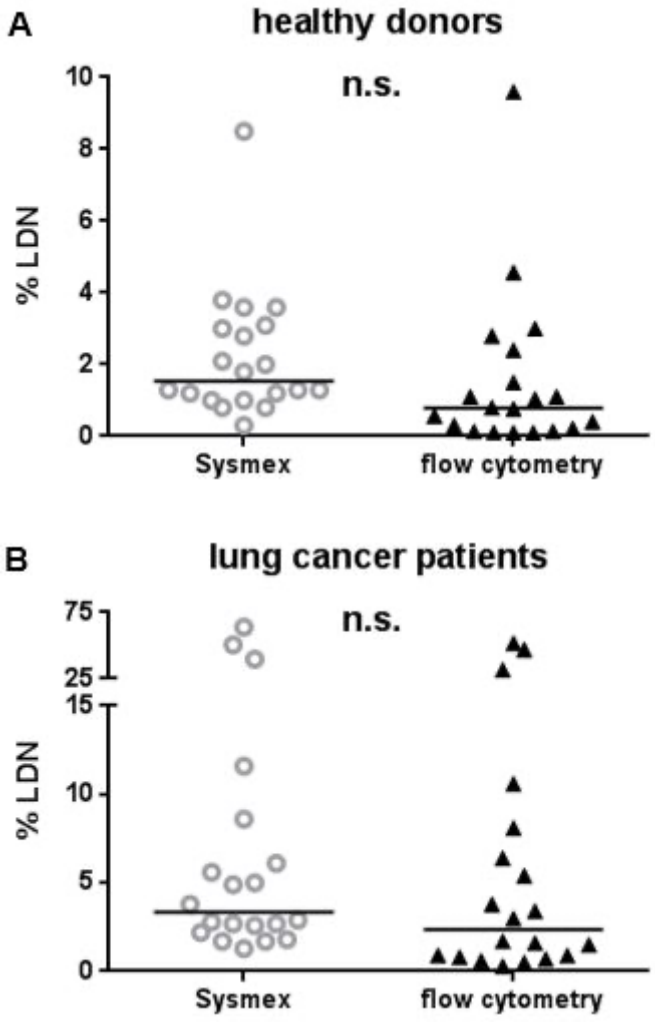

Figure 21: Sysmex XN-350 vs CytoFLEX S: a technical comparison. The results for measured percentual LDN ratios in PBMCs in **(A)** healthy donors and **(B**) patients with lung cancer were directly compared between the two measuring methods. CytoFLEX S-measured samples shown in black, Sysmex XN-350-measured samples shown in grey. LDN: low density neutrophils; n.s.: not significant

To further investigate similarities between the two measurement methods, percentual ratios of LDNs were compared between healthy donors and patients with lung cancer. Both sample sets showed significant differences when measured utilizing the Sysmex XN-350 (*p=0.0022*; Figure 22A) as well as when measured with the CytoFLEX S (*p=0.0068*; Figure 22B).

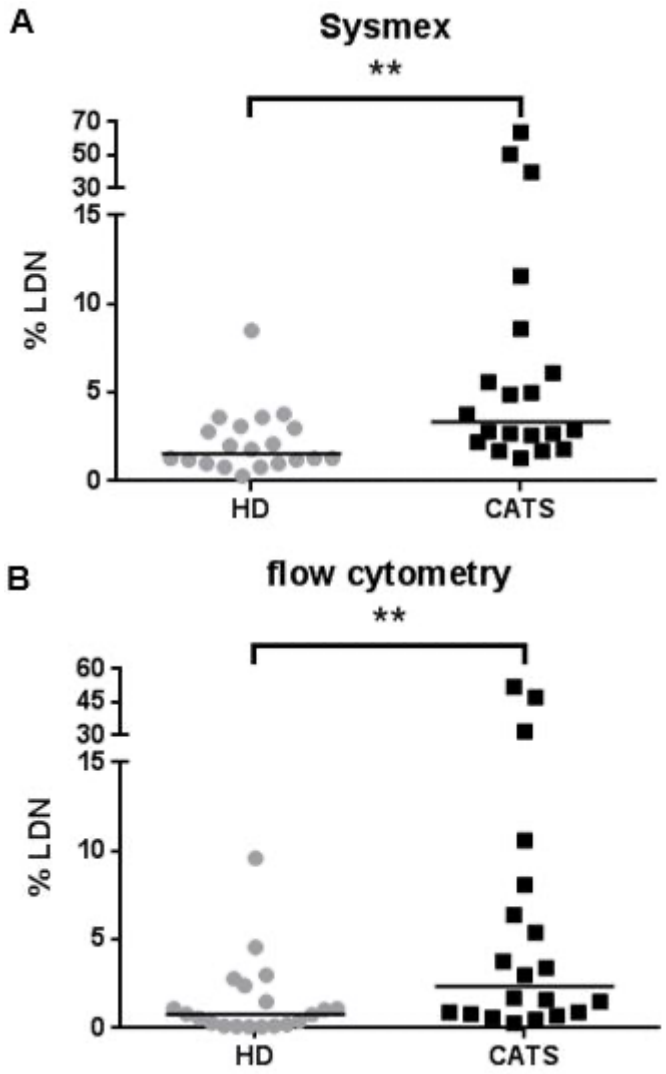

Figure 22: Percentual ratios of LDN in healthy donors vs patients with lung cancer: comparing results from Sysmex XN-350 and CytoFLEX S. Measurements of percentual LDN ratios from healthy donors and patients with lung cancer obtained using a **(A)** Sysmex XN-350 and a **(B)** CytoFLEX S were directly compared. CATS samples shown in black, HD samples shown in grey. LDN: low density neutrophils; HD: healthy donors; CATS: recruited patients with lung cancer within the Vienna Cancer and Thrombosis Study; **: $p < 0.01$

4. Discussion

When the human body is invaded by microorganisms, neutrophil granulocytes are the first responders to counteract intruders and protect the system from potential harm. They do so by utilizing three distinct response mechanisms, namely phagocytosis, degranulation and the formation of neutrophil extracellular traps (NETs).

Originally thought to be homogenous, it is now known that neutrophils can be categorized into distinct subsets. They can be subdivided either according to their state of maturation and activation by investigating the expression of surface markers CD16 and CD62L or according to their density utilizing a density gradient based cell isolation method. With this method of isolation, high density neutrophils (HDNs) are located within the layer of polymorphonuclear leukocytes (PMNs) and low density neutrophils (LDNs) can be found within the peripheral blood mononuclear cells (PBMCs).

Since the discovery of NET formation in 2004 (Brinkmann et al. 2004), many new insights into the nature of neutrophils have been uncovered. NETs have become the focus of different research topics, including but not limited to autoimmune diseases, HIV, cancer and thrombosis. Connections to and their role in those diseases have shed new light on how certain diseases come into existence, progress or resolve due to the involvement of neutrophils. Distinct aspects of these diseases, such as the tumour microenvironment in patients with cancer, are known to significantly influence neutrophils. Here we hypothesised that neutrophils undergo changes in neutrophil subsets, activation potential, DNA release as well as NET formation and release according to NET marker H3Cit. The aim of this study was to investigate these potential differences in neutrophils from patients with newly diagnosed lung cancer compared to healthy volunteers.

In the context of cancer, neutrophils with different densities have been accredited opposing attributes. HDNs show tumour-protective properties and no impairments in normal neutrophil functions, whereas LDNs were found to act pro-tumorigenic, immunosuppressive, show impaired neutrophil functions and can even promote angiogenesis and metastatic seeding (Liu et al. 2017). Hence, we hypothesized that LDNs occur in larger quantities in patients with lung cancer compared to healthy individuals.

When NET formation occurs without a pathogenic trigger within circulation, NETs provide a scaffold for circulating tumour cells to adhere to, thereby furthering metastatic seeding (Jablonska et al. 2017). They also bind and activate platelets, leading to thrombus formation, which is a common and often fatal occurrence in patients with cancer (Fuchs et al. 2010). We hypothesized that LDNs as well as HDNs from patients

with lung cancer have a higher activation potential, release more DNA and show an increased predisposition to undergo NET formation upon stimulation with ionomycin (IO) and phorbol 12-myristate 13-acetate (PMA) as compared to LDNs and HDNs from healthy individuals.

This study found that HDN and LDN cell counts were significantly increased in patients with lung cancer. Both patients' HDNs and LDNs showed significantly decreased granularity at baseline level as well as upon stimulation with IO and PMA, whereas only LDNs from patients with lung cancer were significantly decreased in size as compared to those from healthy volunteers.

Upon stimulation, HDNs and LDNs from patients with lung cancer showed increased activation potential according to the expression of CD11b, but no changes in expression levels of CD66b could be observed.

Changes in neutrophil subset distribution were also found in patient LDNs, as they showed a significant increase in activated neutrophils compared to cells from healthy individuals, without a reduction in newly released and mature neutrophils. No such changes could be seen when comparing HDNs from healthy volunteers to those from patients with lung cancer.

Interestingly, cells from lung cancer patients showed decreased levels of DNA release, with significant differences in PMNs at the unstimulated state as well as upon high dose IO stimulation. Within PBMCs, a significant decrease could also be observed with unstimulated cells, upon low dose IO stimulation and at low and middle dose PMA stimulation.

When investigating differences in NET release, we observed that HDNs from patients with lung cancer showed significantly more H3Cit positivity upon IO stimulation, whereas patient LDNs significantly decreased in H3Cit positivity upon PMA stimulation as compared to cells from healthy volunteers.

Additionally, we investigated certain predictive parameters such as platelet, leukocyte and neutrophil counts as well as factor VIII activity, levels of prothrombin fragment 1+2, fibrinogen and D-dimer. These predictive parameters were found to be elevated in patients with newly diagnosed lung cancer compared to healthy individuals.

Within several of our experimental approaches, a proportion of investigated cells are described as "baseline". As mentioned before, these baseline HDNs and LDNs have been fixed immediately after isolation. Thereby they represent the most accurate picture of circulating cells and how they have been influenced solely by the nature of the disease.

4.1. Observed other aspects

As expected, platelet counts were elevated amongst patients with newly diagnosed lung cancer (Table 6), as this effect has been described quite thoroughly in the literature (Yuan et al. 2020). A highly elevated platelet count, known as thrombocytosis (defined by a platelet count above 350 x 10^9 / L (Khorana et al. 2008)), can have a multitude of origins (Schafer 2004), but several studies have already elucidated the connection between elevated platelet count and cancer. It has been shown that thrombocytosis can be a predictive indicator for future lung cancer occurrence (Hamilton, Peters, et al. 2005) as well as for poor prognosis (Ji et al. 2015). A prospective cohort study conducted in 2017 states that amongst adults with thrombocytosis, 11.6% of male and 6.2% of female study participants developed malignancies. These numbers increased to 18.1% and 10.1%, respectively, if a second raise in platelet count was measured within 6 months after the initial thrombocytosis measurement (Bailey et al. 2017). This predictive aspect also holds true for other types of cancer such as colorectal (Hamilton, Round, et al. 2005), uterine (Walker, Hyde, and Hamilton 2013) and renal cancer (Shephard et al. 2013). In other cancer types however, such as ovarian (Hamilton et al. 2009) and breast cancer (Walker, Hyde, and Hamilton 2014), thrombocytosis is not predictive. However, since in our study we did not investigate cancer-associated changes in platelets, we cannot make any further assumptions on potential disease progression or outcome in our patients based on their platelet count measurements.

Our results additionally showed an increase in factor VIII activity, elevated levels of prothrombin fragment 1+2, increased amounts of fibrinogen as well as higher D-dimer levels in patients with lung cancer (Table 6) compared to healthy individuals. Since the general risk of suffering from thrombotic events is significantly elevated in patients with malignancies (Zwicker, Furie, and Furie 2007), several studies have highlighted the fact that these patients show increased levels of coagulation proteins (such as factor VIII; Vormittag et al. 2009), higher fibrinogen levels (Edwards et al. 1987) as well as an increase in certain biomarkers (such as prothrombin fragment 1+2; Goldenberg, Kahn, and Solymoss 2003). Elevated levels of D-dimer have also previously been reported (Ay et al. 2009). These preceding findings were reflected very well by our observations regarding the aforementioned factors and markers.

We also observed an elevation in leukocyte count in our cohort of patients with lung cancer (Table 6). Elevated leukocyte count, also known as leucocytosis, was found to be predictive for VTE occurrence in patients with cancer (Khorana et al. 2008) and is also associated with increased mortality in some cancer subtypes, including lung carcinoma (Kasuga et al. 2001). However, the observed median leukocyte count in

samples derived from lung cancer patients did not exceed 11 x 10^9 / L, which is considered the baseline value for leucocytosis (Khorana et al. 2008). To make further predictions on potential VTE occurrences and disease progression based on leukocyte count, a follow-up study focusing on individual patients would have to be conducted.

Our observations also showed an increase in absolute and relative neutrophil count in patients with lung cancer (Table 6). This stands in accordance with previous literature, as it has been observed that neutrophils are the most predominant immune cell type in non-small cell lung carcinoma (NSCLC), making up almost 20% of all cells showing CD45 positivity (Kargl et al. 2017). Additionally, as previously stated, G-CSF plays a major role in neutrophil production, release and retention (Borregaard 2010). It is known that cancerous cells are capable of producing G-CSF, thereby influencing neutrophil production and release towards increased cell numbers (Jablonska et al. 2017). In NSCLC specifically, G-CSF is expressed in high quantities and has therefore been suggested as a potential diagnostic tumour marker (Bahar et al. 2010). Even though we did not measure G-CSF levels, we assume that this mechanism is reflected in our data. It also came to no surprise that both HDNs and LDNs occurred in significantly higher numbers in patients with lung cancer compared to healthy individuals (Figure 13A and B), since granulopoiesis as well as the release of neutrophil from the bone marrow are known to be generally upregulated in patients with malignancies (Coffelt, Wellenstein, and De Visser 2016). A study conducted in patients with adenocarcinoma (ADC), the most abundant lung cancer subtype, also revealed that levels of LDNs were highly elevated in ADC patients, especially in advanced tumour stages. This elevation was also shown to be correlated with poor prognosis (Krieg et al. 2020). Several other studies also state that LDNs are associated with and occur more frequently not only in cancer (Wang et al. 2018), but also in other diseases such as HIV (Cloke et al. 2012), systemic lupus erythematosus (SLE) (Denny et al. 2010) and asthma (Fu, Tobin, and Thomas 2014).

We were also able to observe a significant increase absolute PMN count (PMN / mL) in patients with lung cancer (Figure 13C), but interestingly no significant differences in absolute PBMC count (PBMC / ml) were observed (Figure 13D). This might be because even though significantly increased levels of LDNs within the PBMC fraction isolated from patients with lung cancer were observed (as compared to healthy individuals), this increase of LDN levels was not enough to significantly increase overall PBMC levels in those patients.

4.2. Differences in neutrophil cell size and granularity

Our data depicts a significant overall decrease of FSC, which reflects cell size, in LDNs as well as SSC in both HDNs and LDNs from patients with lung cancer (Figure 14). Within our experimental neutrophil activation setup, no significant differences in HDNs could be observed regarding FSC (Figure 15A). Looking at SSC however, which corresponds to a cells' granularity, significant differences were observed at baseline levels and upon stimulation with both IO and PMA (Figure 15B). In LDN FSC, a significant decrease was only observed upon low dose IO induction (Figure 15C), whereas SSC was significantly decreased at baseline and upon IO stimulation (Figure 15D). A recently published review by Mollinedo (2019) argues, that a stepwise activation of mature neutrophils is the cause for them to gradually gain their immunomodulatory properties, which have been awarded to LDNs. They highlight that neutrophils might degranulate during extravasation or through interactions with endothelial or cancerous cells, which could lead to phenotypical changes, changes in surface marker expression levels, general cell functionality and even density (Mollinedo 2019). Alternatively to degranulation-associated changes in neutrophil size and granularity, variations in neutrophil cell volume can also be the result of aquaporin-9 mediated water uptake, leading to an increase in FSC as well as a decrease in SSC (Karlsson et al. 2011). Karlsson et al. (2011) also state that the phosphorylation and cell membrane translocation of aquaporin-9 are important steps in neutrophil activation. Another study conducted in patients with NSCLC also showed that aquaporin-9 was in fact upregulated in patients' tumours as well as within surrounding tissues (Chen et al. 2021). As our data depicts a significant decrease in SSC in both HDNs and LDNs from patients with lung cancer, we assume that both the previously described mechanism of stepwise activation and degranulation as well as the effects of aquaporin-9 on neutrophils could be the underlying factor to our observations. To verify these assumptions however, a subsequent study focusing on aquaporin-9 as well as on specific degranulation-associated markers would need to be conducted.

The same argument as from Mollinedo's review was brought up some years prior by Liu et al. (2017), but with specific focus on why LDNs show a significant decrease in granularity. They also suggested that human LDNs are impaired activated neutrophils which underwent degranulation and observed higher expression levels of neutrophil surface markers such as CD11b (amongst others) on LDNs (Liu et al. 2017). Those molecules are known to be present on granules (Borregaard 2010; Cloke et al. 2012) as well as are associated with the process of degranulation (Nakayama et al. 2001). Since we could observe this decrease in granularity in patients with lung cancer reflected by

measured SSC (Figure 15D), our data supports the suggestion of Liu et al. that LDNs are indeed impaired, activated neutrophils that have undergone degranulation.

Contrary to previous findings concerning the size of LDNs (Denny et al. 2010), our data depicted LDNs from patients with lung cancer to be significantly smaller compared to LDNs isolated from healthy individuals. To our knowledge, as of now there are no studies directly comparing the size of LDNs isolated from healthy individuals to LDNs derived from patients. Denny and colleagues observed cell size of LDNs to be increased (Denny et al. 2010) compared to HDNs, as did Hacbarth and Kajdacsy-Balla several years before. They argued that the decrease in density due to cellular activation by specific soluble factors not only leads to degranulation, but also to an increase in cell size (Hacbarth and Kajdacsy-Balla 1986). However, both studies investigated neutrophils isolated from SLE patients and compared LDNs to HDNs. We investigated LDNs isolated from patients with newly diagnosed lung cancer and compared them to LDNs derived from healthy individuals, no size comparison of HDNs to LDNs was performed in our setup. Also, the underlying disease microenvironment in lung cancer patients might lead to different observations in cell size as seen in the context of SLE by Denny et al. (2010) and Hacbarth and Kajdacsy-Balla (1986). In regards to a previously mentioned study by Karlsson and colleagues (2011), who investigated the role of aquaporin-9 in neutrophil volume modulation, we could not observe the mentioned increase in cell size within our samples. Therefore, we can make no clear assumptions on why LDNs isolated from our patients with lung cancer decrease rather than increase in cell size. Additional research with specific focus on lung cancer would be needed to gain further insights into this phenomenon.

4.3. Effects of PMA and IO induction on neutrophil degranulation

Regarding neutrophil degranulation measured through changes in SSC, we observed that HDNs from patients with lung cancer showed decreased SSC in all conditions, from baseline to unstimulated through different concentrations of both IO and PMA stimulation (Figure 15B). Interestingly, patients' LDNs also showed a significant decrease in SSC in all conditions but PMA stimulation (Figure 15D). The effects of different neutrophil activating agents on the release of their granular content has been studied quite intensively for many years. Especially the role of PMA and calcium ionophore A23187 (a calcium ionophore which acts in a very similar manner to IO) were investigated already in the 1970s. A study conducted by Wright, Bralove and Gallin (1977) measured degranulation upon PMA and A23187 stimulation according to the release of the granule-associated enzyme lysozyme. They found that roughly 50% of the total cellular lysozyme content was released after a 30 min incubation, both for PMA and A23187

separately (Wright et al. 1977). However, this study was conducted using neutrophils from healthy adult individuals that have not been isolated as different subsets. Comparable data from patients with cancer is unfortunately not available. Regarding our data on changes in HDN SSC (Figure 15B), it can be assumed that the decreased granularity in all measured samples from patients with lung cancer occurs as a mixed result of granule release due to the potential interactions of neutrophils with cancerous cells (as mentioned by Mollinedo (2019)) as well as through the effects of PMA and IO described by Wright et al. (1977). In hindsight to our data on patient LDNs (Figure 15D), only baseline, unstimulated and IO-stimulated cells showed decreased granularity compared to LDNs from healthy individuals. This might be due to differences in granular protein release through different activators, as also described by Wright and colleagues. They observed that additionally to lysozyme release, calcium ionophore stimulation lead to the β-glucuronidase externalisation. Since β-glucuronidase only occurs in azurophilic granules, they were capable of distinguishing different mechanisms of granule release triggered by different stimuli, with PMA triggering the release of specific granules and A23187 initiating the release of azurophilic granules (Wright et al. 1977). As Liu and colleagues (2017) argued, LDNs already underwent degranulation, but didn't specify the types of granules extruded. Therefore, it might be possible that within this initial degranulation process mainly specific granules are ejected. Hence, an additional stimulation with PMA would not lead to further excessive degranulation. To verify this hypothesis, however, further studies need to be conducted. No significant differences in HDN cell size between healthy individuals and patients with lung cancer were observed, neither at baseline nor upon stimulation (Figure 15A). It is also unclear, why cellular size of patient LDNs is only reduced upon stimulation with 1 µM IO (Figure 15C) as compared to LDNs from healthy individuals, since no literature about the effects of calcium ionophore stimulation on the size of neutrophils from cancer patients is currently available.

4.4. Effects of PMA and IO induction on CD11b and CD66b expression

In our data it was clearly observable that CD66b expression levels were increased upon stimulation, but without significant differences between LDNs and HDNs (Figure 16A and C, Figure 17A and C). CD66b is a membrane marker for neutrophil degranulation which is released upon cell activation. This upregulated expression is necessary to mediate neutrophil adherence to endothelial cells that have been pre-activated by cytokines (Kuijpers et al. 1992). The effects of various stimuli on freshly isolated neutrophils have been studied before and it was found that stimulation with PMA (Gorbet, Postnikoff, and

Williams 2015) as well as with calcium ionophore A23187 (Yamanaka et al. 1996) results in CD66b upregulation. These findings put our obtained results in accordance with previous literature. Therefore, it can be assumed that the expression of CD66b on neutrophil subpopulations is not affected by lung cancer.

We also observed that expression of CD11b is highly upregulated in both HDNs (Figure 16B and D) and LDNs (Figure 17B and D) from patients with lung cancer upon stimulation with IO and PMA, compared to cells isolated from healthy individuals. CD11b, together with CD18, is part of the Macrophage-1 antigen (Mac-1), a neutrophil membrane heterodimer that mediates neutrophil adhesion to the endothelium by binding fibrinogen (Diamond and Springer 1993). Its upregulation thereby is an important attribute to classify neutrophil activation (Bednarska et al. 2016). As previously observed stimulation with PMA as well as with calcium ionophores such as A23187 leads to an upregulation in surface expression of CD11b and subsequently to neutrophil adhesion to endothelial cells *in vitro* (Vedder and Harlan 1988). A study conducted with isolated peripheral blood neutrophils from patients with advanced epithelial ovarian cancer (EOC) showed that Mac-1 expression is drastically enhanced in neutrophils from those patients compared to neutrophils from healthy individuals. It is hypothesized that in solid tumour pathology, Mac-1 plays a crucial role as the key adhesion molecule mediating endothelial tumour cell adhesion and thereby facilitating cancer progression. Interestingly, the same study also found that Mac-1 expression differed dramatically depending on the type of EOC, where undifferentiated carcinomas showed a dramatic increase compared to other histological EOC types (Bednarska et al. 2016). Unfortunately, no comparable studies focusing on lung cancer are available to date. However, our data underlines that the findings of Bednarska et al. (2016) might also hold true in lung cancer pathology. However, since none of these studies differentiated between HDNs and LDNs, it must be assumed that their results are comprised of a combination of both subtypes. In our observations, no significant differences at the CD11b basal level (baseline) could be observed between patients and healthy individuals.

4.5. Changes in neutrophil subset distribution

When investigating neutrophil subset distribution according to Pillay et al. (2012), we could not observe any statistically significant differences in HDNs from healthy individuals to HDNs from patients with lung cancer (Figure 18A). Within the LDN fraction, patients with lung cancer showed significantly increased levels of activated ($CD16^{high}/CD62^{low}$) neutrophils without affecting the distribution of mature ($CD16^{high}/CD62^{high}$) and newly released ($CD16^{low}/CD62^{high}$) neutrophils (Figure 18B),

compared to neutrophils from healthy individuals. A study conducted with samples from patients with head and neck squamous cell carcinoma (HNSCC) concluded similar results (Millrud et al. 2017). They found that HNSCC patients displayed increased levels of activated (but not mature and newly released) neutrophils in their blood compared to a healthy control cohort. However, as their method of neutrophil isolation did not separate high from low density neutrophils, it must be assumed that these results reflect a mix of both the HDN and LDN subset.

Millrud et al. additionally observed a significantly elevated expression of Mac-1 receptors CD18 and CD11b, thereby providing means to further distinguish the activated neutrophil subset from other subsets. This elevated expression also suggests an increased capacity to migrate, which was confirmed via investigation of IL-8-facilitated migration (Millrud et al. 2017). IL-8 is secreted from tumour cells in high levels (Chen et al. 1999), leading to chemotaxis of activated neutrophils, which were predominantly found within histological samples from HNSCC tumours. It was also observed that during IL-8-induced migration, levels of activated neutrophils increased, whereas simultaneously the other two subsets decreased, thereby suggesting that IL-8 plays an important role in the induction of neutrophil maturation (Millrud et al. 2017). The IL-8 increase through secretion by tumour cells might be responsible for the significant elevation in $CD16^{high}/CD62^{low}$ neutrophil levels observed in our study, but additional experiments focusing on IL-8 specifically would be needed to strengthen this assumption.

The study from Millrud et al. also investigated the effect of activated neutrophils on the anti-tumour immune response. Several *in vitro* experiments concluded that activated neutrophils can inhibit tumour cell migration and proliferation as well as induce apoptosis of tumour cells, thereby attributing them anti-tumour properties. They also observed that the survival rate of patients with high proportions of activated neutrophils was significantly higher compared to those with lower levels (Millrud et al. 2017). Since our results showed increased levels of activated neutrophils only within the LDN fraction (Figure 18B) and LDNs were previously credited to exhibit pro-tumour properties (Liu et al. 2017), further investigations would be needed to form a clear statement on whether $CD16^{high}/CD62^{low}$ neutrophils are beneficial for the patient or rather facilitate tumour development. It is highly possible that this question needs to be answered individually for different types of malignancies.

4.6. Effects of PMA and IO induction on levels of released DNA

It was interesting to observe that DNA release in PMNs occurred significantly more intense in unstimulated and high dose IO treated samples from healthy individuals than in those from patients with lung cancer (Figure 19A). Similar results could be observed in isolated PBMCs, where cells from healthy donors released their DNA in significantly larger amounts when left unstimulated, upon low dose IO and low to middle dose PMA treatment (Figure 19B). This came to our surprise, as this effect was more likely to be expected from patient-isolated cells. In the literature it is described that especially G-CSF, which is expressed by a variety of tumours (Jiang et al. 1999; Kaira et al. 2008), leads to neutrophil activation and subsequent DNA release. A study conducted by Demers et al. (2012) using tumour-bearing mice states that DNA is released either at late tumour stage or upon a so-called “second hit”, such as due to bacterial LPS in the course of a minor infection (Demers et al. 2012). As the patients included in our study were all newly diagnosed and not within the late stage of their disease, we expected to trigger this “second hit” with PMA and IO stimulation, but could not observe the previously described results. This might be due to the fact that Demers and colleagues worked in mice and focused specifically on citrullinated histones by measuring levels of H3Cit, which was not possible with our photometric analysis method of DNA release. It might also be possible that the “second hit” had already occurred in-vivo before blood was drawn and hence could not be induced upon stimulation ex-vivo. This would also explain the significantly increased amount of released DNA from unstimulated cells derived from healthy donors. Even though peripheral blood neutrophils were also considered in the study by Demers et al. (2012), literature investigating solely PBMC DNA release in the course of cancer pathology is unfortunately not available.

The data gathered in this DNA release setup should correspond to the release of NETs to the surroundings. However, since our samples were measured photometrically, no specific NET markers could be considered. Therefore, there was no possibility to differentiate between DNA released from neutrophils or from other cells contained within the PBMC fraction during measurement and analysis, as it would not have been suitable to further purify isolated cells to only investigate neutrophils. Additional purification protocols such as the StemCell untouched neutrophil isolation kit (STEMCELL Technologies Inc., Vancouver, CA) (Thomas et al. 2015) would have potentially falsified the results as neutrophils might have become even more pre-activated during further purification. Although, since the PMN fraction consists of mostly HDNs, it can be assumed that the vast majority of released DNA originates from neutrophils. Within the PBMC fraction however, other cells may have contributed to the results by releasing their

own DNA. Eosinophils, mast cells and macrophages have previously been described to also form extracellular traps in a process described as ETosis (Guimarães-Costa et al. 2012), which for some cell types can also be triggered via PMA. Mast cells were shown to release their extracellular traps after just 10 min of very low dose PMA induction (25nM) (Von Köckritz-Blickwede et al. 2008), eosinophils formed their traps after 20 min of IL-5 and interferon-γ priming (Yousefi et al. 2008). The process of macrophage extracellular DNA release is referred to as METosis, a rather rapid process that leads to the formation of macrophage extracellular traps (METs) within 30 min (Doster et al. 2018). Also, in monocytes, DNA release has been studied before. Upon stimulation with PMA, calcium ionophore A23187 and two other NETosis associated biological stimuli (platelet activating factor and zymosan), monocytes were reported to produce ETs within 3 h of stimulation (Granger et al. 2017). Since we stimulated the cells for 3 h, these mentioned timepoints of trap release from cells other than neutrophils would fall into this time range. Hence, parts of the detected released DNA within the PBMC fraction might originate from cells other than neutrophils, with monocytes possibly being the most relevant amongst these, as they make up between 10 - 20% of PBMCs (Kleiveland 2015).

4.7. Effects of PMA and IO induction on NET release

Contrary to our results obtained in the DNA release setup, HDNs isolated from patients with lung cancer showed increased levels of H3Cit upon stimulation with IO (Figure 20A). Patients' LDNs however were observed to not undergo NET release more frequently than those from healthy individuals upon IO induction and were even significantly less likely to release NET DNA when stimulated with PMA (Figure 20B). As this flow cytometric analysis specifically quantified levels of extruded DNA by measuring the MFI of citrullinated NET DNA, it represents a more precise insight into the actual nature of citrullination-mediated NET release of isolated neutrophils from patients with lung cancer as compared to our photometrically measured samples in the DNA release setup. The current literature states that different stimuli lead to different NET formation pathways (Kenny et al. 2017). Calcium ionophores such as IO and A23187 are known to induce the histone citrullination pathway via PAD4. PAD4 is responsible for the conversion of arginine to citrulline residues on histones, leading to the loss of the positive charge of histones and subsequently to rapid chromatin decondensation (Li et al. 2010) in preparation for subsequent NET DNA expulsion. As we were able to observe an increase in H3Cit positivity on HDNs derived from patients with lung cancer (Figure 20A), our findings support the notion of Li et al. (2010). PMA however has been described to act in a distinctively different manner. PMA leads to the induction of NOX2 during the

NOX2-dependent NET formation pathway, which subsequently increases ROS and MPO-related activity. The translocation of NE and MPO to the nucleus promotes DNA decondensation, and eventually rupture of the neutrophil granula and nuclear envelope, resulting in the DNA strands being decorated by granular and cytosolic proteins prior to DNA release into the extracellular space in the form of NETs (Papayannopoulos et al. 2010). It is still controversial if PMA is capable to induce histone citrullination (de Bont et al. 2018). Multiple studies suggest that PMA is not capable to induce histone deamination (Kenny et al. 2017; Neeli and Radic 2013) because the induction of NET formation through PMA occurs without the activation of PAD4 (Douda et al. 2015). It is even suggested, that PMA itself is a potent inhibitor of PAD4 (Neeli and Radic 2013). However, several studies found that citrullination of histone H3 can in fact be induced by PMA, both in myeloma-bearing and tumour-free mice neutrophils (Li et al. 2020) as well as in human neutrophils derived from peripheral blood (Pejler et al. 2021). Our results clearly show increased levels of H3Cit upon PMA stimulation at least in LDNs from healthy individuals (Figure 20B) and are thereby supportive of the hypotheses supporting PMA as a citrullination-inducing agent.

With our experimental setup, the previously described “second hit” (Demers et al. 2012) could clearly be induced in HDNs utilizing IO as a stimulant. Hence, our results indirectly support the hypothesis by Demers and colleagues that the production of certain cancer-derived factors (such as G-CSF) or inflammation-related cytokines prime neutrophils, might make them more prone to NET formation. A recent study using murine lung cancer cells also showed that extracellular ribonucleic acid (RNA) derived from cancerous cells were able to indirectly activate neutrophils (Li et al. 2019). These RNAs induced Il-1β release from respiratory epithelial and endothelial cells. The release of this cytokine in succession activated neutrophils to release DNA content in form of NETs. This was not only shown *in vitro*, but also in an *in vivo* setup where mice were injected with murine lung cancer cells, resulting in neutrophil activation measured via CD11b expression (Li et al. 2019). Unfortunately, this study did not differentiate between HDNs and LDNs but showed a rather general picture regarding neutrophils in lung cancer. Therefore, it can be assumed that in lung cancer, tumour derived RNAs are an important player in indirect neutrophil activation and subsequent NET release and may also play a role in our experimental setup.

Furthermore, a possibility why LDNs from patients showed significantly lower levels of citrullinated H3 might be due to an early release of certain cancer-associated factors, such as MPO and NE, necessary for PMA induced NET formation. As stated by Liu and colleagues (2017), LDNs are degranulated activated HDNs. Assuming this degranulation process mainly releases azurophilic granules, a great amount of both

MPO and NE would already have been extruded prior to cell isolation and could therefore not be involved in PMA-induced NET formation. This would favour previous findings regarding general cancer pathology, as both NE and MPO are released during cancer disease progression. In lung cancer specifically it has been observed that levels of NE and MPO in bronchoalveolar lavage fluid as well as in serum were significantly increased as compared to healthy individuals (Vaguliene et al. 2013). This additionally strengthens our assumption that these factors might have already been extruded by LDNs before isolation and furthermore supports the notion by Liu and colleagues (2017) on the pro-tumorigenic properties of LDNs in general. This extrusion additionally benefits the tumour, as NE has proinflammatory effects and is (amongst other granule proteins) involved in the suppression of T-cell activation (Treffers et al. 2016). In the case of MPO, even though it is stated that it shows anti-tumour activity (Masucci, Minopoli, and Carriero 2019), it is also known that it can act proinflammatory and thereby has pro-tumour properties (Ostrand-Rosenberg 2008). To verify the assumption concerning azurophilic granule release, further investigation on the type of granules extruded during LDNs development is needed.

When studying a chronic myelogenous leukaemia mouse model, Demers et al. (2012) showed that neutrophils were more prone to undergo NET formation compared to cells from control mice. This was accredited to tumour cell released G-CSF, which is capable of pre-activating neutrophils, so that upon a “second hit”, NET formation is initiated. When simulating a minor infection in mice bearing tumours, large quantities of chromatin were released, resulting in a highly pro-thrombotic state. This was also observed in mouse models of lung and mammary carcinoma. Thereby, they concluded NET formation as a prominent cause for cancer associated thrombus formation (Demers et al. 2012). It was interesting to observe that in our study, patient HDNs were more prone to undergo NET formation than their healthy counterparts upon a “second hit”, leaving the question if NET release from patient LDNs requires other stimuli or if G-CSF priming of patient HDNs is more distinct then in LDNs.

One study conducted with isolated neutrophils from breast cancer patients showed that LDNs from patients showed an increased capability of NET formation when stimulated with A23187 in a full glucose medium, compared to HDNs. When deprived of glucose, LDNs were still capable to form NETs in a greater extent than HDNs (Hsu et al. 2019). This leads to the assumption that under nutrient-poor conditions such as cancer, LDNs are responsible for most of the extruded NET DNA and thereby the culprits in promoting cancer associated thrombosis. Taking together our individually obtained results, where absolute numbers in LDN MFI were higher than their HDN counterparts, this assumption might hold true.

4.8. Differences between two technical principles of cell counting

When technically comparing an automated haematology analyser such as the Sysmex XN-350 to a flow cytometer such as the CytoFLEX S in regards to the percentual occurrence of LDNs within our samples, both systems showed comparable results that did not significantly differ from one another (Figure 21). To understand why, the working principles of both systems have to be evaluated. Automated haematology analysers work in multiple principles to detect cells. Systems using electrical impedance work via two electrodes that are placed in an isotonic solution, separated by a glass tube with a small aperture. An electric current is generated between the electrodes and when a cell passes through the aperture, a change in electrical resistance occurs. This change is measured as a pulse, where the height of the pulse corresponds to cell volume. The width of the pulse is proportional to time each cell needs to pass the aperture. For this to work, the cell suspension has to be highly diluted, so that only a minimal number of cells can pass the aperture at the same time. Fluorescence, light absorption (measured via spectrophotometry) and electrical conductivity are also used in automated haematology analysers (Dayyal 2017). Systems like the Sysmex XN-350 use light scatter and hydrodynamic focusing for detection (TradeMed 2020). Hydrodynamic focusing forces cells to take one path for volume measurement. Light scatter is measured using a laser which focuses on the flow cell, hitting bypassing cells. The laser light scatters in various directions, making it possible to detect FSC and SSC of each individual cell and thereby qualifying and quantifying each event as the correct cell type (Dayyal 2017).

Flow cytometers such as the CytoFLEX S also use light scatter as one working principle, but with the addition of multiple lasers with different colour filters, so that a large number of additional parameters (besides FSC and SSC) can be assessed simultaneously, if the sample has been prepared accordingly (Thermo 2020).

Due to the similar working principles of both used systems, our results came as expected. However, it must be noted that the Sysmex XN-350 automatically delivers the final results without the need of any special adjustments to the device before measurement. With the CytoFLEX S, several adjustments (such as inputs of the correct wavelengths and gating of areas to investigate) need to be conducted manually prior to each measurement, which therefore leaves this system potentially more vulnerable to human error. Taken together, when all necessary manual adjustments for the CytoFLEX S have been set and tested, this system is capable of providing more detailed information about counted cells, but the Sysmex XN-350 can as well be used to give accurate information about cell numbers, making further analysis based on cell counts accurately possible. This is reflected in our findings, as both methods of analysis

provided similar results for LDN cell counts. This assures not only the validity of data gathered from measurements with the Sysmex XN-350 but also verifies that our gating strategy for LDN identification was chosen correctly.

4.9. Two different cell counting techniques applied on LDN counting

Using our two systems to compare percentual LDN rations between healthy donors and patients with lung cancer, both the Sysmex XN-350 (Figure 22A) and the CytoFLEX S (Figure 22B) revealed a significant increase in LDNs in lung cancer patients. As discussed before, both systems reflect accurate results due to their similarity in working principles. Our observed increase of LDNs in patients with lung cancer using two different methods of cell counting stands in accordance to several studies which have identified the presence of LDNs in patients with cancer (Lecot et al. 2019; Mackey, Coffelt, and Carlin 2019; Sagiv et al. 2015; Silvestre-Roig et al. 2019; Wang et al. 2018). However, so far there is no data available that compares the ratio of isolated LDNs from healthy individuals to LDNs from patients with malignancies.

5. Conclusion

This study investigated the potential differences in neutrophils isolated from patients with newly diagnosed lung cancer regarding neutrophil subset distribution, changes in neutrophil activation potential, DNA release and H3Cit positivity, as compared to neutrophils isolated from a matched cohort of healthy individuals.

Levels of both HDNs and LDNs were significantly increased in patients with lung cancer. SSC was significantly decreased in both HDNs and LDNs isolated from patients with cancer, whereas a decrease in FSC was observable in LDNs. Upon stimulation with IO and PMA, a decrease in cellular granularity could be observed in HDNs with both stimuli, whereas LDNs only decreased in SSC without stimulus and upon IO stimulation. Stimulation had almost no impact on cell size in both HDNs and LDNs, with only the exception of a significant decrease in LDN FSC upon low dose IO induction.

When stimulated, CD66b levels were increased in both HDNs and LDNs from patients with lung cancer and healthy individuals, without significant differences between the two cohorts. However, CD11b expression levels were expressed in higher levels on HDNs and LDNs isolated from patients with lung cancer upon stimulation. Therefore, we hypothesize that neutrophils within malignant systems tend to be activated more easily than those derived from healthy individuals.

Subset distribution did not differentiate between healthy individuals and patients with lung cancer in HDNs, but a significant increase in activated neutrophils in LDNs could be measured. Due to conflicting literature additional studies would be needed to clearly state if LDNs are in fact pro-tumorigenic or might exhibit anti-tumour characteristics under certain circumstances or within specific types of cancer.

PMNs from patients with lung cancer released significantly less DNA when left unstimulated and upon induction with high dose IO. Furthermore, PBMCs released significantly less of their DNA when not stimulated, upon low dose IO as well as when induced with low and middle dose PMA. HDNs from patients with lung cancer only showed a significant increase in H3Cit positivity upon stimulation with both doses of IO. Interestingly, patient LDNs depicted a significant decrease in H3Cit positivity as compared to LDNs from healthy individuals, but only upon PMA induction.

A technical comparison of the Sysmex XN-350 cell counter with CytoFLEX S flow cytometer in regard to percentual occurrences of LDNs showed no significant differences between the two systems verifying Sysmex XN-350 for cell count analysis.

Taken together, this study was able to show that LDNs do in fact occur in larger quantities in patients with lung cancer as compared to healthy individuals. It was also clearly observable that both HDNs and LDNs isolated from patients have a higher

potential of activation upon *in vitro* stimulation based on expression levels of neutrophil activation marker CD11b. However, with our results we were not able to support the hypothesis that HDNs and LDNs isolated from patients with lung cancer have a general tendency to release more DNA as well as show an increased predisposition to undergo NET formation *in vitro*. Additional studies with different experimental setups would be needed to draw a more complete picture about the *in vivo* nature of neutrophil subsets in the context of lung cancer.

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