Role of Neutrophil Extracellular Traps and Necroptosis in Abdominal Aortic Aneurysm Disease Development and Progression

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a life-threatening vascular disease, involving complex molecular and pathophysiological mechanisms. Usually, either an increase of the aortic diameter to more than 3 centimeters, or a 1.5 fold enlargement of the healthy, non-dilated aortic lumen are diagnosed as AAA. As only surgical therapies are currently available for treating the disease, understanding the mechanisms and developing novel treatment options are of utmost importance.

Impaired remodeling of the aortic wall is one of the most typical characteristics of AAA disease. Depletion of medial smooth muscle cells (SMCs) is the main reason that leads to segmental expansion. SMCs are primary cellular components of the blood vessel wall. They play an important role in maintaining vessel structure, as well as regulating vessel function. Under pathological environmental factors and pathological conditions, SMCs lose their original characteristic of a contractile phenotype, while acquiring the characteristics of other cells. Another noticeable reason accounts for the depletion of SMCs is programmed cell death (PCD), which is considered a pre-designed cell death pathway. Currently, PCD is divided into three types: apoptosis, autophagic cell death and necroptosis. Apoptosis is a

caspase-dependent cell death pathway. An increasing amount of research has proven that apoptosis of SMCs is important for AAA progression. However, studies using caspase inhibitors could not reveal a therapeutic benefit to prevent aneurysm development. This suggests that there must be other factors that influence the destiny of SMCs. Necroptosis was for a long time recognized as a random cell death process. This opinion has changed dramatically after identifying a mechanism, involving receptor-interacting protein kinases 1 and 3 (RIPK1, 3) and its substrate, the mixed lineage kinase-like domain protein (MLKL) as crucial landmarks in the necroptosis pathway. Data from my own thesis presented here reveal that RIPK1, RIPK3 and MLKL levels are significantly increased in diseased human AAA samples compared to non-dilated controls. High level of RIPK3 expression could also be detected in human AAA samples compared to healthy human aortic tissue through immunohistochemical analysis. Hence, the necroptosis pathway appears to be involved in AAA development and disease progression. Neutrophils are the first line of defense of our immune system in response to infections, through releasing cellular contents, which include DNA and proteins termed neutrophil extracellular traps (NETs). Studies have demonstrated the existence of NETs in human atherosclerotic lesions, as well as AAA tissues. In addition, limitation

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of NET production has been suggested to restrain AAA growth. The stimulation of neutrophils with phorbol myristate acetate (PMA) leads to the induction of NET release. In my thesis project, the treatment of SMCs with NETs contributed to SMCs apoptosis, being assessed using a kinetic live-cell imaging system. Further, NET treatment also induced the upregulation of several necroptosis marker genes in SMCs (such as RIPK1, RIPK3 and MLKL). This further strengthens the notion that necroptosis in SMCs might trigger AAA progression.

In conclusion, limited SMC survival appears as one of the specific characteristics in AAA progression. Necroptosis and apoptosis of SMCs are contributing to aneurysm dilatation, and NETs seem to propagate these effects. Targeting NETs could thus be a promising future therapeutic strategy to limit AAA disease.

LIST OF ABBREVIATIONS

AAA: Abdominal aortic aneurysm

AoSMCs: Aortic smooth muscle cells

CABG: Coronary artery bypass grafting

CCL2: chemokine ligand 2

CIAPs: Cellular inhibitors of apoptosis

CTSS: Cathepsin S

CXCR4: C-X-C chemokine receptor type 4

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic Acid

EVAR: Endovascular aneurysm repair

FACS: Fluorescence activated cell sorting

FADD: Fas-associated death domain

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

H3Cit: Citrullinated histone 3

IL: Interleukin

LDL: Low-density lipoprotein

LncRNAs: Long non-coding RNA

miRNAs: MicroRNAs

MLKL: Mixed lineage kinase-like domain protein

MPO: Myeloperoxidase

ncRNA: Non-coding RNAs

NE: Neutrophil elastase

NETs: Neutrophil extracellular traps

NFKB: nuclear factor kappa-light chain-enhancer of activated B
cells

PAD4: Protein-arginine deiminase 4

PBS: Phosphate buffered saline

PCD: Programmed cell death

PCI: Percutaneous coronary interventions

PDGF: Platelet-derived growth factor

PMA: Phorbol myristate acetate

RIPK1: Receptor interacting protein kinase 1

RIPK3: Receptor interacting protein kinase 3

ROS: Reactive-oxygen species

RPLP0: Ribosomal protein lateral stalk subunit P 0

Smac: Second mitochondria-derived activation of caspase

Smad: Small mothers against decapentaplegic

SMMHC: Smooth muscle heavy chain

TGFβ: Transforming growth factor β

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor family receptor

TRADD: TNF receptor-associate death domain

TRAF: TNFR-associated factor

VSMCs: Vascular smooth muscle cells

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1 INTRODUCTION

1.1   Abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) was first described in Ancient Rome. AAA refers to partial aortic enlargement caused by blood flow exerting pressure on weak parts of the aortic wall. Typically, an aortic diameter bigger than 3 centimeters or more than 1.5 times of the non-dilated aortic lumen are diagnosed as AAA(Kent, 2014). A series of risk factors are responsible for inducing AAA formation, for example male gender, smoking, increased alcohol consumption, hyperlipidemia, arterial hypertension, genetic inheritance, diabetes, obesity, and infection(Baird et al., 1995; Brown and Powell, 1999; Clifton, 1977; Greenhalgh and Powell, 2008; Wittels, 2011).
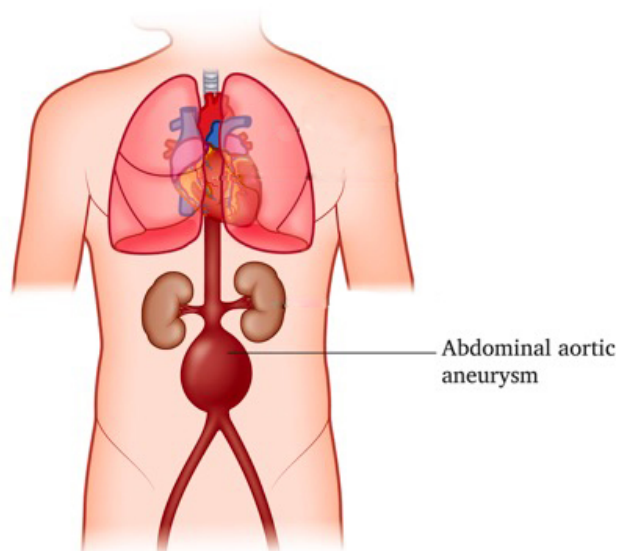
Figure 1.1. Abdominal aortic aneurysm(Adapted from Wanhainen et al., 2019).

In most clinical cases, ruptured AAAs are followed with severe clinical symptoms such as abdomen or back pain, loss of consciousness, low blood pressure and even death, making disease discovery challenging(Li et al., 2018). In the aortal media and intima, typical pathological characteristics are found in the aortic wall, including cholesterol crystals, foam cells, thrombosis, calcifications and adventitia inflammatory infiltrate in the adventitia; however, increased levels of metalloproteinases and proteases appear to be the most crucial factor leading to degradation of the medial elastin and reduction of the adventitial coherence(Chamberlain et al., 2010). Consequently, the aorta becomes more sensitive and fragile to damage from blood flow accelerations. The cleavage of decorin by the serine protease granzyme B also contributes to AAA by disrupting collagen and decreasing adventitial coherence(Ang et al., 2011; Chamberlain et al., 2010). Ultrasound and computed tomography (CT) scans are popular and sensitive detection methods in clinical diagnosis. Currently, surgical repair is the only method for treating AAA; however, it is accompanied by a perioperative relatively high mortality risk of approximately 2-5%(Golledge et al., 2006). Compared with traditional open surgery, endovascular aneurysm repair (EVAR) has the advantage of lower mortality risk, shorter hospital stay and fewer periprocedural complications(Thomas et al., 2014). Smoking cessation, medical therapy to reduce arterial hypertension and high cholesterol levels are also advised by physicians and guidelines. The high mortality and limited treatment methods have attracted research attention worldwide. It is apparent that there is

an urgent need for a deeper understanding of the molecular mechanisms involved to address AAA progression with improved methodology.

1.2   Atherosclerosis

The main underlying condition for the majority of cardiovascular diseases, including aortic aneurysm, is atherosclerosis in the vascular system. Atherosclerosis is the narrowing of the arteries caused by plaque accumulated between the endothelium and tunica media in the vessel wall, which leads to stiffening of the artery walls. The damaged endothelium initiates the inflammatory process and attracts circulating monocytes to attach to the internal wall of the artery. Over time, monocyte-derived macrophages and smooth muscle cells (SMCs) absorb oxidized low-density lipoprotein (LDL) and transform into foam cells(Robbins et al., 2013). High lipid content further propagates inflammation. Meanwhile, SMCs from the medial layer proliferate and migrate into the intima and constitute the fibrous cap of the developing plaque. Over time, plaques become bigger, and are comprised of fatty substances, calcifications, thrombosis and inflammatory cells(Shankman et al., 2015). Several factors greatly influence atherosclerosis, for example, genetic factors, hypertension, cigarette smoking, high plasma cholesterol and high blood glucose(Ridker, 2002). The characteristics of the plaque determine its fate, i.e. rupture or stability, which can then lead to severe outcomes. Atherosclerosis also contributes to various complications, including ischemic heart disease and

heart failure, kidney failure, aneurysm formation, stroke and arrhythmia(Bennett et al., 2016).

The coronary arteries ensure the blood supply of the heart. Atherosclerosis of the coronary arteries that lead to ischemia and altered blood flow are very common in the clinical setting. Typical symptoms are chest pain, breathlessness, sweating, queasiness, dizziness, palpitations, weakness and light-headedness. New diagnostic and prognostic methods are also used in clinical practice, including proteomics, metabolomics and genetic approaches(Doring et al., 2013). Patients are advised to change their lifestyle, control their blood pressure, lower their blood cholesterol levels and accept anti-platelet therapy or even surgery to treat the condition.

1.3   Smooth muscle cells

Vascular SMCs are primary cellular components of the blood vessel wall. SMCs play an important role in maintaining vessel wall structure, as well as regulating vessel properties. The most prominent function of SMCs is contraction(Siow and Pearson, 2001). SMCs express contractile proteins and other molecules that are responsible for the contraction and elastic effects(Rzucidlo et al., 2007). They also synthesize extracellular matrix and influence the development and progression of vascular diseases through complicated mechanisms. SMCs are always recognized as multifunctional and versatile cells. Traditionally, intimal SMCs were considered beneficial cells in stabilizing the fibrous cap in

atherosclerosis; however, an increasing number of studies have revealed the detrimental function of SMCs in vascular disease formation and progression(Low et al., 2018). Intimal thickening caused by SMC migration and proliferation after percutaneous coronary interventions (PCI) and coronary artery bypass grafting (CABG) is a common phenomenon in cardiovascular patients(Ghista and Kabinejadian, 2013). Meanwhile, intimal SMCs are recognized as originating from the media(Bochaton-Piallat and Back, 2018). Affected by various environmental factors, SMCs can undergo obvious shifting from the contractile to synthetic phenotype(Siow and Pearson, 2001). This mechanism has been shown to play a crucial role in vascular disease development and progression.

1.4 Non-coding RNAs

It has recently been established that the vast majority of the human genome has no protein coding function(Consortium, 2012). This group of transcripts is termed non-coding RNAs (ncRNAs)(Birney et al., 2007; Shahrouki and Larsson, 2012; Washietl et al., 2007). NcRNAs were initially regarded as useless junk RNA(Palazzo and Lee, 2015); however, this recent negative attitude towards ncRNAs has changed dramatically. An increasing number of studies have demonstrated that ncRNAs are closely involved in vital molecular activities, such as replication(Zhang et al., 2011), enhancing or limiting transcription events, fine-tuning gene expression(Farh et al., 2005; Poole et al., 1998), the maintenance of chromosome structure(Park et al., 2002), cellular development,

differentiation, hormone regulation(Knoll et al., 2015). Through these multiple mechanisms they can influence the progression and development of numerous human diseases(Shahrouki and Larsson, 2012),(Leung and Natarajan, 2014). The connection between ncRNAs and their involvement in triggering disease progression make it possible to therapeutically alter their expression, and to thus hopefully develop novel diagnostic and treatment strategies.

1.4.1 Classification of ncRNAs

NcRNAs are separated into groups according to the number of nucleotides: ncRNAs shorter than 200 nucleotides belong to the group of small ncRNAs(Paralkar and Weiss, 2013), ncRNAs longer than 200 nucleotides belong to the long ncRNA (lncRNA) subgroup(Ma et al., 2013). Mature microRNAs (miRNAs) are defined as 18-20 nucleotide long single-stranded RNAs(Bartel, 2004). They act on the post-transcriptional level and suppress RNA expression(Bartel, 2009). They have been shown to mediate disease conditions and to serve as valuable diagnostic markers(Owens, 1995). In contrast, lncRNAs influence gene expression through more complex molecular mechanisms and interventions(Beermann et al., 2016). For example, lncRNAs with different nucleotide compositions may still exert the same molecular function if they exhibit the same three-dimensional structure(Pang et al., 2006). LncRNAs can be produced in any locus of the human genome, including introns, exons, enhancers and intergenic regions, and even overlap with one or more introns and exons on

the same or opposite strand(Thum and Condorelli, 2015).

LncRNAs play a role in transcription by interacting with transcription factors - not only in the nucleus but also in cytoplasm. LncRNAs also regulate mRNA processing and post-transcriptional control, and affect cell survival and apoptosis(Mercer et al., 2009). Furthermore, lncRNAs are crucially involved in specific organ maturation, which also includes cardiac and vascular development(Klattenhoff et al., 2013; Lorenzen et al., 2014). Until now only very few of them have been investigated for their specific function in mechanisms that trigger disease progression. Studies in the Vascular lab that I participated in, identified functional relevance for the lncRNA H19 in AAA development and disease progression(Li et al., 2018), which will be discussed in the results section.

1.5 Neutrophil extracellular traps

Neutrophils are the most abundant type of white blood cells circulating in the human bloodstream, meanwhile, they are the immune system's first line of defense in response to infection. Apart from pathogen elimination via phagocytosis, neutrophils also release cellular contents termed neutrophil extracellular traps (NETs) by an induced death program called NETosis(Urban et al., 2009). NETs are web-like structures comprised of DNA, globular antimicrobial peptides, histones and cytoplasmic proteins(Doring et al., 2017), thus provide a high concentration of antimicrobial components, which trap and

kill microbes.

During NETosis, reactive oxygen species (ROS), neutrophil elastase (NE) and myeloperoxidase (MPO) are vital mediators in this process(Brinkmann et al., 2004). ROS operate on peptidyl arginine deiminase 4 (PAD4), contributing to histones modification and chromatin decondensation, then, NE and MPO entry to the nucleus, accelerate the decondensation of chromatin. After that, neutrophil nuclear membranes are degraded, leading to nucleoplasm and cytoplasm fusion(Fuchs et al., 2007; Yang et al., 2016). After this, the cytoplasmic membranes are continually disrupted, expelling the cell contents to the neutrophil exterior(Vorobjeva and Pinegin, 2014), which is recognized for being different from apoptosis and necrosis(Steinberg and Grinstein, 2007).

NETosis is divided into two forms according to different stimuli, time-consumption, and ultimate consequences and effects. The classical suicidal NETosis takes several hours; on the contrary, vital NETosis can be completed in a short time, i.e. several minutes. Suicidal NETosis is usually initiated through complement receptors with diverse ligands, leading to the release of calcium from the endoplasmic reticulum, which stimulates NADPH oxidase and activates the suicidal NETosis pathway. With regard to vital NETosis, DNA-filled vesicles are expelled via nuclear blebbing without disrupting the plasma membrane(Jorch and Kubes, 2017). During this rapid process, neutrophils remain intact and are able to fulfill their antimicrobial function even beyond their lifespan.

NETs may act as a physical barrier that blocks spread of the pathogens, prevent pathogen migration and spreading(Brinkmann et al., 2004); in addition, due to components such as lysozymes, proteases and antimicrobial peptides, NETs eventually attack intruders, including bacteria, fungi(Urban et al., 2006), parasites and viruses(Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007). However, the exposure of self-DNA with granule proteins may lead to inflammatory conditions, such as atherosclerosis through stimulation of antigen presenting cells, i.e., plasmacytoid dendritic cells (pDC) and monocytes(Lande et al., 2011). Studies also showed the existence of NETs in atherosclerotic lesions, including in mice and in human atherosclerosis(Megens et al., 2012). Through interactions with platelets, NETs are involved in thrombus formation(Fuchs et al., 2010), thus contribute to atherosclerosis and other cardiovascular diseases. Moreover, an increasing amount of studies found that NETs are not only involved in cardiovascular diseases, but also associated with acute and chronic inflammation, autoimmune diseases and even malignant tumors(Pinegin et al., 2015).

1.6 Programmed cell death

Programmed cell death (PCD) is a designed cell death pathway with complicated background and mechanisms(Fuchs and Steller, 2011). PCD is involved in a wide range of human disorders, including diseases of the nervous system, malignant tumors and diseases of the immune system. Researchers in the field divide PCD

into three types: apoptosis, autophagic cell death and necrotic cell death(Edinger

and Thompson, 2004), also being termed necroptosis.

Comparison of PCD Pathways

Healthy Cell

nucleus

	Caspase-Dependent	Cathepsin-Dependent	RIP3 and MLKL mediated
Molecular Mechanism			
Morphological Characteristics	Chromatin condensation DNA fragmentation Cell shrinkage Shedding of apoptotic bodies	Double-membraned vesicles (autophagosomes) Autophagolysosomes containing captured material	Swelling of organelles(ER & mitochondrial) Cell Swelling Rupture of plasma membrane Lysis of cell
PCD pathway	Apoptosis (PCD type I)	Autophagic Cell Death (PCD type II)	Necroptosis (PCD type III)

Figure 1.2. The three different types of programmed cell death (apoptosis, autophagic cell death, and necroptosis)(Adapted from Halonen, 2015).

Apoptosis is the most studied and best understood PCD pathway. It is

characterized by cellular suicide and implied through perfectly designed

pathways(Kerr et al., 1972). With its characteristic of causing no damage to the

host, apoptosis helps eliminate pathogens and adverse materials, thus it is very

crucial to continuous viability of an organism(Elmore, 2007). Necrosis has long

been regarded as a casual reactivity when cells are exposed to severe damage.

However, an increasing number of studies have asserted that necrosis is actually

orchestrated through very specific and distinct signaling pathway, which is part of the reason that it has been renamed 'necroptosis'(Chan et al., 2015; Sun and Wang, 2014). Autophagy involves cellular component degradation and renewal(Mizushima and Komatsu, 2011). In some pathological conditions but also physiological conditions, autophagy can induce cell death(Mizushima et al., 2008; Tsujimoto and Shimizu, 2005), which is termed autophagic cell death. All three death pathways are not clearly separated, as some overlap exists among the associated pathways, presenting cells with various choices when facing crucial occasions(Nikoletopoulou et al., 2013).

2   AIMS OF THE STUDY

2.1 To clarify the role of apoptosis and necroptosis in AAA progression.

2.2 To investigate the influence of neutrophils and NETs on AAA.

2.3 To study the mechanisms of NETs regulating SMC death.

2.4 To provide novel therapeutic ideas for AAA.

3   MATERIALS AND METHODS

3.1 Neutrophils isolation

Materials:

Polymorphprep (Axis-Shield; 1114683)

PBS (phosphate buffer saline, Sigma)

HBSS (Hank's balanced salt solution, Life Technologies; 14025100) + Ca + Mg + 25 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, Gibco; 15630-056)

PMA (Phorbol 12-myristate 13-acetate) (stock 1.62mM)

DMEM (Dulbecco's modified Eagle's medium) + 10% FCS (fetal calf serum) + pyruvate + 0.2mG/mL G418

6-well plate

Methods: Blood was drawn in six Ethylenediaminetetraacetic Acid (EDTA) tubes, and pipetted in to eight 15-mL tubes with 6 mL blood layered on top of 6 mL of Polymorphprep (prewarmed). It was then spun for 35 min at 600 g without break and acceleration, and the lower white band (neutrophils) was collected in a 50-mL tube (four 15-mL tubes/ 50-mL tube) using 1-mL tips; tips were changed each time they entered the tube. The 50-mL tube was filled to 50-mL with PBS, and spun for 5 min 300 g, then the buffer was removed, the pellet resuspended in 20 mL of HBSS with 25 mM HEPES, and the neutrophils were counted. Fluorescence-activated cell sorting (FACS) showed 90-98% purity. We

collected 51.000.000 neutrophils with 95% purity, and 2.000.000 cells were used for the NET release assay.
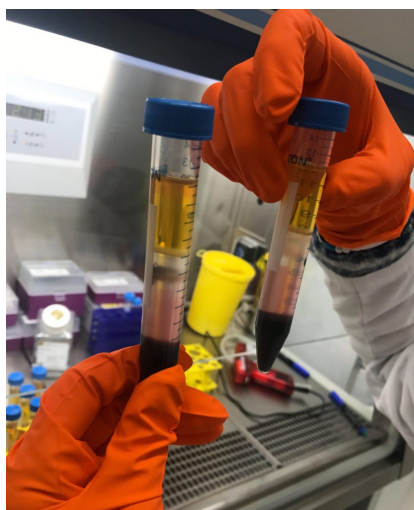
Figure 3.1. Sorting neutrophils from the layered blood samples of healthy donors.

3.2 NET-fragment isolation

Methods: Neutrophils (1.000.000 cells/mL) were placed in HBSS with 25mM HEPES, and 2mL of neutrophil suspension was pipetted in to each well of 4 x 6-well plates and seeded for 30 min at 37°C. NETosis was stimulated by adding 1 mL 300 nM PMA in HBSS 25 mM, incubating for 4 h at 37°C, washing with PBS (Phosphate buffered saline), adding 500uL human SMC medium containing 10 μ/mL AluI and incubating for 30 min at 37°C, The supernatant was collected and pooled, and spun at 300 g for 5 min; the supernatant was collected and frozen for later use. The NET concentration was measured with a Qubit fluorometer (Life Technologies, Invitrogen by Thermo Fisher Scientific), and the NETs were sonicated before use (5 seconds at 21% amplitude).

3.3 Isolation of endothelial cells and smooth muscle cells from human aortas

Materials:

Medium for ECs: EC basal media with growth supplements (ECBM2, PromoCell, c-22111) and antibiotics (Pest). For digestion, 1 mg/ml Collagenase A (allergenic, irritant) in 1 U/ml dispase (Stemcell Tech) was added.

Medium for SMCs: DMEM/F-12 in 5% FCS and antibiotics. For digestion, 1.4 mg/mL collagenase A was added. We used 12.5 cm^2 cell culture flasks for ECs and 25 cm^2 cell culture flasks for SMCs. We prepared 0.2% gelatin from 2% Gelatin A solution (Sigma) and diluting it in Milli-Q water. The 12.5 cm^2 flask (BD Falcon) was covered with 0.2% gelatin and the flask was placed in an incubator 37°C for about 1 h, and washed with PBS twice.

Tissue preparation: The aorta was transported from the operation unit to the lab in HBSS or PBS buffer. Under a fume hood, the aorta was washed 2-3 times to remove the blood. Collagenase A (1 mg/mL, Sigma/Roche 11088793001, 0.23 U/mg) was dissolved in 10 mL dispase(1 U/mL) in DMEM/F-12 (Stemcell Tech 07923-100ml) and heated at 37°C to dissolve. The Collagenase solution was filtered using a syringe and sterile filter. The adventitia was separated from the aorta using forceps. The aorta was placed in a tissue culture dish containing collagenase/dispase, endothelial side face-down to immerse it in the collagenase/dispase. The aorta was incubated for 20-25 min at 37°C in an incubator, during which the dish was moved once or twice. Next, the aorta was

transferred inside a fume hood. The specimen was turned endothelial side up and the endothelial surface was scraped very carefully, using a sterile scalpel, at least 5-7 times. Care was taken not to scrape too strongly to avoid detaching SMCs from the aortic wall. Using a 1-ml pipette, the collagenase solution was poured several times onto the aortic surface to detach ECs with flow. The collagenase solution containing ECs was transferred to a 50-ml tube. Then, the surface of the aorta was rinsed in the same manner with warm PBS an additional 2-3 times and collected in the same tube. The aorta was immediately transferred to a tissue culture plate containing medium prepared for SMC isolation. The tissue was cut into small pieces to aid digestion. The culture plate was placed back in an incubator, or placed upside down/EC side down in medium and incubated for 3 h at 37°C. SMCs were obtained with firmer scraping, and strained using a 100 µM sterile cell strainer (yellow) to remove debris. The cells were centrifuged (400 g/5 min), and 2.5 ml ECBM2 was added to the cell pellet, which was transferred to a gelatinized T12.5 flask. The next morning (or late the same day, after 6 h) the cells were washed with warm PBS twice and fresh ECBM2 was added. The medium was changed every 48/78 h. When the cells were confluent, the cultures were split in two T25 flasks, and cultured until confluent again before they were frozen in three tubes (total 3 mL) in FBS (fetal bovine serum) /10% DMSO (dimethyl sulfoxide) at around 600,000 cells per tube. (When wake up, seed one tube of cells in T75 flask). The cells were trypsinized (TrypLE Express, LifeTech), pre-warmed, washed in PBS and incubated at 37°C for 5 min

to detach. Then, medium was added, and the cells were centrifuged (300 g, 5 min) and resuspended before being plated or frozen. If plated, the dish was moved once or twice every hour. After about 4-6 h, the aorta would have dissolved into a cell suspension of SMCs. The cells were washed twice with DMEM/F-12 containing 5% serum and seeded in a T 25 flask. Fresh medium was added after 72 h, and changed thereafter every 48 or 72 h.
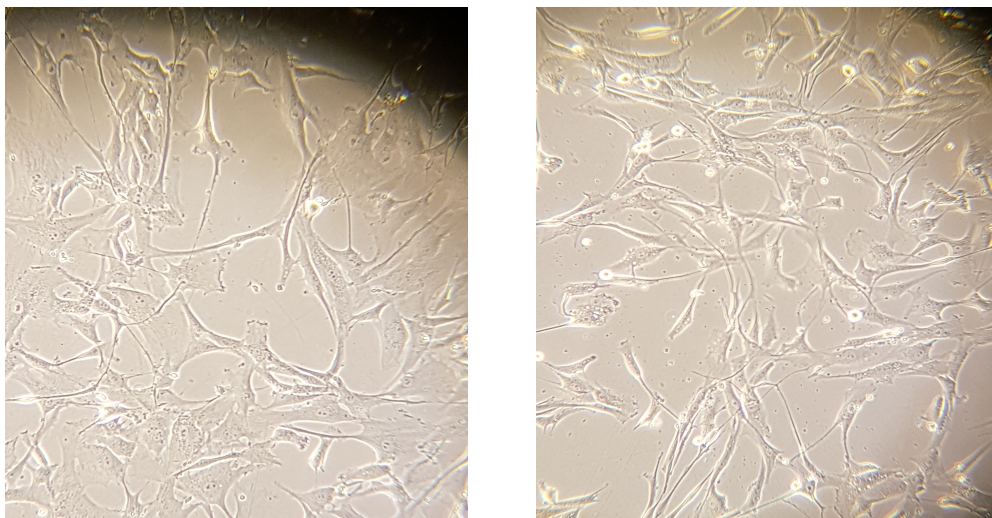
Figure 3.2. SMCs isolated from a carotid artery. (LEICA DM4000 B microscope)

3.4 Cell culture and transfection

SMCs were propagated in growth media (Lonza) with 5% fetal bovine serum per standard protocols. Lipofectamine RNAiMAX (Invitrogen) reagent was used for transfection of small antisense oligonucleotides at the concentration of 20 nM. And Lipofectamine 3000 (Invitrogen) reagent was used for vector transfection at the concentration of 50 ng/ml.

3.5 RNA and protein isolation

Materials:

RNeasy Mini Kit (Qiagen)

70% Ethanol (VWR)

Absolute ethanol (VWR)

8 M Urea

2 M DTT (dithiothreitol)

Methods: The cell monolayer was disrupted by adding 350 μL buffer RLT (RNeasy Lysis Buffer from RNeasy Mini Kit), and pipetting up and down. If isolation could not proceed, the cells were frozen at -80°C degree so that isolation could be attempted on another occasion. The 70% ethanol (350 μL) was added, and mixed well by repeatedly pipetting up and down. The sample was not centrifuged. The lysate was transferred to a RNeasy Mini spin column (placed in a 2-mL collection tube), which was closed and centrifuged. Typically, experiments were run in triplicate or quadruplicate, the flow-through from biological replicates was pooled in a 15-mL Falcon tube and placed on ice. 700 μL buffer RW1 was added to the column and spun, and the flow-through was collected as described above. 500 μL buffer RPE was added to the column and spun, and the flow-through was collected as described above. 500 μL buffer RPE (Washing Buffer from RNeasy Mini Kit) was added to the column and spun, this time for 2 min, before the flow-through was collected as described above. The column was dried by exchanging the 2-mL collection tube for a new one and

centrifuging at full speed for 1 min. Any residual flow-through was collected as described above. The column was placed in a 1.5-mL collection tube and 30 μL RNase-free water was added to the middle of the membrane of the column and incubated for 5 min. It is crucial for the RNA yield that the RNase-free water covers the membrane, ensuring that none adheres to the wall of the column. The column was centrifuged at 12,000 μL for 1 min to collect RNA. The tubes were labelled carefully using stickers from a labelling machine. All tubes were kept on ice at all times and centrifugation was carried out in cold room. The combined flow-through was incubated at -20°C for at least 24 h; it was recommended to leave the protein to precipitate for 72 h. Aliquots (1.5 mL) of the flow-through was placed in a 1.5-mL Eppendorf tube and spun for 15 min at 15,000 g to pellet the protein. The supernatant was discarded and another 1.5 mL of flow-through was added to the same tube and spun, and the process was repeated until all flow-through had been centrifuged and all proteins had been pelleted. The protein pellet was washed twice by adding cold absolute ethanol, incubating at -20°C for 20 min and then spinning again for 15 min at 15,000 g, which was repeated twice. The pellet was dissolved in 50 μL 8 M urea + 50 mM DTT. The urea + DTT buffer was freshly made at all times.

3.6 RNA quantification and gene expression

Methods: RNA was extracted as directed in a TRIzol-based (Invitrogen) RNA isolation protocol. RNA was quantified with a Qubit™ RNA HS Assay Kit

(Invitrogen by Thermo Fisher Scientific) with a Qubit 3 Fluorometer (Life Technologies, Invitrogen by Thermo Fisher Scientific); RNA quality was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies) and 260/280 ratios > 1.8 were required. The RNA was used for sequencing or reverse transcription with a high-capacity RNA-to-cDNA (complementary DNA) kit (Thermo Fisher Scientific) according to the manufacturer's instructions using a Mastercycler (Eppendorf).

3.7 Quantitative reverse-transcription polymerase chain reaction

Quantitative PCR (qPCR) was performed with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) with a StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies). TaqMan Gene Expression Assays (Applied Biosystems by Thermo Fisher Scientific) for *RIPK1* (receptor-interacting protein kinase 1), *RIPK3, MLKL* (mixed lineage kinase-like domain protein), *RPLP0* (ribosomal protein lateral stalk subunit P 0), *GUSB* (glucuronidase beta), *CTSS* (cathepsin S), *PDGFB* (platelet-derived growth factor beta) and *TNF* (tumour necrosis factor) were used. All probes were normalized to *GUSB* and *RPLP0* as endogenous controls. Amplification was calculated using an ABI PRISM 7900HT (Applied Biosystems). All fold changes were calculated by the comparative threshold cycle (ΔΔCt) method and are expressed as the mean ± SEM.

3.8 Immunohistochemistry staining for slides

Methods: Paraffin-embedded slides were deparaffinized with xylene and 99%, 96% and 70% ethanol, at 5 min each. The slides were washed twice in PBS plus 0.025% Triton X-100 with gentle agitation. Frozen sections were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 30 min at room temperature. Paraffin bedding slides skip. Circles were drawn around the tissue with a hydrophobic PAP pen. The samples were blocked in 5% normal serum with 1% BSA (bovine serum albumin) in PBS for 30 min at room temperature. The slides were drained for a few seconds and the area around the sections was wiped with tissue paper. Primary antibody diluted in PBS with 1% BSA was applied and incubated overnight at 4°C or for 1 h at room temperature. The samples were rinsed twice with PBS (0.025% Triton X-100) with gentle agitation. If a horseradish peroxidase (HRP) conjugate was used for detection, the slides were incubated in 0.3% H_2O_2 in PBS for 15 min, and rinsed twice with PBS (0.025% Triton X-100) with gentle agitation. If a biotin-labelled secondary antibody was used, samples were blocked an with avidin/biotin blocking kit (Reagent A × 10 minutes, wash, then Reagent B × 10 minutes). The enzyme-conjugated secondary antibody diluted in PBS with 1% BSA was applied and incubated for 1 h at room temperature, and the sample was rinsed twice with PBS (0.025% Triton X-100) with gentle agitation. For the Elite ABC kit: 100 μL A was added to 5 mL PBS, mixed well, then 100 μL B was added to it, allowed to stand for 30 min before use. For the ABC AP kit: 100 μL A was added to 10 mL PBS, mixed well, then 100 μL B was added to it. Rinsing was done twice with PBS (0.025% Triton X-100) with

gentle agitation. The samples were developed with the substrates for 10 min at room temperature or under microscopy for desirable. Then, the sample was rinsed in running tap water for 5 min and counterstained haematoxylin for 10 s. Then, it was rinsed in running tap water for 5 min, dehydrated, cleared and mounted.

3.9 Immunofluorescence staining on frozen sections

Materials:

PBS-T: PBS + 1× 0.02% Tween 20 (1 L PBS, 200 μL Tween 20)

Blocking solution: 5% normal horse serum/PBS

Normal horse/goat serum (Jackson ImmunoResearch)

Mounting anti-fade media: ProLong

Acetone

PAP pen (To create a hydrophobic barrier and use less precious reagents)

Methods: Frozen sections were air-dried for 30 min at room temperature, fixed with acetone for 10 min at room temperature and washed once with PBS. Circles were drawn using PAP pen around the tissue, and the tissue was incubated with blocking solution for 1 h at room temperature before being incubated with the primary antibody (Abcam) diluted in blocking solution at 4°C. The dilution used was based on the primary antibody. The sample was washed three times with PBS-T at 5 min each time, washed three times with PBS at 5 min each time, and incubated with fluorescent secondary antibody (Invitrogen) diluted in blocking

solution (1:500) for 1 h at room temperature. Then, the sample was washed three times with PBS-T at 5 min each time, washed three times with PBS at 5 minutes each time, and incubated with or DAPI (4′ 6-diamidino-2-phenylindole, 1:5000; nuclei staining) for 15 min at room temperature. Finally, the samples were washed once with PBS-T for 5 min, then washed once with PBS for 5 min before the slides were mounted using anti-fade media.

3.10 Immunofluorescence for cells

Multi-colour immunostaining key points:

Materials:

First antibody (Abcam): Different species

Second antibody (Invitrogen): A third resource (different from the first antibody)

Block: Serum second antibody resource or third resource

The antibodies were incubated either simultaneously (in a mixture) or sequentially (one antigen after another). If incubating simultaneously, then the antibodies should be of different species. For the secondary antibodies, sequential incubation was advisable. After fixation, the sample could be stored in PBS for a few weeks at 4°C.

Methods: Coverslips were sterilized under UV light for 15 min (this step was skipped if the coverslips had been commercially sterilized). Treated or untreated cells were grown on the glass coverslips in 24-well plates. The cells were washed

three times with ice-cold PBS and fixed in 4% PFA in PBS (pH 7.4) for 30 min at room temperature. For permeabilization, if the target protein was localized intracellularly, samples were incubated for 10 min with PBS containing 0.2% Triton X-100. However, this step is not appropriate for membrane-associated antigens, as it destroys membranes. The cells were washed in PBS twice at 5 min each. The cells were incubated with 2% serum from the species in which the secondary antibody was raised in (here, goat serum) for 30–60 min at room temperature to block nonspecific antibody binding [alternative blocking solution: 3% BSA in PBS-T (PBS + 0.1% Tween 20)]. For immunostaining, cells were incubated in antibody diluted in blocking buffer in PBS in a humidified chamber or overnight at 4°C (or for 1 h at room temperature). The cells were washed in PBS twice at 5 min each, then incubated with the secondary antibody (1:1000, Alexa Fluor® 488/546/633) in PBS for 1–2 h at room temperature in the dark. The secondary antibody solution was decanted and the cells were washed three times with PBS at 5 min each in the dark. For counterstaining, cells were incubated in 0.1–1 µg/mL Hoechst or DAPI (nuclear staining) (1:5000–20,000) for 5 min, and then washed in PBS three times at 5 min each. Skip Step 12 and 13 if mounting with medium gold anti-fade with DAPI. The coverslips were mounted with a drop of mounting medium (Gold Antifade without DAPI), dried in the dark at room temperature for 10 min, and sealed with nail polish. The slide was photographed or stored in the dark at -20°C. Step 8, 10, 12: In a dish holding a wet paper towel and plastic membrane, drop one solution (50–100 µL),

flip the glass coverslips onto it. Other steps can be performed in the well plates.

3.11 In vitro studies

Methods: Human aortic SMCs (hAoSMCs) were propagated in SMC growth medium (SmGM-2; Lonza) as directed by standard protocols (Lonza; passage no. 4–5). The cells were seeded on 6-well plates, followed by 24-h treatment with or without NETs treatment, and then the IncuCyte Zoom System (Essen BioScience) was used for dynamic, live-cell imaging of proliferation, migration, apoptosis (IncuCyte caspase-3/7 reagent) and necroptosis assessment or for RNA analysis, as indicated. For fluorescent staining, the cells were seeded on round cover slides in 12-well plates.

3.12 Kinetic assessment of apoptosis in human aortic SMCs

The IncuCyte Zoom System (Essen Biosciences) was used for real-time assessment of hAoSMC status. It is a real-time quantitative live-cell imaging and analysis device that may visualize and quantify cell activities over time through automatically gathering and analyzing images continually within a standard laboratory incubator. The system allows us to conduct kinetic measurements of living cells and provides insight into active real-time biological processes. During my experiment, a caspase-3/7 apoptosis reagent (Essen Biosciences) was added before the plate was monitored in the IncuCyte with phase/fluorescence and a 2h/imaging pattern. Images were auto-collected for 72 h and then analysed

using the IncuCyte software package.

3.13 Human tissue sample acquisition

Human samples were harvested during surgery and stored in the Munich Vascular Biobank (MVB), which was approved by the local Hospital Ethics Committee. The MVB includes patients who underwent surgical repair for AAA, carotid artery stenosis, peripheral aortic disease, as well as abdominal aortic samples from organ donor controls. Experiments were performed in accordance with the rules of the Declaration of Helsinki. Approval for studies on human tissue samples was obtained under informed consent, and complied with all guidelines and policies at the Klinikum rechts der Isar, Technical University Munich.

From January 2004 to December 2018, tissue from over 2700 patients was collected, including 1567 carotid plaques, 481 AAA specimens, 703 peripheral aortic disease samples, 64 peripheral aneurysms and 80 thrombus samples. In addition, serum was also collected from over 4400 patients, including 1394 high-grade carotid stenosis patients, 1380 AAA patients, 1702 peripheral aortic disease patients. 102 healthy aortic tissue samples were also collected as control group from Department of Trauma Surgery. Furthermore, clinical data of all patients was also acquired. Providing evidences to understand the link between tissue characterizations and patients' medical history.

After surgical excision, fast and accurate tissue processing is important. Usually,

the selected segments of the tissue were frozen in liquid nitrogen directly and then stored at -80°C, or fixed in formalin (minimum of 24h) and decalcified using EDTA, followed with embedding in paraffin and finally stored at room temperature. Afterward, the tissue contributes to histological and RNA analysis.

3.14 Statistics analysis

Data are presented as mean ± SEM, unless stated differently. Groups were compared using Student's t-test. Normality was tested to ensure that parametric testing was appropriate. Reverse transcription-polymerase chain reaction (qRT-PCR) results were determined using the original frequency domain regression (FDR) method of Benjamini and Hochberg, with Q = 1%. Each row was analysed individually, without assuming a consistent SD (standard deviation). A value of $P<0.05$ was considered statistically significant.

3.15 Ethic vote

The human samples used in this study were based on the Munich Vascular Biobank and were approved by the Ethics Committee at Technical University of Munich. (project number: 2799/10)

4   RESULTS
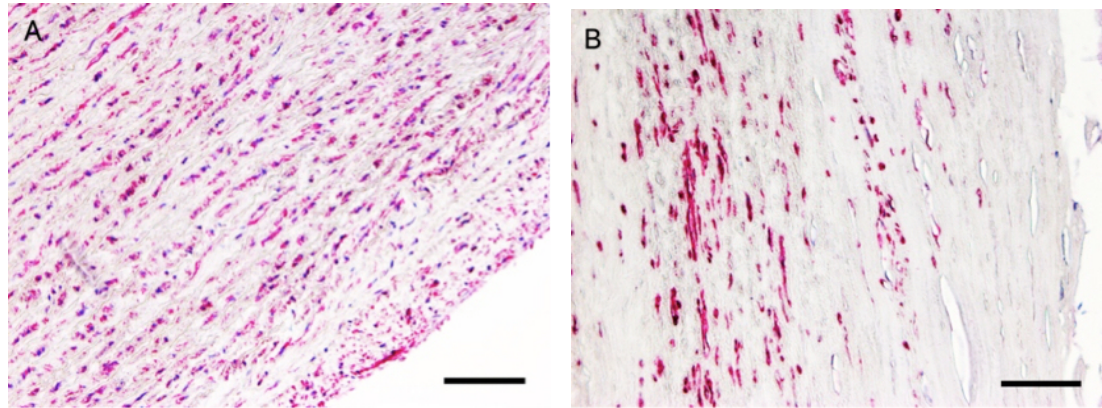
4.1 SMCs depletion in AAA.

Figure 4.1. SMC depletion in AAA sample compared to that in control aorta sample. (A) Immunohistochemistry for SMC alpha actin (SMA) in control human aorta sample. (B) Immunohistochemistry for SMA in AAA human sample. Abdominal aortic aneurysm (AAA), smooth muscle cells (SMCs).

There was an obvious decrease of the SMC alpha actin (SMA) marker in human AAA tissue in comparison with healthy human aortic tissue (Figure 4.1.). As one of the most specific characteristics of AAA, SMC depletion exerts a huge influence on the progression of this disease, as it thins out the aortic wall and weakens its structure and elasticity. Understanding the underlying mechanism would provide novel ideas for preventing or treating the disease.

4.2 Neutrophils purification and NETs concentration.
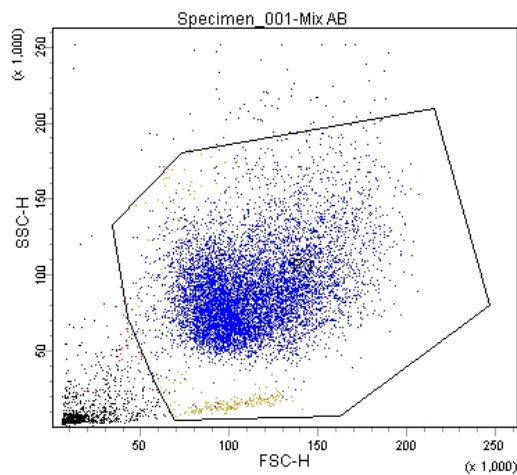
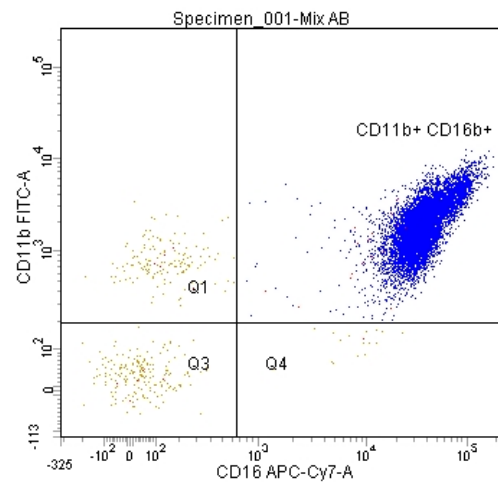A.   B.

Figure 4.2.1. Neutrophil purification measured by FACS. (A) Light scatter plot; a region has been drawn around neutrophils. (B) Plot of two fluorescences, neutrophils are coloured blue. Fluorescence-activated cell sorting (FACS); forward scatter (FS); side scatter (SS).

Figure 4.2.1. shows two dot plots of four parameters data derived from human peripheral blood cells. FS is most sensitive to the size of the cell, while SS is most influenced by the optical homogeneity. A region has been marked around the neutrophils in the light scatter cytogram (labelled in blue). The cells were labelled with anti-CD11b-FITC-A and anti-CD16-APC-CY7-A, which are surface markers of neutrophils. There are at least three sub-populations in the plot of CD11b versus CD16b, blue dot plots represent CD11b+ and CD16b+ cells, which are neutrophils and the largest subpopulation. This indicates that neutrophil purity is sufficient to conduct latter research.

Donor 1	3440 ng/mL	Donor 2	3000 ng/mL
Donor 3	2580 ng/mL	Donor 4	2180 ng/mL
Donor 5	1784 ng/mL	Donor 6	4040 ng/mL
Donor 7	3920 ng/mL	Donor 8	3760 ng/mL
Donor 9	2020 ng/mL	Donor 10	1414 ng/mL
Donor 11	1192 ng/mL	Donor 12	1190 ng/mL
Donor 13	3680 ng/mL	Donor 14	3678.7 ng/mL
Donor 15	4408.7 ng/mL	Donor 16	3870.8 ng/mL

Table 4.2.2. Concentration of NETs from 16 donors measured with a Qubit fluorometer (Life Technologies, Invitrogen by Thermo Fisher Scientific). Neutrophil extracellular traps (NETs).

The donors are group members from Munich Vascular Biology Lab, with their informed consent, six EDTA tubes of blood was drawn from each donor, followed by isolation of neutrophils. The number of neutrophils differs among participants, and they are not equally sensitive to PMA stimulation, which results in different concentrations of NETs. As shown in table 4.2.2., NET concentration varies from 1192 ng/mL to 4408.7 ng/mL. For the latter experiment, in which we stimulated SMCs with NETs, we chose NETs with high concentrations in order to trigger maximum changes in the stimulated cells.

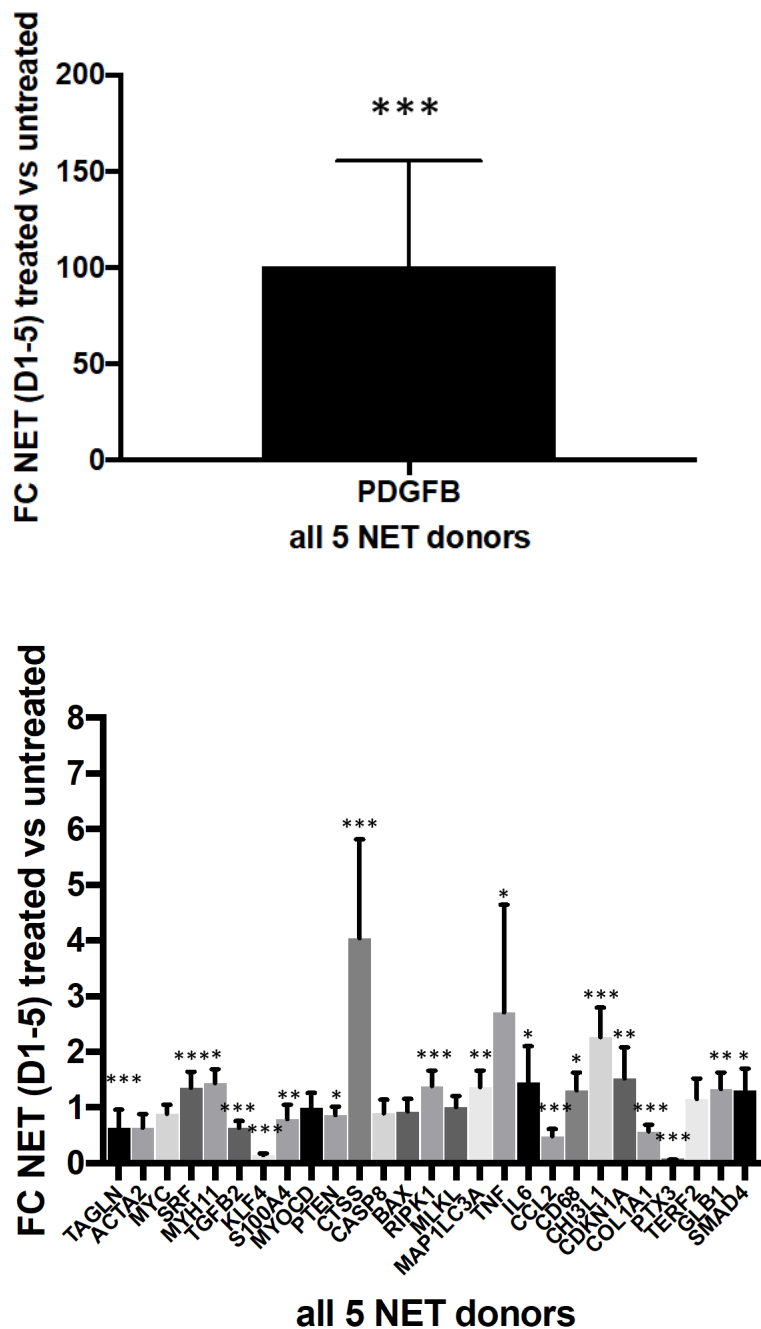## 4.3 NETs treatment changed several genes in human aortic SMCs.

Figure 4.3.1. NET treatment for 24 h altered gene expression in human aortic SMCs. Shown is a data summary of NETs isolated from five donors. (*p < 0.05, **p < 0.01, ***p< 0.001). Neutrophil extracellular traps (NETs); smooth muscle cells (SMCs); platelet-derived growth factor (PDGF); cathepsin S (CTSS); receptor interacting protein kinase 1 (RIPK1); mixed lineage kinase-like domain protein (MLKL).

NET treatment altered various genes in human SMCs (Figure 4.3.1.), which can influence the fate of SMCs through diverse mechanisms. We have chosen a panel of genes that are relevant for SMC-related pathologies, including genes that are related to phenotypic switching (dedifferentiation) like *PDGF*, *CTSS*, *RIPK1*, which underwent substantial expression changes upon NET stimulation. PDGF is part of a family of growth factors that regulate cell proliferation, differentiation and migration(Kim et al., 2007). PDGF is also closely associated with blood vessel formation, as well as the genesis of fibroblasts, SMCs and endothelial cells(Kim et al., 2010). High expression of PDGF is known to be involved in cardiovascular diseases, including atherosclerosis(Lin et al., 1995). CTSS is a lysosomal enzyme expressed by some epithelial cells, and can furthermore cleave extracellular matrix (ECM) proteins(Díez, 2010), regulate blood vessels permeability and trigger angiogenesis(Wang et al., 2006), RIPK1 and RIPK3, as well as their substrate MLKL are identified as crucial markers and mediators of necroptosis. Overall, treating SMCs with NETs seems to influence SMC dynamics and fate through diverse and complex mechanisms.

Summarized below are important functions of the genes being chosen for our panel to study SMCs upon NET stimulation:

Gene	Known function
TAGLN	Early marker of SMC differentiation

ACTA2	Vascular contractility and blood pressure homeostasis
MYC	Cell cycle progression, apoptosis and cellular transformation
SRF	Cell proliferation and differentiation
MYH11	Contractile protein in SMCs, able to convert chemical energy into mechanical energy through the hydrolysis of ATP
TGFB2	Recruitment and activation of SMAD family transcription factors that regulate gene expression
KLF4	Normal development of the barrier function of skin; control the G1-to-S transition of the cell cycle following DNA damage
S100A4	Cell cycle progression and differentiation
MYOCD	Cardiogenesis and differentiation of the SMC lineage
PTEN	Tumor suppressor
CTSS	Cleave ECM proteins, regulate blood vessel permeability and angiogenesis
CASP8	Cell apoptosis
BAX	Mitochondrial apoptotic process
RIPK1	Inflammation and cell death in response to tissue damage, pathogen recognition
MLKL	TNF-induced necroptosis
MAP1LC3A	Mediates the physical interaction between microtubules and components of the cytoskeleton

TNFα	Cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation
IL6	Inflammation and the maturation of B cells
CCL2	Immunoregulatory and inflammatory processes
CD68	Phagocytic activity
CHI3L1	Inflammation and tissue remodelling
CDKN1A	Encodes a potent cyclin-dependent kinase inhibitor, a regulator of cell cycle progression at G1
COL1A1	Encodes the pro-alpha1 chains of type I collagen
PTX3	Regulation of innate resistance to pathogens, inflammatory reactions
TERF2	Encodes a telomere specific protein; senescence marker
GLB1	Generate mature lysosomal enzyme; senescence marker
SMAD4	Tumor suppressor and inhibition of epithelial cell proliferation
PDGF	Genesis of fibroblasts, SMCs and endothelial cells

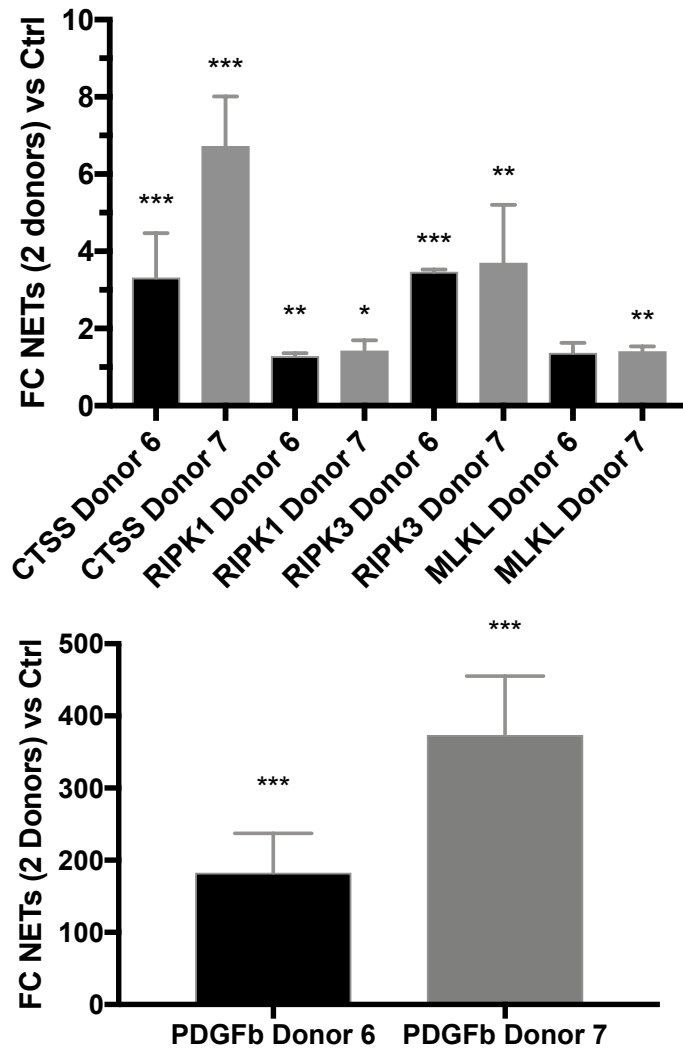Table 4.3.2. Important functions of genes in qPCR experiment.

Figure 4.3.3. NET treatment for 24 h upregulated several genes in human SMCs: *CTSS*, *RIPK1*, *RIPK3*, *MLKL*, *PDGFB*, and *RPLP0* corrected. Neutrophils were isolated from two donors, followed with PMA stimulation. (*p < 0.05, **p < 0.01, ***p < 0.001). Smooth muscle cells (SMCs); phorbol 12-myristate 13-acetate (PMA); cathepsin S (*CTSS*); ribosomal protein lateral stalk subunit P 0 (*RPLP0*); receptor-interacting protein kinase 1 (*RIPK1*); receptor-interacting protein kinase 3 (*RIPK3*); mixed lineage kinase-like domain protein (*MLKL*); platelet-derived growth factor (*PDGF*).

The concentration of NETs from Donor 6 and Donor 7 are 4040 ng/mL and 3920 ng/mL respectively. SMCs of passage 6 were treated with NETs at a concentration of 500 ng/mL for 24 h. Then cells were collected and analyzed.

With the knowledge of previous qPCR results, PDGF, CTSS and RIPK1 are chosen for the analysis this time. RIPK3 and MLKL are as mentioned downstream mediators of RIPK1, and more specific markers of the necroptosis pathway. Based on thesis results, NETs seem to influence the SMCs fate through inducing programmed cell death via the necroptosis pathway.
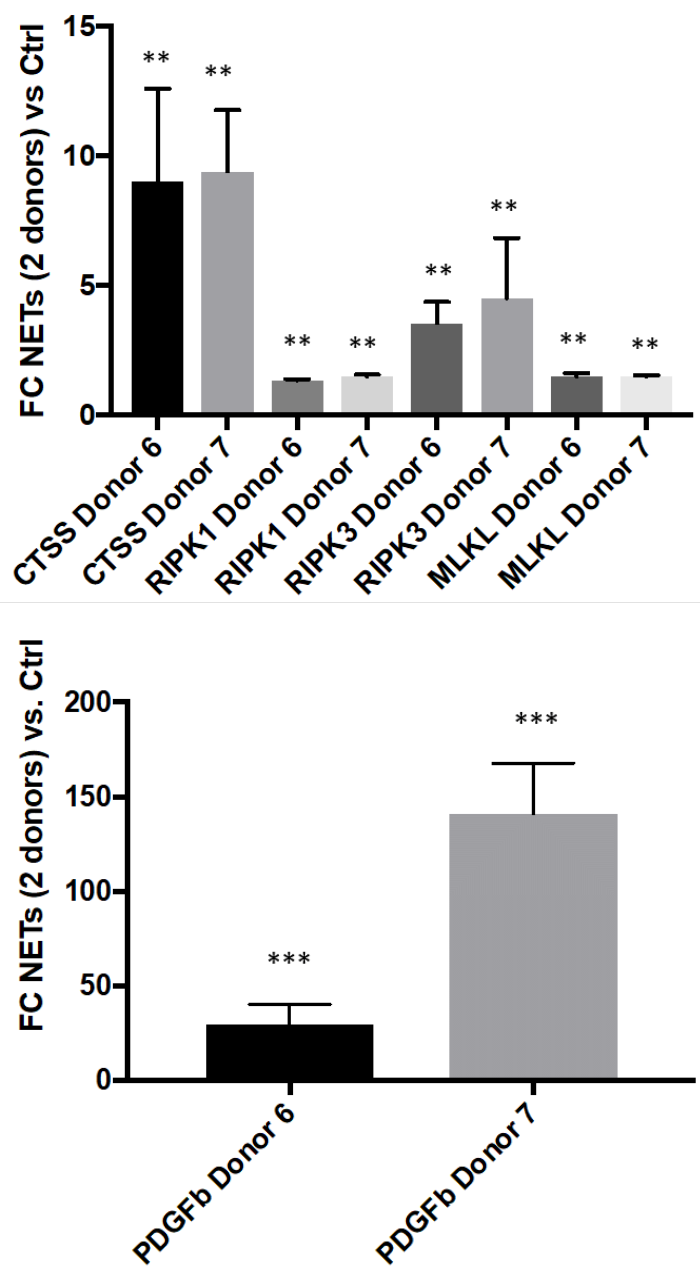

Figure 4.3.4. Repetition of 24h NET (NETs from Donor 6 and Donor 7) treatment on

human SMCs. *RPLP0* corrected. (*p < 0.05, **p < 0.01, ***p < 0.001). Neutrophil extracellular traps (NETs); ribosomal protein lateral stalk subunit P 0 (*RPLP0*); smooth muscle cells (SMCs); cathepsin S (*CTSS*); receptor-interacting protein kinase 1 (*RIPK1*); receptor-interacting protein kinase 3 (*RIPK3*); mixed lineage kinase-like domain protein (*MLKL*); platelet-derived growth factor (*PDGF*).

NETs-treated SMCs show increased *CTSS*, *PDGFB*, *RIPK1*, *RIPK3* and *MLKL* mRNA levels. This repetition experiment shows the same trend as the previous one, confirming that NETs influence the destiny of SMCs through induction of the necroptosis pathway. Increases of *PDGFB* and *CTSS* may indicate that NETs further trigger proinflammatory conditions.
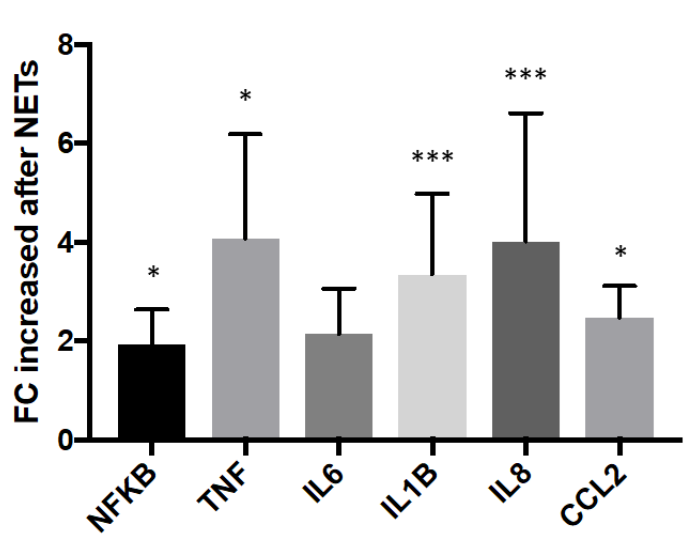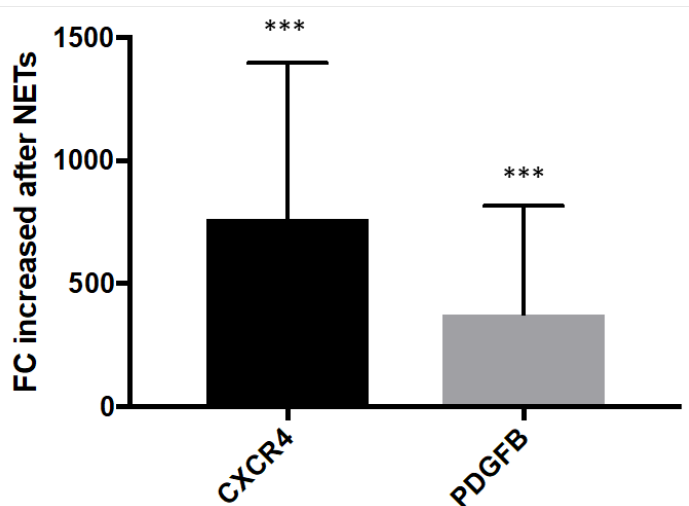
Figure 4.3.5. The 24 h NET treatment altered the inflammation-related genes in human aortic SMCs. Shown is a data summary of NETs isolated from two donors. (*p < 0.05, **p < 0.01, ***p < 0.001). Interleukin (*IL*); platelet-derived growth factor (*PDGF*); tumour necrosis factor alpha (*TNFα*); nuclear factor kappa-light chain enhancer of activated B cells (*NFKB*); chemokine ligand 2 (*CCL2*); C-X-C chemokine receptor type 4 (*CXCR4*).

NET treatment of human SMCs influenced their viability through various mechanisms. The treated SMCs had increased *PDGFB*, *IL8*, *IL1B*, *CCL2*, *NFKB*, *TNFα* and *CXCR4* mRNA levels - a sign that NETs can influence SMCs through inflammation-related mechanisms. NFκB regulates cytokine production and immune response to harmful stimuli, while ROS, TNFα and IL-1β are inducers of NFκB activity, which then controls multiple genes and pathways involved in inflammation. TNFα regulates various cell activities, such as the immune response and inflammation, and IL1β is an important mediator in the inflammatory response and is associated with multiple cellular activities, including cell proliferation, differentiation, and apoptosis. IL8 is involved in attracting neutrophils to sites of infection and stimulating phagocytosis. CCL2 is

implicated in recruiting T cells, monocytes and dendritic cells to inflammation sites. CXCR4 is able to conduct chemotactic effects for lymphocytes. All these inflammatory genes in SMCs are altered after NETs treatment, indicating that NETs also influence SMCs activity through an enhancement of inflammation.
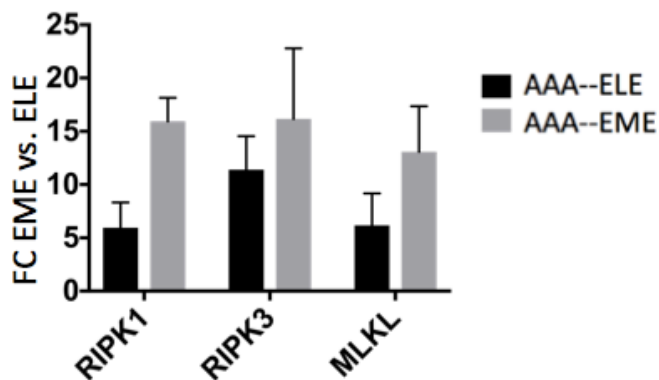
A.
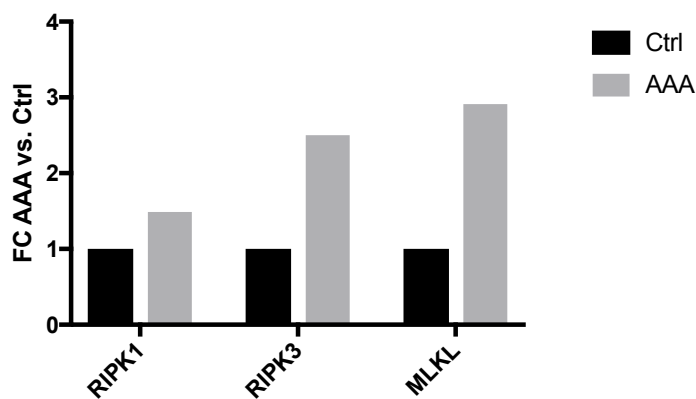
B.

Figure 4.3.6. (A) Upregulation of *RIPK1*, *RIPK3* and *MLKL* in human AAA samples compared to normal control aortic samples. Grey bar: AAA group; black bar: normal control group. (B) Upregulation of *RIPK1*, *RIPK3* and *MLKL* in human AAA samples from emergency surgery (EME) compared to human AAA samples from elective surgery (ELE). Grey bar (EME): AAA samples from emergency surgery patients; black bar (ELE): AAA samples from elective surgery patients. Receptor-interacting protein kinase 1 (RIPK1); receptor-interacting protein kinase 3 (RIPK3); mixed lineage kinase-like domain protein (MLKL); abdominal aortic aneurysm (AAA).

There was an obvious increase in *RIPK1*, *RIPK3* and *MLKL* mRNA levels in human AAA tissue compared to human healthy aortic tissue (Figure 4.3.5.). In comparison with tissue from AAA patients without severe disease, tissue from patients with ruptured AAA had even higher *RIPK1*, *RIPK3* and *MLKL* mRNA levels. These findings indicate that the necroptosis pathway is important in AAAs, but also that the levels of *RIPK1*, *RIPK3* and *MLKL* may also indicate the severity of disease acceleration.

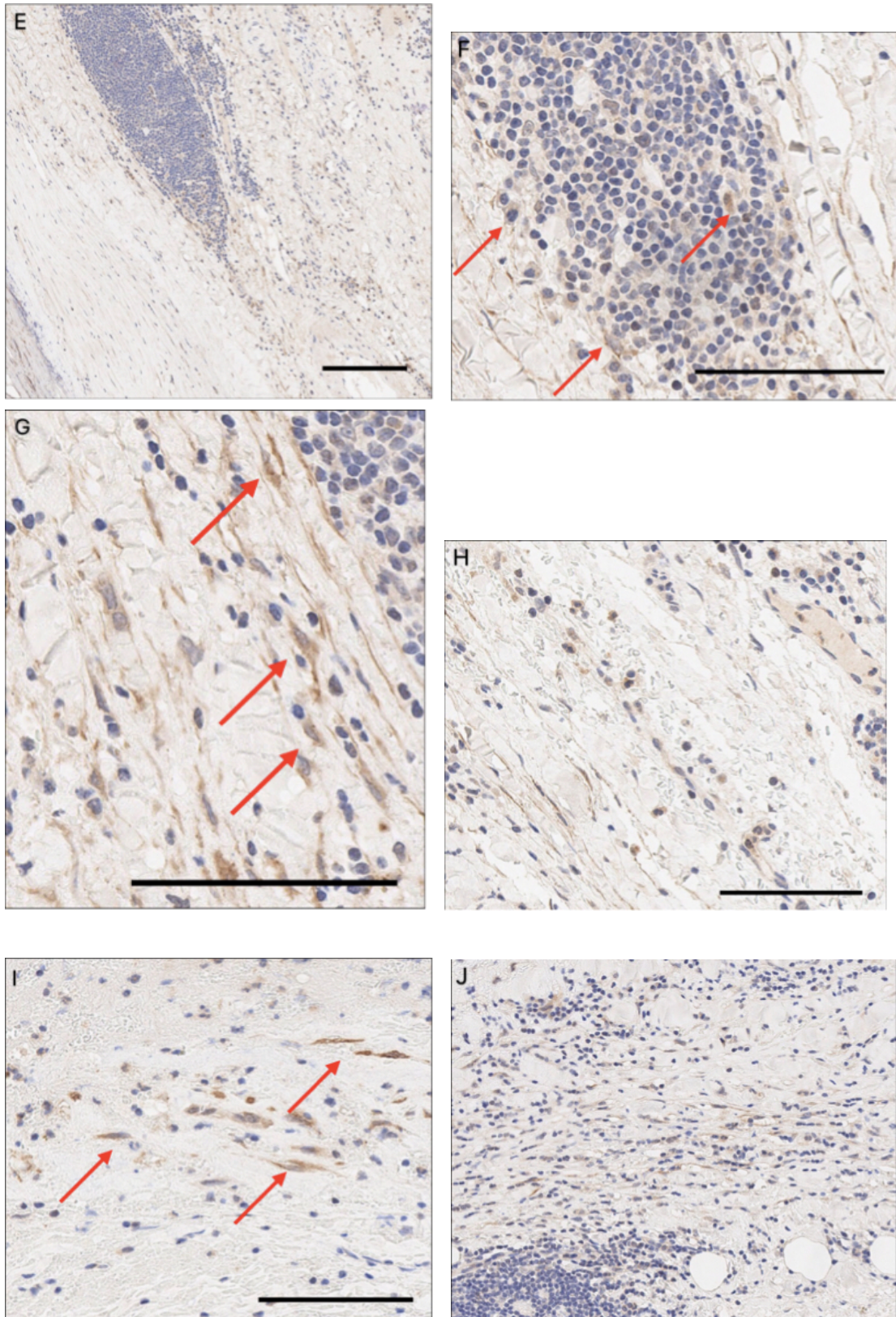## 4.4 RIPK3 expression in human aortic tissue.
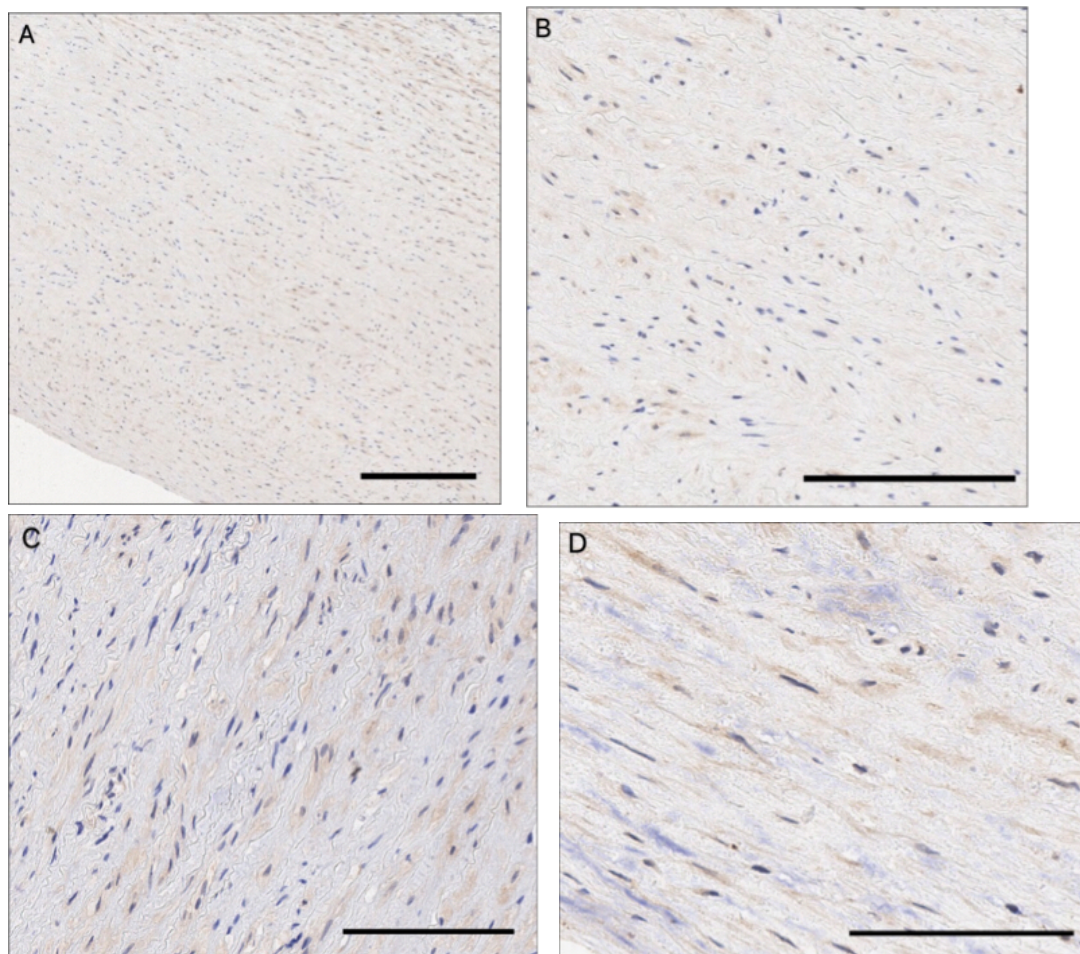
Figure 4.4. RIPK3 expression in human aorta vessel wall. (A–D) Low RIPK3 expression in human healthy aortic tissue. (E, F) Increased RIPK3 expression in human aortic aneurysm tissue. (G, H) High RIPK3 expression in tissue adjacent to human aortic aneurysm tissue. (I, J) High RIPK3 expression in angiofibroblasts of human aortic

aneurysm tissue. Receptor-interacting protein kinase 3 (RIPK3).

RIPK3 is a crucial mediator in the necroptosis pathway. Thus, it was used in our experiments as a detection marker of this pathway through immunohistochemistry (Figure 4.4.). No obvious RIPK3 positive staining is detected in healthy aortic tissue; however, the aortic aneurysm tissue had clear RIPK3 positive staining, indicating the existence of the RIPK3-dependent cell death mechanism in AAA disease. In accordance with the increased *Ripk3* mRNA level in aneurysm tissue from AAA patients, staining of RIPK3 in human AAA tissue could also be easily observed. In the former experiment, I treated SMCs with NETs and obtained upregulation level of *RIPK3* and *MLKL*, but in this staining experiment, high expression of RIPK3 is also observed in what seems to be angio-fibroblasts in AAA patients. This indicates that in human disease, necroptosis-associated mediators are not specific to SMCs, but appear to be overall important for AAA expansion also in other cell types.

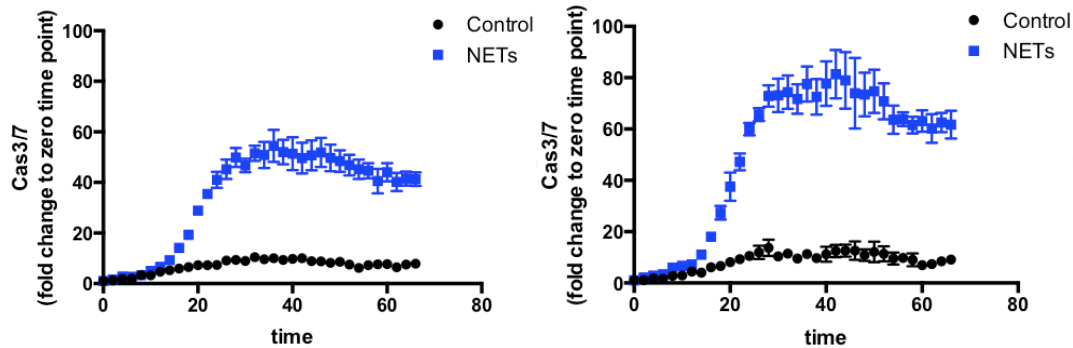## 4.5 NETs treatment induce apoptosis of human aortic

SMCs.

Figure 4.5.1. Caspase-3/7 signal measured by IncuCyte Zoom System. Black dots: Caspase signal in live control human SMC group. Blue dots: Caspase signal in NETs-treated SMC group. Smooth muscle cell (SMC); neutrophil extracellular traps (NETs).

SMCs of Passage 6 were grown in a 6 well plate format. NETs from Donor 6 and Donor 7 were added to the plate. At the same time, a caspase-3/7 apoptosis reagent (Essen Biosciences) was added, and then the plate was monitored in the IncuCyte live cell imaging apparatus (Figure 4.5.1.). After analysing the data with the help of the IncuCyte software package, no obvious differences are detected in the first 18 hours between the control group and NET-stimulated group. However, after that time point, we can detect an increase in caspase signalling in the NETs treated group, which peaks approximately 30h after starting the treatment. On the contrary, the caspase signal remains stable in the control group. Thus, NETs influence SMCs death through apoptosis-related mechanisms to a certain extent.
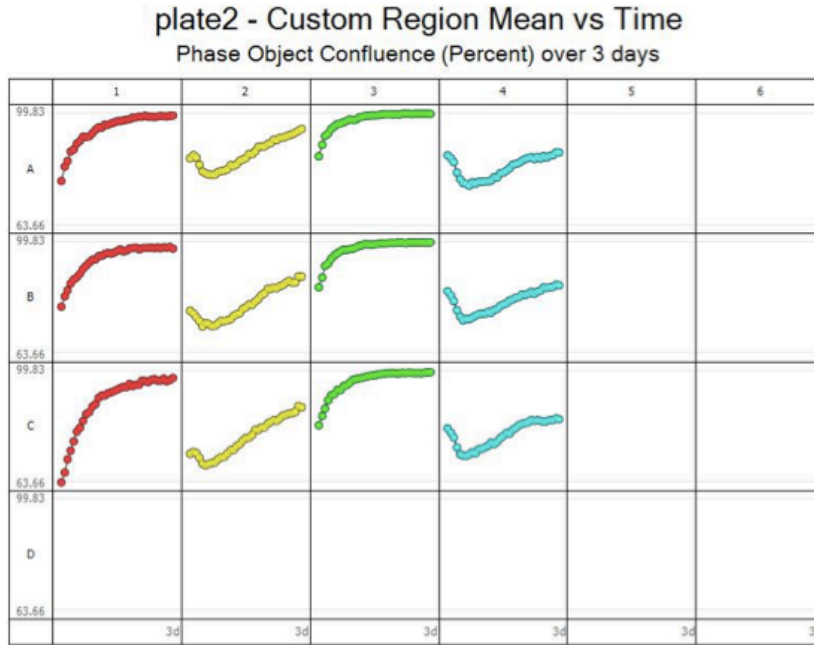
Figure 4.5.2. Phase Object Confluence measured by IncuCyte Zoom System. (A–C 1) Control group; (A–C 2) NET treatment group (Donor 8); (A–C 3) control group; (A–C 4) NET treatment group (Donor 9). Smooth muscle cell (SMC); neutrophil extracellular trap (NET).

NETs diluted in SMC growth medium at a concentration of 500 ng/mL are added into the treatment group. Meanwhile, pure SMC growth medium is added into the control group. In the control group, a stable increase of SMCs was observed (Figure 4.5.2.). However, in the NETs treated group, total SMCs were decreased firstly, followed by a slight increase. Thus, treatment with NETs seems to also influence the proliferation of SMCs.

Figure 4.5.3. Green Object Count measured by IncuCyte Zoom System. (A–C 1) Control group; (A–C 2) NET treatment group (Donor 8); (A–C 3) control group; (A–C 4) NET treatment group (Donor 9). Smooth muscle cell (SMC); neutrophil extracellular trap (NET).

In the control group, the caspase signal remained stable; however, an obvious increase of caspase signal was observed in the NETs-treated group. Thus, treatment with NETs seems to influence SMCs death through the caspase-dependent pathway, which is the apoptosis pathway.

Figure 4.5.4. Live cell images captured with the IncuCyte Zoom System. (A) Green signal indicates caspase-3/7 in NET treatment SMC group, and in control SMC group. Smooth muscle cell (SMC); neutrophil extracellular traps (NETs).

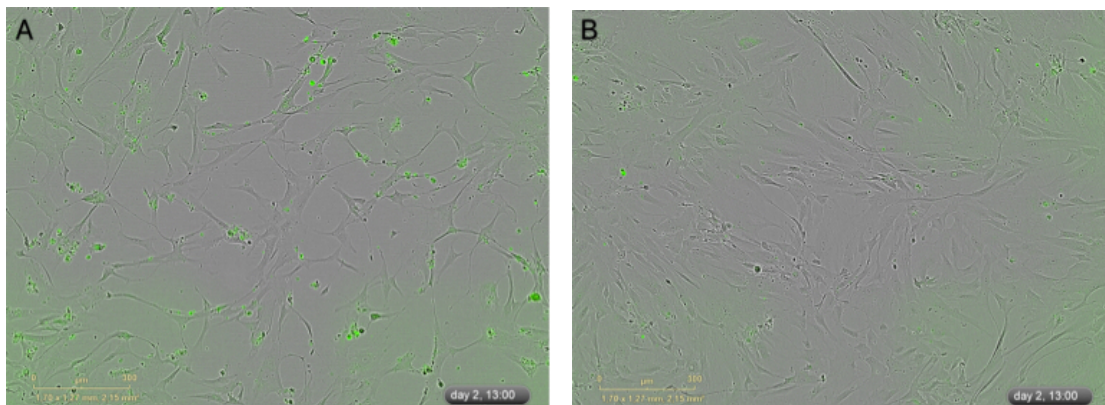Compared to SMCs in the control group, more caspase green signal was observed in the NETs treatment group (Figure 4.5.4.). In accordance with the caspase signal comparison analysed by the IncuCyte software package, we observed the caspase signal visually in both groups. Further, NETs treatment induced SMC death through the apoptosis pathway.

4.6. H19 triggers SMC apoptosis

In one study I participated in during my time in the Vascular Biology laboratory in the Department of Vascular and Endovascular Surgery, the lncRNA H19 was detected as a promising elevated lncRNAs in medial SMCs in dilated aortic tissue. Knockdown of H19 dramatically markedly reduced human SMCs apoptosis, while overexpression of H19 showed the opposite result (Li et al., 2018).
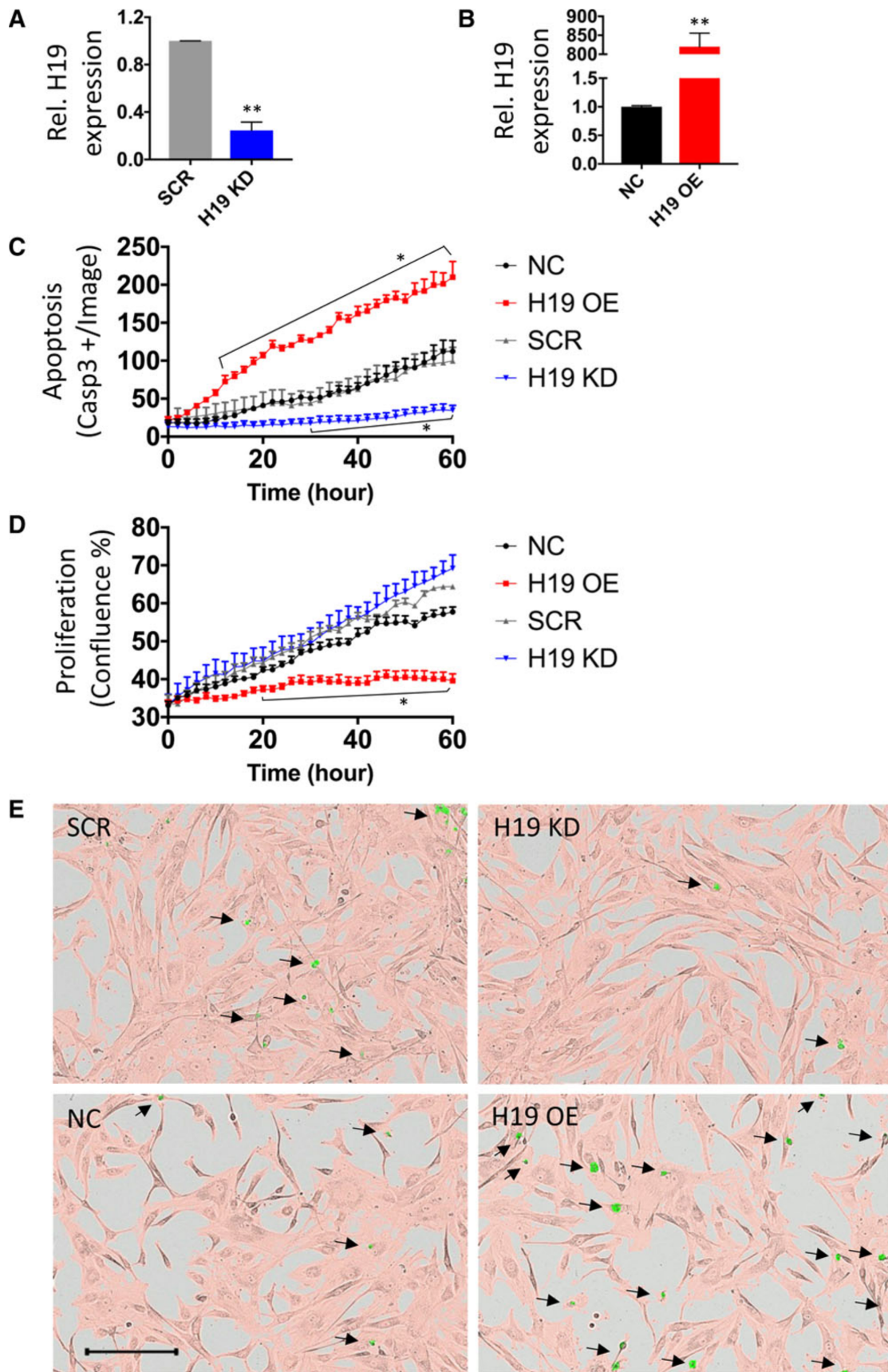
Figure 4.6 H19 promotes apoptotic cell death in vascular smooth muscle cells. (A) Verification of H19 knockdown (KD) effects. (B) Verification of H19 overexpression (OE)

effects. (C) Apoptotic rates of SMCs. (D) Proliferative rates of SMCs. (E) Typical pictures of proliferation and apoptosis of SMCs in different groups (Li et al., 2018).

For deeper investigating the effects of H19 adjustment in SMCs, cells were carefully measured for rates of proliferation and apoptosis. H19 in cultured SMCs was inhibited with LNA-GapmeRs or overexpressed with pcDNA-H19. Overexpression of H19 leaded to increased apoptosis and decreased proliferation of SMCs. On the contrary, Knockdown of H19 restrained SMC apoptosis, without obvious influence on proliferation (Li et al., 2018).

5 DISSCUSSION

AAA remains a life-threatening vascular disease with only surgical therapy or endovascular stenting currently available for large aneurysms. AAA denotes a specific instance of aortic wall remodeling, which is characterized by media degradation and progressive vessel wall weakening and finally rupture(Kent, 2014). The most noticeable pathological changes are observed in the medial and intimal layers, such as foam cells accumulation, cholesterol crystals existence, more calcifications, ulcerations, and thrombosis, and increasing release of metalloproteinases and proteases, moreover, inflammation infiltration is also presented in adventitia. In AAA, elastin and collagen undergo obvious changes in content and structure due to the emergence of elastin- and collagen-degrading proteinases. Accompanied by these alterations, SMC depletion appears to be the most crucial aspect that explains the impaired vessel wall integrity(Thompson et al., 1997). Furthermore, medial SMCs are involved to great extent in maintaining extracellular matrix production and reversing its continual decrease; consequently, in AAA, the paucity of SMCs drives further deterioration of the aorta.

SMCs in the healthy aortic media are usually stable and more inclined to be differentiated(Orekhov et al., 1998), forming contractile units along with the adjacent extracellular components. However, under influences of environmental factors, such as growth factors/inhibitors, mechanical influences, cell-cell and

cell-matrix interactions, extracellular lipids and lipoproteins, and various inflammatory mediators(Owens et al., 2004).(Allahverdian et al., 2014), vascular SMCs still have the potential to change their phenotype, platelet-derived growth factor (PDGF) leads to decreased expression of SMC contractile genes and contractile protein degradation through autophagy pathway(Lacolley et al., 2018). In contrast to PDGF, transforming growth factor (TGF-β) contributes to the opposite effect through small mothers against decapentaplegic (Smad) signaling pathway(Low et al., 2018). After shifting to a synthetic (de-differentiated) phenotype, SMCs may acquire the characteristics of other cells(Shankman et al., 2015), for example, macrophage-like features with an increase inflammatory and phagocytotic features. Meanwhile, SMC-specific markers, such as smooth muscle cell alpha actin, smooth muscle heavy chains (SMMHCs) and others become decreased(Mulvihill et al., 2004). SMCs from healthy media usually exhibit a spindle-like shape, corresponding to the contractile phenotype. However, synthetic phenotypic SMCs have cobblestone-like morphology, corresponding to the synthetic phenotype of the intima-media of the aorta(Allahverdian et al., 2018). Hence, apart from the functional changes during the phenotypic shifting, SMCs still undergo complex structural changes.

In my data, an obvious decrease of the SMC alpha actin marker is detected in human AAA tissue in comparison with healthy human aortic tissue. SMC depletion constitutes major threats to AAA progression. Thus, understanding the

underlying mechanism would offer novel opportunities for treating AAA.

SMC apoptosis has long been recognized as the main reason for triggering of the decreasing SMC population during AAA formation(Thompson et al., 1997). Cellular, biochemical, morphological and molecular changes make it apparent that apoptosis accounts as indispensable underlying mechanism behind SMC depletion. Even under normal physiological conditions, apoptosis contributes greatly to controlling the cell population, modifying cellular composition synthetics, and eradicating damaged cells, let along in pathological conditions. Either the cells sense the stress by intracellular signals, or the cells are activated by extracellular ligands that bind to cell-surface death receptors, the pathway are then initiated.

The apoptosis pathway requires energy(Elmore, 2007); another important element is caspase in the cytoplasm. Generally, 14 caspases are closely involved in apoptosis, playing various roles in different stages, thereby forming the complete pathway(Salvesen and Dixit, 1997). Typically, the apoptosis mechanisms are divided into three groups: the extrinsic pathway, intrinsic pathway, and granzyme pathway(Cullen and Martin, 2009). Stimulation of extrinsic (death receptor-mediated) and intrinsic (mitochondrial) pathways initiate the activity, followed by chromatin condensation, DNA fragmentation, cell shrinkage and shedding of apoptotic bodies(Nelson et al., 2008). The extrinsic pathway is mediated by extracellular ligands binding to cell-surface death receptors, including Fas or TNF family receptor (TNFRs), followed by a

series of chain reactions mediated by Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD), which activate initiator caspase-8 and then executioner caspase-3(Sun et al., 2000). Caspase-3 functions in a crucial step that controls caspase-6 and -7, which finally contribute to cell apoptosis(Reed, 2000). The intrinsic pathway is initiated by intracellular stimulations and depends on the release of proteins from mitochondrial membrane, cells sense the stress including DNA damage, ischemia, ultraviolet (UV) light, inflammation and oxidative stress, which in turn influence mitochondrial membrane potential, consequently inhibiting the anti-apoptotic process in the intrinsic pathway (Lee et al., 2013). Afterward, cytochrome C is released from mitochondrias, and binds to the apoptotic protease activating factor, forming a complex that activates procaspase 9, then activated initiator caspase 9 activates the 'executioner' caspases 3, 6, and 7, leading to cellular apoptosis.

In my study, the IncuCyte Zoom System was used for visualizing and quantifying cellular activities over time. As a real-time quantitative live-cell imaging and analysis device, the system is good at conducting kinetic measurements of living cells and providing thorough insights into active real-time biological processes. During my experiment, after treating SMCs with NETs, the caspase-3/7 signal increased over time, and peaked around 24 h after treatment initiation. We also detected an obvious decrease in the SMC population; however, caspase-3/7 did not increase greatly in the control group. On the contrary, there was an evident

increase in the total amount of SMCs. In summary, treatment with NETs influenced SMCs death through a caspase-dependent pathway, which is the apoptosis pathway.

However, only apoptosis is insufficient in explaining the evident elimination of SMCs. Given that caspase inhibitors have limited influence in preventing aneurysm progression(Yamanouchi et al., 2010), necroptosis is also believed to be closely involved in inducing medial SMC death. RIPK1 and RIPK3 and their substrate MLKL have been identified as vital and representative markers of necroptosis(Galluzzi et al., 2017). Typical characterizations include organelle swelling, cell degradation, inflammation and leakage of cellular components after cell damage(Giampietri and Starace, 2014). Death receptors such as TNFRs accumulate TRADD, TNFR-associated factor 2 (TRAF-2), RIPK1 and cellular inhibitors of apoptosis (CIAPs) as complexes, then second mitochondria-derived activation of caspases (Smac) lead to the separation of RIPK1, presenting the opportunity to form the RIPK1-RIPK3 necrosome, which then activates MLKL, followed by changes to the membrane system and its integrity(Fulda, 2013; Grootjans et al., 2017). In contrast to the non-inflammatory function of apoptosis, necroptosis gives rise to evident damage and inflammation (Dhuriya and Sharma, 2018).

My own data indicated that RIPK1, RIPK3, MLKL levels were increased in human AAA tissue samples compared to human healthy aortic tissue. Moreover, compared with tissue from AAA patients without severe symptoms, much higher

mRNA level of *RIPK1, RIPK3,* and *MLKL* were measured in tissue from patients with ruptured AAA. These findings indicate that necroptosis is popular in AAA disease, the levels of *RIPK1, RIPK3* and *MLKL* may also indicate the severity of disease consequences. Immunohistochemistry staining revealed high RIPK3 expression in human AAA samples, however, no obvious RIPK3 positive staining was observed in healthy human aortic tissue. Both RIPK3 mRNA and protein levels were increased in human AAA samples, therefore we propose that RIPK3-dependent cell death mechanism induces AAA progression.

As the first defense cells against pathogens at the inflammation area, neutrophils are closely involved in pathogen elimination via phagocytosis. Besides the phagocyte function, the discovery of NETs enhances our knowledge of this multifunctional cell type. Recent studies have demonstrated the existence of NETs in lesions of atherosclerosis and arterial thrombi(Doring et al., 2017). In addition, NETs also induce activation of ECs, antigen-presenting cells and platelets, triggering coagulation(Dabrowska et al., 2018). Moreover, NETs can induce endothelial dysfunction and trigger proinflammatory immune reactions(Qi et al., 2017). Overall, NETs play a critical role in the development and progression of atherosclerotic plaque and arterial thrombosis. On the other side, there is evidence that NETs also trigger inflammation and contribute to AAA progression directly(Yan et al., 2016), even in the early stages of AAA growth, and pharmaceutical limitation of NETosis restrains AAA growth in mouse and human tissues. Numerous inflammatory cells are detected in the

aneurysm lesions, among which neutrophils account for a large group. Neutrophil-derived dipeptidyl peptidase I (DPPI) is necessary for neutrophil recruitment to the aortic wall and NET release. NETs activate plasmacytoid dendritic cells (pDCs) and give rise to interferon alpha, exacerbating inflammation and aneurysm progression(Yan et al., 2016). NETs production blockade or pCDs depletion or limitation of interferon alpha activity may work against AAA development. Circulating interferon alpha may become a promising biomarker for predicting AAA progression.

In the present study, NETs treatment induced changes of multiple genes in human SMCs. These genes affect SMC fate through various mechanisms. Noticeably, the upregulation of *RIPK1, RIPK3* and *MLKL* was rather remarkable, being a reminder that NETs partly influence SMCs fate through the necroptosis pathway. PDGF was also upregulated evidently, which is proved to be closely involved in cardiovascular diseases, blood vessel formation and cytogenesis. In addition, level of CTSS also alters a lot, served for degrading ECM, controlling blood vessel permeability and regulating vasculogenesis. NETs treatment also result in upregulation of *IL8*, *IL1β*, *CCL2*, *NFKB*, *TNFα* and *CXCR4* mRNA levels. NFκB regulates cytokine production and immune response to harmful stimuli, ROS, TNFα and IL-1β are normal inducers of NFκB activity, which then controls multiple genes involved in inflammation. TNFα regulates various cell activities, such as immune response and inflammation, IL1β is an important mediator in inflammatory response and is associated with multiple cellular activities, IL8 is

involved in driving neutrophils to infection site and stimulating phagocytosis. CCL2 is implicated in recruiting T cells, monocytes and dendritic cells to inflammation sites. CXCR4 is able to conduct chemotactic effect for lymphocytes. Thus, NETs also influence SMC activities through inflammation-related mechanisms.

To conclude: SMCs depletion is a specific characteristic in AAA. SMC Necroptosis and apoptosis contribute to AAA progression. NETs are present in AAA, exacerbating AAA through SMCs necroptosis and apoptosis and inflammatory mechanisms.

In a published study I participated in during my time in the Vascular Biology laboratory in the Department of Vascular and Endovascular Surgery, the lncRNA H19 was recognized as one of the most dramatically increased lncRNAs in medial SMCs in dilated aortic tissue(Li et al., 2018). Knockdown of H19 remarkably reduced apoptosis of human aortic SMCs, while overexpression of H19 led to the opposite changes. Hypoxia-induced factor 1-alpha (HIF1α) was investigated to change its expression in response to overexpression and knockdown of H19. Knockdown of HIF1α suppressed H19-related SMC death, which indicates that HIF1α operates at downstream level of H19. Decrease of H19 adequately restrained the expression of HIF1α and obviously abolished SMC apoptosis. Co-localized staining of rising H19 and HIF1α, together with apoptotic marker, were detected in SMCs in human aneurysmal tissue. H19 attracts specificity protein (Sp1) to the promoter of HIF1α, and then contributes to transcription of

HIF1α. Followed with association with mouse double minute 2 (Mdm2), thus prohibiting MDM2-related p53 decline. Upregulated apoptotic death of SMCs was related with association between H19 and HIF1α, as well as MDM2 and p53. Thus, increased H19 connects to the promoter of HIF1α and attracts Sp1, which mediates HIF1α transcription. HIF1α associates with Mdm2 and stabilizes p53, which induces SMC apoptosis. So, lncRNA H19 influences SMC survival in AAA progression. Inhibiting the expression of H19 might be a promising treatment for aortic aneurysm disease.

6 CONCLUSION AND FUTURE PLANS

NETs are present in AAA disease. SMC necroptosis and apoptosis are associated with AAA progression. NETs exacerbate AAA through SMC necroptosis and apoptosis pathways.

Future plan: Co-localization of SMC and necroptosis/apoptosis markers through immunohistochemistry and immunofluorescence. Does RIPK3 or MLKL inhibition affect AAA development (in experimental *in vitro* and *in vivo* models)?

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Auszeichnungen

2015	National Scholarship for graduate students
2017	Scholarship from Chinese Scholarship Council

Sprachkenntnisse

Chinesisch	Muttersprache
Englisch	IELTS 7.0
Deutsch	A2.1

Veröffentlichungen

1. H19 induces Abdominal Aortic Aneurysm Development and Progression.
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9 PUBLICATIONS

Li DY, Busch A, Jin H, Chernogubova E, Pelisek J, Karlsson J, Sennblad B, **Liu S**, Lao S, Hofmann P, Bäcklund A, Eken SM, Roy J, Eriksson P, Dacken B, Ramanujam D, Dueck A, Engelhardt S, Boon RA, Eckstein HH, Spin JM, Tsao PS, Maegdefessel L. (2018) H19 induces Abdominal Aortic Aneurysm Development and Progression. Circulation. 2018 Oct 9;138(15):1551-1568.

doi: 10.1161/CIRCULATIONAHA.117.032184.

PMID: 29669788

10 POSTER PRESENTATIONS

Role of Neutrophil Extracellular Traps in Abdominal Aortic Aneurysm Disease Development and Progression. Shengliang Liu, Valentina Paloschi, Zhiyuan Wu, Jaroslav Pelisek, Shen Lao, Hans-Henning Eckstein, Oliver Sohnlein, Lars Maegdefessel. 8th Munich Vascular Conference, 2018.

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